Molecular Mechanisms Underlying the Acquisition, Extinction and Reinstatement of Cocaine-Associated Memory: Relevance to Addiction Processes

Shervin A. Liddie
University of Miami, sliddie@med.miami.edu

Follow this and additional works at: http://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
http://scholarlyrepository.miami.edu/oa_dissertations/1303

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
MOLECULAR MECHANISMS UNDERLYING THE ACQUISITION, EXTINCTION
AND REINSTATEMENT OF COCAINE-ASSOCIATED MEMORY: RELEVANCE
TO ADDICTION PROCESSES

Shervin Albert Liddie

Approved:

Yossef Itzhak, Ph.D.
Professor of Psychiatry
and Behavioral Sciences
and Molecular and Cellular
Pharmacology

Claes Wahlestedt, M.D., Ph.D.
Professor of Psychiatry
and Behavioral Sciences

Philip McCabe, Ph.D.
Professor of Psychology

Juan Young, Ph.D.
Assistant Professor of
Human Genetics

David Wolgin, Ph.D.
Professor of Behavioral Neuroscience
Florida Atlantic University

M. Brian Blake, Ph.D.
Dean of the Graduate School
An important target for combating drug addiction is to understand the neurobiological mechanisms that sub-serve relapse to drug use. Drug addiction is thought to usurp the neural mechanisms of learning and memory. The conditioned place preference (CPP) paradigm which employs the principles of Pavlovian learning is often used to investigate the incentive value of drugs of abuse and the formation of drug-associated memory. One caveat to conditioned reward studies is the use of a fixed daily dose of the addictive drug during training. However, the transition from drug use to addiction in human addicts involves an escalation in drug intake. I posit that a paradigm that can effectively simulate increases in drug intake will better model the human drug use pattern. Evidence suggests that conditioning by escalating doses of cocaine (Esc-C) confers higher magnitude and more persistent cocaine-memory than conditioning by a fixed daily dose of cocaine (Fix-C). Thus, my research objective was to investigate the contribution of different signaling molecules to the acquisition, reconsolidation, extinction and reinstatement of cocaine associated memory developed by Esc-C versus Fix-C. At the cellular level, I found that the formation of Esc-C memory was associated with markedly increased hippocampal expression of the mRNA and protein that codes for
the NR2B subunit of the *N*-methyl-D-aspartate receptor (NMDAR) compared to formation of Fix-C memory, suggesting a positive correlation between the strength of cocaine associated memory and levels of NR2B subunit expression. Pharmacologically, the development (acquisition) of Fix-C and Esc-C memory was attenuated by antagonism of NR2B-containing NMDARs. However, inhibition of the neuronal nitric oxide synthase (nNOS) which is downstream of the NMDAR attenuated the acquisition of Fix-C but not Esc-C memory. This suggests that the acquisition of Fix-C memory is NO-dependent while Esc-C memory is NO-independent. Regarding memory reconsolidation, NR2B antagonism disrupted reconsolidation of both Fix-C and Esc-C memory. However, while reconsolidation of Fix-C memory was NO-dependent similar to acquisition, reconsolidation of Esc-C memory was NO-insensitive. Conversely, inhibition of the extracellular signal-related kinase (ERK) signaling pathway disrupted reconsolidation of Esc-C but not Fix-C memory. With respect to extinction learning, I investigated the use of phosphodiesterase (PDE) inhibitors as cognitive enhancer to facilitate elimination of extinction-resistant Esc-C CPP. I found that specific inhibition of PDE9, which increases levels of cGMP in the hippocampus and amygdala, induced extinction learning and prevented cocaine-primed reinstatement in mice conditioned by Esc-C. This suggests that PDE9 has a prominent role in consolidation of extinction learning. Stress is a major contributor to relapse to drug use in human addicts. I found that stress-induced reinstatement of Fix-C and Esc-C CPP (following their extinction) was unperturbed by ifenprodil. However, stress-induced reinstatement of Fix-C CPP, but not Esc-C CPP, was attenuated by MK-801, 7-NI and the corticotrophin releasing hormone receptor subtype 1 (CRH-R1) antagonist antalarmin. This suggests that stress-induced reinstatement of Esc-
C CPP may engage alternative signaling pathways. Taken together, my studies have shown that variations in the stimulus salience of cocaine reward from a fixed dose to escalating doses engage different neural substrates in the formation of cocaine-associated memory. Furthermore, my studies highlight the importance of understanding the significance of drug memory strength, which could be relevant to the severity of addiction, as it relates to the development of pharmacotherapeutics for the management of addiction.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES AND TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Overview</td>
<td>1</td>
</tr>
<tr>
<td>Epidemiology of cocaine abuse</td>
<td>1</td>
</tr>
<tr>
<td>Molecular and cellular mechanisms of cocaine addiction</td>
<td>3</td>
</tr>
<tr>
<td>The conditioned place preference (CPP) paradigm</td>
<td>5</td>
</tr>
<tr>
<td>Role of glutamate signaling in drug-induced neuroadaptations</td>
<td>7</td>
</tr>
<tr>
<td>Nitric oxide (NO) signaling pathway</td>
<td>7</td>
</tr>
<tr>
<td>Role of nitric oxide in cocaine effects</td>
<td>9</td>
</tr>
<tr>
<td>Research objectives and hypothesis</td>
<td>10</td>
</tr>
<tr>
<td>Figures for Chapter 1</td>
<td>14</td>
</tr>
<tr>
<td>Chapter 2: VARIATIONS IN THE STIMULUS SALIENCE OF COCAINE REWARD</td>
<td>17</td>
</tr>
<tr>
<td>REWARD INFLUENCES DRUG-ASSOCIATED CONTEXTUAL MEMORY</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>17</td>
</tr>
<tr>
<td>Background</td>
<td>18</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>21</td>
</tr>
<tr>
<td>Results</td>
<td>27</td>
</tr>
<tr>
<td>Discussion</td>
<td>32</td>
</tr>
<tr>
<td>Figures for Chapter 2</td>
<td>40</td>
</tr>
</tbody>
</table>
Proposed model for the development of Fix-C and Esc-C memory………… 93
Future directions............................................................................ 95
Silent synapses in cocaine-associated memory strength..................... 96
Site-specific inhibition of signaling targets..................................... 96
Effect of extinction and withdrawal on NR2B................................ 97
Signaling molecules in stress-induced reinstatement of Esc-C CPP……... 98
Fear Conditioning........................................................................... 99
Tables and Figures for Chapter 5.................................................... 101
REFERENCES............................................................................... 105
LIST OF FIGURES AND TABLES

Figure 1.1. Past month illicit drug use among persons aged 12 or older: 2012 (page 14).

Figure 1.2. Model proposing a network of four circuits involved with addiction: reward, motivation/drive, memory, and control (page 15).

Figure 1.3. Nitric oxide (NO) signaling pathway (page 16).

Figure 2.1. The NR2B subunit of NMDA receptor contributes to the development of Esc-C memory (page 40).

Figure 2.2. Acquisition of cocaine memory: antagonism of NR2B-containing NMDA receptors attenuates the acquisition of both Fix-C and Esc-C memory while nNOS inhibition attenuates Fix-C but not Esc-C memory acquisition (page 41).

Figure 2.3. Reconsolidation of cocaine memory: temporal- and dose-dependence of NMDAR channel blockade (page 42).

Figure 2.4. Reconsolidation of cocaine memory: antagonism of NR2B-containing NMDARs disrupts Fix-C and Esc-C memory reconsolidation (page 43).

Figure 2.5. Effect of inhibition of NMDAR downstream signaling on reconsolidation of cocaine memory (page 44).

Table 3.1. Schematic presentation of the timeline and different phases of the extinction experiment (page 61).

Figure 3.1. Effect of different doses of PDE inhibitors on levels of cAMP and cGMP in the hippocampus and amygdala (page 62).

Figure 3.2. Effect of different PDE inhibitors on extinction of cocaine-induced CPP (page 63).

Figure 4.1. The NMDAR antagonist MK-801 attenuates stress-induced reinstatement of Fix-C but not Esc-C CPP whereas the NR2B antagonist ifenprodil has no effect on stress-induced reinstatement of Fix-C and Esc-C CPP (page 81).

Figure 4.2. Inhibition of nNOS signaling disrupts stress-induced reinstatement of Fix-C but not Esc-C CPP (page 82).

Figure 4.3. The nNOS inhibitor 7-NI does not interfere with mobility during the FST (page 83).
Figure 4.4. The CRH-R1 antagonist antalarmin attenuates stress-induced reinstatement of Fix-C but not Esc-C CPP (page 84).

Figure 4.5. Mice not exposed to FST do not spontaneously reinstate place preference (page 85).

Figure 4.6. Plasma corticosterone increases following exposure to FST despite attenuation of stress-induced reinstatement of Fix-C CPP (page 86).

Table 5.1. Effect of different pharmacological agents on Fix-C and Esc-C memory acquisition, reconsolidation and stress-induced reinstatement (page 101).

Table 5.2. Effect of conditioning schedule on expression of NR2B subunit of the NMDAR at the mRNA (Grin2b) and protein (NR2B) levels (page 102).

Figure 5.1. Proposed model for the development of Fix-C and Esc-C memory (page 103).

Figure 5.2. Repeated shocks at increasing intensities elicit higher magnitude and more persistent freezing response than repeated shocks at a fixed intensity (page 104).
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-NI</td>
<td>7-nitroindazole</td>
</tr>
<tr>
<td>BAY-73-6691</td>
<td>PDE9 inhibitor</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine 3’:5’-cyclic monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioned response</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Esc-C</td>
<td>Escalating cocaine conditioning schedule</td>
</tr>
<tr>
<td>Fix-C</td>
<td>Fixed cocaine conditioning schedule</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N(omega)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activating extracellular kinase</td>
</tr>
<tr>
<td>MK-801</td>
<td>Dizocilpine, NMDAR antagonist</td>
</tr>
<tr>
<td>NAC</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NR1</td>
<td>Compulsory subunit of NMDAR encoded by Grin1 gene</td>
</tr>
<tr>
<td>NR2A</td>
<td>Subunit of NMDAR encoded by Grin2a gene</td>
</tr>
<tr>
<td>NR2B</td>
<td>Subunit of NMDAR encoded by Grin2b gene</td>
</tr>
<tr>
<td>pCREB</td>
<td>Phosphorylated cAMP response element-binding protein</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDEi</td>
<td>Phosphodiesterase inhibitor</td>
</tr>
<tr>
<td>pERK</td>
<td>Phosphorylated extracellular signal-related kinase</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble Guanylyl cyclase</td>
</tr>
<tr>
<td>SL327</td>
<td>MEK inhibitor</td>
</tr>
<tr>
<td>STM</td>
<td>Short-term memory</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Overview

Drug addiction is defined as the loss of control over the intense urges to take drugs even at the expense of adverse consequences. Such loss of control may develop as a result of dysregulation of the prefrontal cortex (PFC), cingulate gyrus, and extended amygdala, which may occur following repeated drug use (Koob & Volkow, 2010). Addiction is associated with abuse of opiates, alcohol and psychostimulants such as cocaine acting on mesocorticolimbic pathways. Drug addiction has been a problem faced by mankind for centuries, yet little progress has been made toward developing effective treatment strategies. This thesis will investigate the role of different signaling molecules in the acquisition, extinction and reinstatement of cocaine-induced conditioned place preference in mice, as an animal model of addictive behavior.

Epidemiology of cocaine abuse

Cocaine is a powerful addictive psychostimulant that was first extracted from coca leaves by Albert Niemann in 1859. It was not until the 1800s that cocaine use became popular in the medical community when Sigmund Freud, an Austrian psychoanalyst, promoted cocaine as a cure for depression and sexual impotence. Additionally, in 1886, cocaine’s popularity skyrocketed when John Pemberton added coca leaves as an ingredient to Coca-Cola soft drink. Consequently, patrons experienced euphoria and boosted energy levels which in turn made Coca-Cola a popular drink of choice. However, when the dangers associated with cocaine use became increasingly
evident, public pressure forced the Coca-Cola Company to remove cocaine from the soft
drink in 1903.

Today, abuse of illicit drugs such as cocaine continues to present a significant health and social problem worldwide. A major problem in the treatment of drug addiction is that during periods of abstinence, pervasive thoughts about the drug lead an individual to relapse to drug use (Koob et al., 2004). The 2012 National Survey on Drug Use and Health revealed that an estimated 23.9 million (9.2%) Americans aged 12 or older were current (used in the past 30 days) illicit drug users at the time of the survey. Of these, cocaine was the second most abused illicit drug behind marijuana, with around 1.6 million (0.6%) current users aged 12 or older (Fig. 1.1; SAMHSA, 2013).

As reported by the National Institute of Drug Abuse, in the USA, it is estimated that the costs of drug abuse and addiction values $524 billion a year. Of this, illicit drug use account for approximately $181 billion in health care, loss of productivity, crime, incarceration and drug enforcement. Among illicit drugs, cocaine use is not only responsible for the most hospital emergency department and drug-treatment center visits but is also the most common cause of drug-related deaths (Lange & Hillis, 2001).

Cocaine is a potent central nervous system (CNS) stimulant with effects lasting up to one hour, depending on route of administration. Cocaine has been used as a local anesthetic in nasal (Noorily et al., 1995) and eye surgery. However, the common recreational cocaine use is geared toward experiencing euphoria, stimulation, feelings of confidence and well-being.
Molecular and cellular mechanisms of cocaine addiction

Although addicts are aware of the negative health, social and economic consequences of their drug abuse, they continue to compulsively seek and use illicit drugs. This begs the question “why can’t addicts just quit?” One response to this question is that drugs of abuse changes the brain in such a way that addicts lose control over their ability to stop drug use. Figure 1.2 (taken from Volkow et al., 2003) shows a schematic of how drugs of abuse affect different brain circuits.

The compulsive use of illicit drugs such as cocaine is thought to induce neuroadaptations in brain regions associated with reward-related learning and memory processes. These changes cause a) hyper-sensitization to cues associated with drug-taking, b) impulsive decision making and c) aberrant habit-like learned behaviors so much so that even exposure to adverse consequences do not sway addicts away from drug seeking and taking (Thomas et al., 2008). Drugs of abuse affect the same neural pathways involved in motivation, processing of rewards and decision making. The mesolimbic dopamine system which comprises of the ventral tegmental area (VTA), the nucleus accumbens (NAC), the PFC, hippocampus, amygdala and the bed nucleus of the stria terminalis is generally considered the reward pathway. The rewarding effect of psychostimulants is dependent on the balance between their influence on dopamine (DA), norepinephrine (NE) and 5-hydroxytryptamine (5-HT; serotonin) transmission; the increase in synaptic DA is thought to be the antecedent for their reinforcing and addictive properties of the drugs (Di Chiara, 2000; Wise, 1994). While the overall effect of drugs of abuse in the mesolimbic system is increased levels of catecholamines in the synapse, individual psychostimulants may achieve this effect in different ways.
Cocaine exerts its effects by increasing extracellular monoamine (DA, 5-HT and NE) concentrations by blocking the reuptake of monoamines from the synapse (Rotham et al., 2001). Critically important to reinforcing drug use is cocaine’s ability to bind to and block the pre-synaptic dopamine transporter (Wise, 1984). This blockade results in an accumulation of synaptic dopamine which in turn enhances dopaminergic signaling through increased activation of postsynaptic dopamine receptors.

The neurobiological effects of drugs of abuse on the reward circuitry have been extensively studied. However, what is less clear is why following extended periods of abstinence users remain susceptible to relapse. To this end, relapse remains the primary obstacle to the effective treatment of drug abuse (Stewart, 2000). An individual’s susceptibility to relapse is governed by both genetic and non-genetic factors (Kendler et al., 2003). Studies have implicated that repeated drug use “hijacks” the neural circuitry involved in learning and memory. This hypothesis has emerged from observations where drug-associated cues evoke drug memory, which induces craving during abstinence and precipitates relapse (Robins et al., 2008). A number of studies have addressed the problem of relapse to cocaine seeking induced by drug cues, priming injections and stress using rodent models in conditioned place preference and self-administration paradigms (Erb et al., 1998; Erb and Stewart, 1999; Itzhak and Martin, 2002; Lu et al., 2002; Shaham et al., 2003; Wang et al., 2000). Although the same behavioral phenotype is observed, cocaine priming and stress induced reinstatement of drug seeking seem to be mediated by different neural mechanisms (Aguilar et al., 2009). For example, Sanchez and colleagues (2003) demonstrated that the D1 agonist SKF 81297 attenuated stress-induced but did not affect cocaine-induced reinstatement. Furthermore, Kreibich and
Blendy (2004) demonstrated that different patterns of phosphorylated cAMP response element-binding protein (pCREB) activation in discrete brain regions were observed in stress-induced versus cocaine-induced reinstatement. Thus, a thorough understanding of how elements that precipitate relapse impact brain physiology and neurochemistry is necessary for combating addiction. Specifically, an understanding of how these drug-induced changes affect learning and memory processes will be of valuable importance.

**The conditioned place preference (CPP) paradigm**

Learning and memory mechanisms play a major role in the development of maladaptive behaviors, including drug addiction (Hyman *et al.*, 2006). The conditioned place preference (CPP) paradigm, which employs the principles of Pavlovian conditioning, can model learning and memory processes pertinent to addictive behavior (White & Carr, 1985). In classical conditioning (Pavlov, 1927), an unconditioned stimulus (US) such as cocaine is paired with a neutral context which in turn confers conditioned stimulus (CS) properties to the neutral context. For drug addiction studies, re-exposure to the CS elicits an approach behavior i.e., conditioned response (CR). The three major phases in Pavlovian conditioning are: acquisition, consolidation and retrieval/reconsolidation (Sanchis-Segura & Spanagel, 2006). In animal models, the role of Pavlovian learning in drug-seeking behavior has been implicated in CPP (non-operant behavior) and drug self-administration (operant behavior) studies (Shalev *et al.*, 2002). Relevant to drug addiction, presentation of cocaine- cues to cocaine abusers elicits limbic activation, craving and physiological responses similar to the drugs’ effects, suggesting the emergence of a CR (Childress *et al.*, 1999; Newlin, 1992; Robbins *et al.*, 1999).
CPP is often viewed as “habit learning,” which has a major role in the development of drug addiction (Everitt & Robbins, 2005). The expression of CPP is viewed as a test for reactivity to drug-associated CS; thus, this test has face validity for cue-reactivity in human drug users. The CPP paradigm has also been used to investigate extinction of “drug-seeking behavior” and reinstatement of conditioned response (Itzhak & Martin, 2002; Parker & McDonald, 2000). Interestingly, reinstatement of cocaine CPP (following its extinction) is a drug-specific phenomenon that can be triggered only by drugs that share a similar mechanism of action with that of cocaine (Itzhak & Martin, 2002). Thus, the reinstatement of place preference, like the reinstatement of drug self-administration in animal models, has face validity for the study of relapse.

While the expression of CPP is generally thought to result from incentive-driven conditioned behavior, alternative interpretations suggest that this approach behavior may be mediated a) as a consequence of reinforced behavior and b) as a result of pharmacologically-induced treatment effects (Huston et al., 2013). Naturally the possibility exists that all three processes may contribute to the establishment of CPP. However, the delineation of the contribution of each factor may aid in the development of more targeted therapies since different brain regions may mediate each process. In any event, the manifestation of each process listed above will depend on the ability of test subjects to remember the respective associations learnt during conditioning. Hence, learning and memory processes remain an integral part of assessing drug memory in the CPP paradigm.
The role of glutamate in drug-induced neuroadaptations

Historically, dopamine has been considered the major neurotransmitter involved in the effects of cocaine and other drugs of abuse. However, more recent studies have shown that the excitatory neurotransmitter glutamate plays a central role in the behavioral and neurochemical effects of cocaine. Neuronal changes associated with cocaine use can convert cocaine-induced signals into long-term alterations in neuronal functions through synaptic plasticity (Hyman et al., 2006).

Glutamate signaling through the N-methyl-D-aspartate receptor (NMDAR) plays a central role in synaptic plasticity and learning and memory processes (Collingridge, 1987). Early reports suggested that the NMDAR was involved in the behavioral effects of psychostimulants as administration of NMDAR antagonists prevented the development of sensitization to cocaine (Vanderschuren & Kalivas, 2000). Since then, numerous studies have shown that antagonism of NMDAR disrupted acquisition, expression and reconsolidation of cocaine-associated memory (Kelley et al., 2007; Alaghband & Marshall, 2013). Additionally, repeated cocaine exposure causes increases in extracellular glutamate in the NAC and VTA (Kalivas & Duffy, 1998; Pierce et al., 1996; Reid & Berger, 1996). Since cocaine affects glutamate signaling, it therefore directly or indirectly affects synaptic plasticity and learning and memory processes. Perhaps cocaine’s ability to modulate glutamate signaling contributes to the compulsive and habit-like learned behaviors associated with drug seeking and taking.

Neuronal nitric oxide (NO) signaling pathway

Nitric oxide (NO) has a role in learning and memory mechanisms (Susswein et al., 2004). The major source of NO arises from the conversion of L-arginine to L-
citrulline by the enzyme nitric oxide synthase (NOS). Three major isoforms of NOS have been identified. Neuronal (nNOS) and endothelial (eNOS) are both constitutively expressed, and calcium-dependent. The inducible isoform (iNOS) is present primarily in microglia and macrophages and is not constitutively expressed (Bredt & Snyder, 1994). nNOS has three major alternatively spliced transcripts: nNOSα (150-kDa), nNOSβ (136-kDa) and nNOSγ (125-kDa) (Brenman & Bredt, 1997). Targeted deletion of mouse nNOSα resulted in a 95% reduction in NOS catalytic activity suggesting that nNOSα accounts for the majority of nNOS catalytic activity in the brain (Huang et al., 1993).

nNOS is linked via the postsynaptic density protein PSD95 to the NR2B subunit of the N-methyl-D-aspartate (NMDA) type of glutamate receptors and is activated upon NMDAR stimulation (Christopherson et al., 1999; Sattler et al., 1999). Activation of ionotropic NMDARs increases calcium influx. The subsequent binding of calcium to calmodulin activates nNOS and increases NO production. As a diffusible gas, NO influences several neurons in the area around the synapse (Kiss & Vizi, 2001) as it can diffuse a few hundred micrometers from its point of synthesis (Gally et al., 1990). NO produced from the catalytic activity of nNOS also acts as a retrograde messenger to activate soluble guanylate cycalase (sGC) in the pre-synaptic neuron. The resulting increase in cyclic guanosine monophosphate (cGMP) facilitates glutamate release; a process important for early-phase long-term potentiation (E-LTP) and short term memory (STM) (Fig. 1.3).

Locally (in the post-synaptic neuron), NO activates sGC to generate the second messenger cGMP (Garthwaite et al., 1988). cGMP then a) triggers adenylate cyclase (AC) to generates the second messenger cyclic adenosine monophosphate (cAMP) and b)
directly activates protein kinase G (PKG). Elevated cAMP stimulates protein kinase A (PKA) which phosphorylates cAMP response element-binding protein (CREB). PKG on the other hand phosphorylates extracellular signal-related kinases 1 and 2 (ERK 1/2) which translocate into the nucleus to phosphorylate CREB (pCREB) (Impey et al., 1998). CREB then binds to CREB-binding protein (CBP) to mediate CREB-dependent gene transcription (Guan et al., 2002) which is required for late-phase LTP (Fig. 1.3).

**Role of nitric oxide in cocaine effects**

The observation that nNOS is linked to NMDAR subunits led to studies on the role of nNOS in the effects of cocaine. Chronic cocaine administration increased NOS activity in cerebral cortex, cerebellum, midbrain, hypothalamus, hippocampus, amygdala and spinal cord of Swiss-Webster mice (Bhargava & Kumar, 1997). Acute systemic administration of cocaine facilitated NO efflux in the rat prefrontal cortex via a nNOS-dependent mechanism since this effect was attenuated by the nNOS inhibitor 7-nitroindazole (7-NI) (Sammut & West, 2008). The increase in NO efflux following acute cocaine administration is mediated through interaction between DA D1 receptor and NMDAR in the dorsal striatum (Lee et al., 2011).

Evidence supporting the involvement of NO signaling in cocaine reward emerged from cocaine self-administration studies. The non-selective NOS inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME) dose-dependently suppressed both the maintenance of cocaine self-administration and the absolute reward magnitude of cocaine (Pulvirenti et al., 1996). Additionally, L-NAME significantly reduced drug-seeking behavior following abrupt cessation of drug availability as well as attenuated the reinstatement of cocaine self-administration in response to a priming injection of cocaine (Orsini et al., 2002).
Likewise, the nNOS inhibitor 7-NI prevented cocaine-induced alterations in medial prefrontal cortex excitability and decreased cocaine self-administration in rats (Collins & Kantak, 2002) supporting the role of nNOS in these effects.

NO signaling has a role in cocaine-induced associative learning. Results from CPP experiments support the role of NO signaling in the motivational effects of cocaine. Studies from our laboratory have shown that both pharmacological (7-NI) and genetic (nNOS KO) manipulations of nNOS provided resistance to cocaine-induced CPP (Itzhak et al., 1998). It was also shown that cocaine CPP can be extinguished by disrupting drug-associated memory reconsolidation; a process whereby retrieval of a previously stored memory becomes labile and subject to manipulation. This process was found to be nNOS-dependent because a) administration of 7-NI to WT mice upon retrieval of cocaine-CPP impaired further expression of place preference, and b) administration of the NO donor molsidomine to nNOS KO after retrieval of cocaine-associated memory prolonged CPP expression, suggesting disruption and strengthening of memory reconsolidation, respectively (Itzhak & Anderson, 2007). A subsequent experiment showed that not only does treatment with MK-801 or 7-NI independently, disrupt cocaine-associated memory reconsolidation, but they also provided resistance to reinstatement of CPP following a priming dose of cocaine (Itzhak, 2008). Overall, results suggest the role of NO signaling in cocaine reward and cocaine-associated memory; thus manipulation of this pathway may afford resistance to cocaine-seeking behavior.

**Research objectives and hypothesis**

An important target for combating drug addiction is to understand the neurobiological mechanisms that sub-serve relapse to drug use (Mantsch et al., 2010).
Drug addiction is thought to usurp the neural mechanisms of learning and memory (Hyman, 2005) and affect long term plasticity as a result of changes in gene expression (McClung & Nestler, 2008). Memory of the rewarding effects of drugs of abuse is stored in the brain even after prolonged periods of abstinence. This memory can resurface when an individual is re-exposed to priming doses of the drug itself, drug-related cues or to certain stressors (Shaham et al., 2003).

The CPP paradigm represents a valuable tool for investigating the incentive value of drugs of abuse and the reinstatement of drug seeking behavior following extinction. Traditional CPP studies employ a fixed dose regimen of the drug during training. However, since the transition from drug use to addiction involves an escalation in drug intake (Gawin, 1991), I posit that a paradigm that can effectively simulate increases in drug intake will better model the human drug use pattern. Research investigating differences in the pattern of cocaine dosing in a CPP paradigm have shown that the schedule of cocaine administration, rather than the dose of cocaine, has a significant impact on the development of drug associated memory (Itzhak & Anderson, 2012; Conrad et al., 2013). This suggests that different mechanisms may govern the formation of cocaine-associated memory that was developed by different schedules of cocaine administration during conditioning. As such, the generalization of the involvement of specific signaling molecules in the formation of cocaine-associated memory may be misleading. Therefore an investigation of the contribution of different signaling molecules to diverse types of drug-memory will be beneficial to identify a potent pharmacotherapy for addiction management. Although the NMDAR and downstream signaling molecules have been implicated in relatively ‘weak’ cocaine-associated
memory, it is unclear if the same molecular factors will be involved in ‘strong’ cocaine-associated memory.

_I hypothesize that mice conditioned by the traditional fixed daily dose of cocaine (Fix-C) versus mice conditioned by escalating doses of cocaine (Esc-C) will show differential expression of specific genes and their corresponding proteins. I further hypothesize that the acquisition, extinction and reinstatement of cocaine-associated memory developed by Fix-C and Esc-C engage different signaling molecules._

In Chapter 2 of this thesis, the molecular mechanisms underlying the effect of conditioning schedule on the acquisition and reconsolidation of cocaine-associated memory developed by Fix-C and Esc-C are described. Specifically, the contribution of different genes and their proteins to the development of Fix-C and Esc-C memory as well as pharmacological approaches for attenuating the acquisition and reconsolidation of Fix-C and Esc-C memory were investigated. Results generated from this chapter were accepted for publication in the following manuscript:


In Chapter 3, extinction of Esc-C memory following repeated unreinforced exposures to the training context was investigated. Specifically, the effect of different phosphodiesterase (PDE) inhibitors on extinction learning was investigated. Results from this chapter were published in the following paper:

In Chapter 4, the effects of a) conditioning schedule and b) different pharmacological agents on attenuation of stress-induced reinstatement of CPP were investigated. Results from this chapter are currently being prepared for a manuscript.

Finally, Chapter 5 is a general discussion and review of the central findings in this thesis. The significance of my findings as well as potential future lines of research stemming from my findings is discussed.
Figure 1.1. Past month illicit drug use among persons aged 12 or older: 2012. Illicit Drugs include marijuana/hashish, cocaine (including crack), heroin, hallucinogens, inhalants, or prescription-type psychotherapeutics used nonmedically. The number of persons who used illicit drugs in the past month was 23.9 million. The number of persons who used cocaine in the past month was 1.6 million. Taken from SAMHSA, 2013.
Figure 1.2. Model proposing a network of four circuits involved with addiction: *reward, motivation/drive, memory, and control*. These circuits work together and change with experience. Each is linked to an important concept: saliency (reward), internal state (motivation/drive), learned associations (memory), and conflict resolution (control). During addiction, the enhanced value of the drug in the reward, motivation, and memory circuits overcomes the inhibitory control exerted by the prefrontal cortex, thereby favoring a positive-feedback loop initiated by the consumption of the drug and perpetuated by the enhanced activation of the motivation/drive and memory circuits. Taken from Volkow *et al.*, 2003.
Figure 1.3. Nitric oxide (NO) signaling pathway. NO produced in the post synaptic cell following NMDAR activation stimulates sGC in both pre- and post-synaptic nerve terminals. Stimulation of sGC generates the second messenger cGMP. In the presynaptic terminal, cGMP facilitates vesicle release of glutamate which is required for the early phase of LTP (E-LTP) and short term memory (STM). cGMP activates PKG which phosphorylates ERK1/2. pERK1/2 can translocate to the nucleus where it phosphorylates CREB. ERK1/2 and CREB-dependent gene transcription are required for late phase LTP (L-LTP) and long term memory. Similar pathways are activated in postsynaptic nerve terminals. Increased cGMP signaling stimulates AC to produce cAMP, which activates PKA, which translocates to the nucleus and phosphorylates CREB. Taken from Liddie et al., 2013.
Chapter 2

Variations in the stimulus salience of cocaine reward influences drug-associated contextual memory

Summary

Drugs of abuse act as reinforcers because they influence learning and memory processes resulting in long-term memory of drug reward. We have previously shown that mice conditioned by fixed daily dose of cocaine (Fix-C) or daily escalating doses of cocaine (Esc-C) resulted in short- and long-term persistence of drug memory, respectively, suggesting different mechanisms in acquisition of cocaine memory. The present study was undertaken to investigate the differential contribution of N-methyl-D-aspartate receptor (NMDAR) subunits in the formation of Fix-C and Esc-C memory in C57BL/6 mice. Training by Esc-C resulted in marked elevation in hippocampal expression of Grin2b mRNA and NR2B protein levels compared to training by Fix-C. The NR2B-containing NMDAR antagonist ifenprodil had similar attenuating effects on acquisition and reconsolidation of Fix-C and Esc-C memory. However, the NMDAR antagonist MK-801 had differential effects: a) higher doses of MK-801 were required for post-retrieval disruption of reconsolidation of Esc-C memory than Fix-C memory, and b) pre-retrieval MK-801 inhibited extinction of Fix-C memory but it had no effect on Esc-C memory. In addition, blockade of NMDAR downstream signaling pathways also showed differential regulation of Fix-C and Esc-C memory. Inhibition of neuronal nitric oxide synthase (nNOS) attenuated acquisition and disrupted reconsolidation of Fix-C but not Esc-C memory. In contrast, the mitogen-activating extracellular kinase (MEK) inhibitor SL327 attenuated reconsolidation of Esc-C but not Fix-C memory. These results suggest that NMDAR downstream signaling molecules associated with consolidation and
reconsolidation of cocaine-associated memory may vary upon changes in the salience of cocaine reward during conditioning.

**Background**

The role of learning and memory in the reinforcing effects of addictive drugs continues to garner much attention. Persistent drug-seeking behavior and the inability to extinguish such maladaptive behavior develop when drugs of abuse exert control over neural substrates and signaling pathways that encode long-term memory (LTM) (Hyman *et al.*, 2006). Recently, disruption of persistent drug memory has taken the forefront as a possible treatment strategy for addiction. Memory reconsolidation is the process whereby previously consolidated memories, upon retrieval, become labile and are thereby susceptible to disruption. A number of studies have shown that cocaine-associated memories are vulnerable to disruption upon retrieval of such memories (Miller & Marshall, 2005; Kelley *et al.*, 2007; Tronson & Taylor, 2013).

The conditioned place preference (CPP) paradigm, which employs the principles of Pavlovian learning, can model learning and memory processes pertinent to addictive behavior (White & Carr, 1985). One caveat in CPP studies is the use of a fixed daily dose of the drug reinforcer during training. Given that the transition from drug use to drug addiction involves escalation in drug intake, we posit that investigation of the outcome of escalating doses of cocaine during conditioning is relevant to the human practice of drug use and the development of addictive behavior. Our laboratory (Itzhak & Anderson, 2012) has shown that conditioning by escalating doses of cocaine (Esc-C) produced higher magnitude and more persistent CPP than conditioning by fixed daily doses of
cocaine (Fix-C). This phenomenon was not dose-dependent but rather schedule-dependent; these results were recently confirmed by others (Conrad et al., 2013).

While some studies have used drug self-administration to investigate escalation in drug exposure, results have shown only a modest escalation in drug self-administration; about 1.2-1.5-fold increase over a two-week period (Ahmed & Koob, 1998; Perry et al., 2006; Anker et al., 2012). However, others interpreted these results as a correlate of long access duration to the drug and not escalated drug intake (Knackstedt & Kalivas, 2007). We posit that investigation of mechanisms involved in formation of drug memory can be better modeled in the CPP paradigm by introducing significant changes in the stimulus salience of the drug reward which apparently facilitates contextual learning.

The $N$-methyl-D-aspartate receptor (NMDAR) plays a central role in synaptic plasticity and learning and memory processes (Collingridge, 1987). With respect to cocaine effects, the NMDAR antagonist MK-801 disrupted acquisition, expression and reconsolidation of cocaine-associated memory (Kelley et al., 2007; Alaghband & Marshall, 2013). Functional NMDARs typically exists as heterotetramers between two compulsory NR1 subunits and two modulatory NR2 subunits (NR2A-D) (Zhou, 2009). The NR2 subunits of NMDARs are thought to have a major role in learning and memory (Furukawa et al., 2005). The abundance of specific NR2 subunit appears to be developmentally regulated where NR2B subunits predominate during early brain development while NR2A levels increase progressively with development (Yashiro & Philpot, 2008). The key functional properties of NMDARs are thought to be mediated by the particular NR2 subunits that comprise the receptor channel (Monyer et al., 1994). Indeed, compared to NR2A-containing NMDARs, NR2B-containing NMDARs display
longer decay time constant (Cull-Candy & Leszkiewicz, 2004) and carry greater calcium current per unit charge (Sobcyk et al., 2005). Additionally, it has been reported that NR2A- and NR2B-containing NMDARs are coupled to different downstream signaling pathways (Chen et al., 2007).

One downstream effector of calcium influx through NMDAR is neuronal nitric oxide synthase (nNOS), which catalyzes the production of nitric oxide (NO) (Brenman & Bredt, 1997). Calcium-dependent nNOS is linked via the post synaptic density protein PSD-95 to the NR2 subunit of the NMDAR; thus its selective localization renders it functionally coupled to the NMDAR (Christopherson et al., 1999; Sattler et al., 1999). Relevant to cocaine effects, NO has been implicated as a major contributor to the initiation and maintenance of cocaine CPP and behavioral sensitization (Bhargava & Kumar, 1997; Itzhak et al., 1998; Balda et al., 2006). Another downstream molecule activated by calcium entry through NMDAR is extracellular signal-regulated kinase (ERK) (Sweatt, 2001). One of many regulators of the ERK signaling cascade is RasGRF1, a Ras-specific GDP/GTP exchange factor (GEF) and Ras activator that specifically binds the NR2B subunit of NMDARs. Hence, NR2B-containing NMDARs are mediators of the NMDAR-dependent ERK signaling via Ras-Raf-MEK-ERK signaling (Krapivinsky et al., 2003). With respect to cocaine effects, inhibition of mitogen-activating extracellular kinase (MEK), the ERK kinase, has been shown to disrupt cocaine-associated contextual memory (Miller & Marshall, 2005; Valjent et al., 2006).

Because the NMDAR and its downstream molecules have a role in learning and memory and cocaine effects, this study was undertaken to elucidate the involvement of
NMDAR subunits and downstream signaling pathways in acquisition and reconsolidation of Fix-C and Esc-C memory.

Materials and Methods

Subjects

Male C57BL/6J mice (8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in groups of 5/cage with food and water ad-libitum and were acclimatized to the vivarium for one week before experiments began. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and was approved by the University of Miami Animal Care and Use Committee.

Drugs

Cocaine HCl and (+)-MK-801 hydrogen maleate (dizocilpine) were dissolved in saline (0.9% NaCl). The NR2B antagonist ifenprodil [α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol(+)-tartrate salt] was dissolved in distilled water. Traxoprodil [(1S,2S)-1-(4-hydroxy-phenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol], another NR2B-containing NMDAR antagonist (Chenard et al., 1995), and the nNOS inhibitor 7-nitroindazole (7-NI) were dissolved in a 1:3:6 mixture of DMSO, polyethylene glycol and distilled water, respectively. The MEK inhibitor SL327 was dissolved in 40% DMSO (Atkins et al., 1998). All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Drugs and vehicles were administered intraperitoneally (i.p.) in a volume of 0.1ml/10g.
**Conditioning procedure**

Place preference was monitored using custom-designed Plexiglas cages (Opto-Max Activity Meter v2.16; Columbus Instruments) as previously described (Liddie et al., 2012). The training context consisted of two compartments. One compartment had black and white striped walls and a white floor covered with stainless steel grid; the other compartment had black walls and smooth black floor. Each compartment was scanned by 7 infrared beams. A null zone 8 cm wide was assigned at the interface of the two compartments to ensure that only full entry into each compartment is registered as ‘real’ time spent in each zone. On the first day, mice were habituated (15min) to the training context; preconditioning compartment-preference/aversion was determined. Pre-conditioning average times spent in the black, striped and null zones during 1200 seconds were 456±12, 516±13 and 217±9 seconds, respectively. Half the subjects showed slight preference for one side or the other. Accordingly, mice were paired with cocaine in the less preferred compartment. Although this may be viewed as a biased design, half of the mice were paired with cocaine in the black compartment and the other half were paired with cocaine in the black and white-striped compartment making this design ‘partially biased’. Following habituation, mice were conditioned over 4 days by a) 11.25mg/kg (Fix-C) or b) 3, 6, 12 and 24mg/kg; one dose per day (Esc-C) as we previously described (Itzhak & Anderson, 2012). Doses were chosen to control for total amount of cocaine administered over 4 days. Post-conditioning average time spent in the null zone during 1200 seconds CPP test was 192±7 seconds. Likewise time spent in the null zone following pharmacological treatments, pre- and post-CPP, was not significantly different than vehicle treatment (201±9 seconds).
Experiment 1: Contribution of NMDAR subunits in formation of Fix-C and Esc-C memory

To determine whether NMDAR subunits were differentially regulated in mice conditioned by Fix-C and Esc-C (Itzhak & Anderson, 2012) we performed quantitative real-time polymerase chain reaction (qPCR) and immunoblot analyses (n=3-4 mice/group). Bilateral hippocampus was dissected 24h after conditioning and subsequently analyzed. In the hippocampus NR2A and NR2B are the predominant NR2 subunits that comprise the NMDAR (Monyer et al., 1994). The NMDAR subunits NR1, NR2A and NR2B are encoded by the genes Grin1, Grin2a and Grin2b, respectively. We focused on the hippocampus because of its role in spatial/contextual memory.

Experimental groups for Fix-C and Esc-C included the following: Coc-Paired: saline was given in one compartment at mornings and cocaine in the other compartment 3h later. Coc-Unpaired: saline was given in one compartment at mornings and 3h later mice were re-exposed to the conditioning apparatus in the absence of drug; cocaine was administered (30min later) in the home cage. Saline-Paired: saline given in both compartments. For qPCR the saline, paired and unpaired (Fix-C and Esc-C) groups were analyzed whereas for western blot analyses, only the saline controls and paired groups of Fix-C and Esc-C were analyzed.

qPCR

Twenty-four hours following conditioning sessions, mice were tested for CPP and were sacrificed 20 min later. Bi-lateral hippocampus was dissected and stored in RNAlater (Qiagen). Equal amounts of total RNA were reverse-transcribed and subjected to qPCR analysis (n=4 mice/group). Custom designed qPCR arrays (Qiagen) were used to evaluate
changes in gene expression profiles in response to different experimental conditions. Cycle threshold (Ct) values were used to compare differences in expression levels among the groups. Since saline groups from both Fix-C and Esc-C experiments received the same treatment and there was no significant difference between them, they were combined to serve as control.

**Immunoblot Analysis**

Twenty-four hours after conditioning, mice were tested for the expression of place preference (20min) then sacrificed. Bi-lateral hippocampus was dissected and flash frozen on dry ice. Tissues were homogenized on ice in 200µl RIPA buffer (4.5mM Tris-HCl pH7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (Boston Bioproducts, Ashland, MA) supplemented with Complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN), 1mM EDTA and 1mM PMSF. Samples were centrifuged at 4°C for 10 min. The supernatant was collected and protein concentration was measured using DC Protein Assay Kit (Bio-Rad, Hercules, CA). Equal volumes of laemmli sample buffer (Bio-Rad) were added to each sample aliquot. Prior to electrophoresis, samples were boiled at 94°C for 5 min and 15µg of total protein was subjected to electrophoresis on 4-20% SDS–polyacrylamide gels (Bio-Rad). Proteins were subsequently transferred to Immobilon-FL PVDF Transfer Membrane (Millipore, Billerica, MA). Membranes were blocked with 3% BSA in 0.1% Tween-supplemented Tris-buffered saline (TBS-T) for 1 h at room temperature (RT) then probed for NR1 (1:4000; 5704S; Cell Signaling), NR2A (1:4000; 4205S; Cell Signaling), NR2B (1:4000, 06-600; Millipore) and β-tubulin (1:40000; 05-559; Millipore) overnight at 4 °C. After incubation, membranes were washed 3X for 10 min with TBS-T and then incubated (1hr
at RT) with HRP-linked secondary antibodies. The signal was visualized using an enhanced chemiluminescent (ECL) substrate for HRP enzyme (Bio-Rad). Band intensities were quantified using UN-SCAN-IT gel analysis software (v6.1, Silk Scientific Inc., Orem, UT). Relative density units were expressed as total target protein/beta-tubulin.

**Experiment 2: Acquisition of Esc-C and Fix-C memory**

The effect of the NR2B antagonist ifenprodil on acquisition of place preference was investigated because of the observed increase in NR2B subunit expression in the hippocampus. Additionally, because inhibition of nNOS was shown to block Fix-C memory acquisition (Itzhak *et al.*, 1998) and since nNOS activity is regulated by calcium entry through NMDAR (Christopherson *et al.*, 1999; Sattler *et al.*, 1999) we investigated the effect of the nNOS inhibitor 7-NI on acquisition of Fix-C and Esc-C memory. Thirty minutes prior to each cocaine session, mice received a) vehicle, b) ifenprodil (10mg/kg) or c) 7-NI (25mg/kg). Mice were tested for place preference (15min) 72hr later (day 8).

**Experiment 3: Reconsolidation of Fix-C and Esc-C memory**

3a) **Effect of the NMDAR antagonist MK-801**

Because the NMDAR antagonist MK-801 disrupted reconsolidation of cocaine-associated memory acquired by a Fix-C schedule (Kelley *et al.*, 2007; Alaghband & Marshall, 2013), we investigated the effect of MK-801 on reconsolidation of Fix-C and Esc-C memory. Following conditioning, on day 8, mice (n=6-10/group) received MK-801 (0.1 or 0.3mg/kg) pre- or post-retrieval of place memory. For pre-retrieval, drug was given 30 min prior to CPP expression test (15min) and for post-retrieval drug was given
following a 6 min CPP expression test. Mice were then tested for place preference expression after 4 days.

3b) Effect of NR2B subunit antagonists ifenprodil and traxoprodil

Next, we investigated the effects of ifenprodil and traxoprodil on Fix-C and Esc-C memory reconsolidation. On day 8, mice were re-exposed to the conditioning apparatus for 6 min (memory retrieval by a short CPP test) and immediately thereafter they received ifenprodil (10mg/kg) or traxoprodil (10mg/kg). To test whether subsequent reductions in CPP following ifenprodil treatment was due to disruption of memory reconsolidation, ifenprodil was administered a) in the home cage in the absence of memory retrieval or b) 6 h after memory retrieval (outside the reconsolidation window). Mice were then tested for place preference expression 4 days following the first memory retrieval session.

3c) Effect of the nNOS inhibitor 7-NI and the MEK inhibitor SL327

To investigate the role of NMDAR downstream signaling in memory reconsolidation, the effects of the nNOS inhibitor 7-NI and the MEK inhibitor SL327 were investigated. Mice were conditioned by Fix-C and Esc-C schedules and after 72 h mice were re-exposed to the conditioning apparatus for 6 min (memory retrieval by a short CPP test). Vehicle, 7-NI (25mg/kg) or SL327 (30mg/kg) were administered immediately thereafter. Mice were then tested for place preference expression 4-7 days following the first memory retrieval session.

Data Analysis

Data for behavioral testing were analyzed using two-way ANOVA [(group x time) or (MK-801 dose x time; experiment 3)] followed by Student-Newman-Keuls post hoc test. qPCR data were analyzed using $2^{-\Delta\Delta CT}$ method as described by Livak and
Schmittgen (2001). Differences in expression levels in qPCR and western blot analyses were analyzed by one-way ANOVA. Student’s t-test was also used to compare differences between two groups. Data analysis was done using Sigma Stat version 3.1 (Systat Software Inc.).

Results

Experiment 1: Contribution of NMDAR subunits in formation of Fix-C and Esc-C memory

Mice that received cocaine paired to the training context demonstrated significant place preference while mice that received either saline injections or cocaine unpaired to the training context did not (Fig. 2.1A; day 6). Two-way ANOVA showed there was a significant group effect [for Fix-C:F(2,48)=7.422; p=0.002; for Esc-C:F(2,54)=12.258; p<0.001]; a significant time effect [for Fix-C:F(1,48)=10.201; p=0.002; for Esc-C:F(1,54)=30.231; p<0.001] and a significant group x time interaction [for Fix-C:F(2,48)=4.323; p=0.019; for Esc-C:F(2,54)=8.898; p<0.001]. A comparison of the magnitude of CPP on day 6 between Esc-C-paired and Fix-C-paired found that Esc-C showed significantly higher CPP than Fix-C (t=2.355; p=0.031) – thus confirming our previous findings (Itzhak & Anderson, 2012).

With respect to NMDAR subunit mRNA expression, one-way ANOVA comparing Grin2b expression among the saline control group, and the Fix-C and Esc-C paired and unpaired groups showed a significant group effect [F(4,15)=6.473; p=0.003; Fig. 2.1B]. Grin2b was significantly up-regulated in the paired Esc-C group compared to all other groups; no significant difference in Grin2b expression between the unpaired groups (Fix-C
and Esc-C) was observed (Fig. 2.1B). Levels of Grin1 and Grin2a mRNA were unchanged across all groups (data not shown).

Because the unpaired conditions showed no significant differences in Grin2b mRNA expression, western blot analyses were conducted using only the paired conditions (Fix-C and Esc-C). Western blot analyses of NR2A, NR2B and the compulsory NR1 subunits were carried out. NR2B was significantly up-regulated in Esc-C compared to Fix-C and saline-treated mice. One-way ANOVA revealed an overall significant group effect \[F(2,8)=12.664; p=0.003\]. Figure 2.1C shows an approximate 2.5-fold increase in total NR2B in the hippocampus of Esc-C compared to Fix-C and approximately 5-fold increase compared to saline-treated mice. Student’s t-test showed Fix-C was also significantly up-regulated approximately 2.3-fold over saline-treated mice (\(t=2.684; p=0.044\)). There were no significant differences in expression levels of NR1 and NR2A across the groups although there appeared to be a trend toward reduction in NR2A where saline>Fix-C>Esc-C (Fig. 2.1D, E).

**Experiment 2: Acquisition of Esc-C and Fix-C memory**

We investigated whether systemic administration of the NR2B-containing NMDAR antagonist ifenprodil would attenuate the acquisition of Fix-C and Esc-C memory. Two-way ANOVA analysis of the effect of ifenprodil treatment on cocaine-associated memory expression showed that for Esc-C, there was a significant group effect \(F(1,30)=6.678; p=0.015\), a significant time effect (test day) \(F(1,30)=121.882; p<0.001\) and a significant group x time interaction \(F(1,30)=7.586; p=0.01\). For Fix-C, there was a significant time effect \(F(1,28)=46.351; p<0.001\), however, there was no significant group effect \(F(1,28)=1.894; p=0.18\) and no significant group x time interaction \(F(1,28)=4.001; p=0.051\).
Post hoc analyses showed that administration of ifenprodil 30 min before each cocaine administration session significantly reduced the expression of place preference in both Esc-C (p=0.004) and Fix-C (p=0.024) compared to saline-treated animals (Fig 2.2B, C; day 8).

We next investigated whether inhibition of nNOS influences acquisition of Esc-C memory. Pretreatment with the nNOS inhibitor 7-NI blunted the development of place preference for mice conditioned by Fix-C [F(1,26)=4.908; p=0.036; group effect] but not Esc-C (p=0.995) (Fig 2.2D, E), suggesting NO-independent formation of Esc-C memory. There was a significant time effect for both Fix-C [F(1,26)=64.991; p<0.001] and Esc-C [F(1,26)=150.662; p<0.001], however, there was no significant group x time interaction for neither Fix-C nor Esc-C groups (p>0.05).

**Experiment 3: Reconsolidation of Fix-C and Esc-C memory**

Administration of MK-801 before the CPP test (pre memory retrieval) prevented a reduction in place preference for Fix-C [F(1,48)=8.904; p=0.004; group effect] but it had no effect on Esc-C (Fig 2.3B,C). For Fix-C, there was a significant time effect [F(2,48)=48.437; p<0.001] and a significant group x time interaction [F(2,48)=3.246; p=0.048]. For Esc-C, there was a significant time effect [F(2,33)=85.508; p<0.001] but no significant group x time interaction (p>0.05). The results suggest that the first exposure to the CPP context may elicit extinction in the Fix-C but not Esc-C group and MK-801 inhibited the extinction in the Fix-C group.

In the next experiment MK-801 was administered 6 min following the first CPP test (post memory retrieval). A dose of 0.1mg/kg MK-801 attenuated subsequent expression of Fix-C. Two-way ANOVA showed a significant time effect [F(2,39)=43.151;
p<0.001] but no significant group effect nor group x time interaction (p>0.05). Post hoc analysis showed that on day 12, MK-801-treated animals showed reduced CPP compared to saline-treated mice (p=0.012), suggesting that MK-801 disrupted memory reconsolidation. With respect to Esc-C, two-way ANOVA comparing the dose effect of MK-801 on disruption of reconsolidation showed a significant group (MK-801 dose) effect [F(2,66)=5.747; p=0.005], a significant time effect [F(2,66)=83.735; p<0.001] but no significant group x time interaction. Results suggest that while a low dose (0.1mg/kg) MK-801 was sufficient to disrupt Fix-C memory reconsolidation, a higher dose of MK-801 (0.3mg/kg) was required for disruption of Esc-C memory reconsolidation (Fig. 2.3E; day 12).

In all subsequent reconsolidation experiments drugs were administered following a 6 min CPP test (post memory retrieval). For both Fix-C and Esc-C, the 6 min exposure to the context was sufficient to express the acquisition of CPP (Pre-CPP vs Retrieval; p<0.001; Fig 2.4). Ifenprodil (group effect) significantly disrupted reconsolidation of both Fix-C and Esc-C memory [for Fix-C:F(1,48)=4.123; p=0.048; for Esc-C:F(1,39)=11.832; p=0.001; Fig 4B, C]. There was a significant time effect [for Fix-C:F(2,48)=31.171; p<0.001; for Esc-C:F(2,39)=25.697; p<0.001] but no significant group x time interaction. Administration of ifenprodil either in the home cage in the absence of memory retrieval or 6h after memory retrieval had no effect on Esc-C CPP (Fig 4D), suggesting that ifenprodil disrupted memory reconsolidation. To further validate the involvement of NR2B in Fix-C and Esc-C memory reconsolidation, the effects of traxoprodil was investigated. Traxoprodil is another NR2B receptor antagonist which has previously been tested in clinical trials for the treatment of depression (Preskorn et al.,
dyskinesia and Parkinsonism (Nutt et al., 2008). Administration of traxoprodil following memory retrieval significantly reduced subsequent expression of CPP in both Fix-C and Esc-C (Fig 2.4E, F). Two-way ANOVA showed a significant group effect for Esc-C \[F(1,42)=9.466; p=0.004\] but not Fix-C \[F(1,42)=2.555; p=0.117\]. There was a significant time effect for both Esc-C \[F(2,42)=75.191; p<0.001\] and Fix-C \[F(2,42)=35.280; p<0.001\] but no significant group x time interaction in either case. Post hoc analysis showed that traxoprodil-treated mice displayed significantly lower CPP scores than vehicle-treated mice for both Esc-C \(p<0.001\) and Fix-C \(p=0.005\) on day 12.

We then sought to isolate the contribution of different signaling molecules downstream of the NMDAR in cocaine-memory reconsolidation. Post-retrieval administration of the nNOS inhibitor 7-NI reduced the magnitude of subsequent CPP that was acquired by Fix-C but not Esc-C schedule (Fig. 2.5B, C), suggesting disruption of Fix-C \[F(1,45)=6.558; p=0.014\] but not Esc-C \[F(1,48)=0.0166; p=0.898\] memory reconsolidation. There was a significant time effect for Fix-C \[F(2,45)=42.089; p<0.001\] and Esc-C \[F(1,48)=29.907; p<0.001\] but no significant group x time interaction. The disruption of Fix-C memory reconsolidation by 7-NI is consistent with our previous studies (Itzhak & Anderson, 2007).

We then investigated the involvement of ERK in reconsolidation of Fix-C and Esc-C memory. The ERK kinase (MEK) inhibitor SL327 disrupted Esc-C but not Fix-C memory reconsolidation (Fig 2.5D, E). Two-way ANOVA showed no significant group effect for Esc-C \[F(1,36)=3.904; p=0.056\] nor Fix-C \[F(1,51)=0.873; p=0.354\]. However, there was a significant time effect \{for Esc-C: \(F(2,36)=49.884; p<0.001\); for Fix-C: \(F(2,51)=63.774; p<0.001\)\} and a significant group x time interaction for Esc-C
[F(2,36)=3.727; p=0.034] but not for Fix-C [F(2,51)=0.344; p=0.71]. Post hoc analysis found a significant reduction in CPP on day 12 for Esc-C (p=0.014) but not Fix-C (p>0.05).

Taken together, these results suggest that Fix-C memory engages NO signaling while Esc-C memory may bypass the dependence on NO and engages the NMDAR-dependent ERK signaling pathway.

Discussion

Previous studies have indicated the significance of variations in the daily schedule of cocaine within the conditioning phase, rather than the dose of cocaine, on the development of drug-associated memory (Itzhak & Anderson, 2012; Conrad et al., 2013). While conditioning by fixed daily doses of cocaine resulted in relatively low magnitude of place preference and rapid extinction, conditioning by escalating doses of cocaine resulted in higher magnitude of place preference, and resistance to extinction by re-exposure to nonreinforced context (Itzhak & Anderson, 2012; Liddie et al., 2012). Given these observations, the present study was undertaken to investigate whether Fix-C and Esc-C memory engaged different neural pathways in the formation and reconsolidation of cocaine-associated memory.

Since we were interested in the effect of cocaine-context-associated learning following Fix-C and Esc-C schedules, we focused on the expression levels of NMDAR subunits. The NMDAR is thought to mediate synaptic plasticity and learning and memory (Collingridge, 1987) and different subunits are coupled to specific downstream signaling molecules (Chen et al., 2007). Therefore, the investigation of NMDAR subunits expression could elucidate differences between Fix-C and Esc-C memory (Itzhak & Anderson, 2012). We found that conditioning by Esc-C results in increased hippocampal
expression of both Grin2b mRNA and NR2B protein, suggesting an induction of both transcription and translation of NR2B subunits of the NMDAR. However, in the Fix-C group we did not detect an increase in Grin2b mRNA and only an increase in NR2B protein was observed. The lack of increase in Grin2b mRNA in the Fix-C group is unclear but studies have shown varied correlation between mRNA and protein levels; a result of biological factors which influence transcription and translation processes (Nie et al., 2006). Other studies have shown NR2B protein up-regulation in rat nucleus accumbens following repeated cocaine administration (Huang et al., 2009) and in rat hippocampus following morphine-induced CPP (Ma et al., 2006). Interestingly, however, we observed that the increase in expression levels of NR2B in mice conditioned by the Esc-C schedule was higher than in mice conditioned by the Fix-C schedule. This finding may be relevant to the strength of Esc-C memory compared to Fix-C memory.

Evidence suggests that the NR2B subunit of NMDAR has potential to carry greater calcium current per unit charge (Sobcyk et al., 2005) which may confer a greater influence on downstream signaling cascades that affect synaptic plasticity and learning and memory. For example, rats over-expressing NR2B in the cortex and hippocampus showed improved performance in a number of learning and memory tasks (Wang et al., 2009), while pharmacological or genetic blockade of the NR2B subunit in the cingulate cortex of mice impaired the formation of contextual fear memory (Zhao et al., 2005). Additionally, recent reports suggest that repeated cocaine administration generates silent synapses (Huang et al., 2009). Silent synapses contain higher levels of NR2B-containing NMDARs compared to neighboring synapses and are capable of undergoing rapid metaplasticity to strengthen synapses (Lee et al., 2010). Hence, it is plausible that the
increased expression of NR2B in the present study may have contributed to the
development of a more ‘stable’ Esc-C memory compared to Fix-C. A trend toward a
reduction in levels of NR2A (Fig 2.1D) may indicate a switch in NMDAR subunit
composition in Esc-C mice where NR2B replaces NR2A.

Because of the changes in expression of NR2B, we investigated whether selective
antagonism of NR2B-containing NMDAR could disrupt cocaine-associated memory. The
NR2B antagonist ifenprodil a) attenuated acquisition and b) reduced subsequent place
preference expression when administered following memory retrieval in both Fix-C and
Esc-C groups. The former result is in accordance with others who showed that ifenprodil
prevented the development of cocaine (Kiraly *et al.*, 2011) and morphine (Ma *et al.*, 2006) CPP following fixed administration schedules. The latter result was likely due to
disruption of memory reconsolidation since administration of ifenprodil either a)
following retrieval but outside the reconsolidation window or b) in the absence of
memory retrieval, had no effect on subsequent CPP expression (Fig. 2.4D). The absence
of place preference in the acquisition and reconsolidation experiments was not due to an
aversive influence of ifenprodil since a dose of 10mg/kg ifenprodil is neither rewarding
nor aversive (Suzuki *et al.*, 1999). While we cannot completely rule out extinction
learning, it is feasible to assume that ifenprodil disrupted memory reconsolidation since
we have previously shown that mice conditioned by Esc-C do not exhibit extinction with
few unreinforced exposures to the CPP context (Itzhak & Anderson, 2012). We
corroborated our findings by demonstrating that another NR2B antagonist traxoprodil
was similarly effective at disrupting the reconsolidation of both Fix-C and Esc-C
memory. Taken together, we demonstrate that NR2B-containing NMDARs play a
behaviorally significant role in the development and reconsolidation of cocaine-associated memory independent of the schedule of conditioning.

However, the NMDAR antagonist MK-801 had differential effects on reconsolidation of Fix-C and Esc-C memory and it appears that the dependence of NMDAR in the process of memory reconsolidation is temporally mediated. First, post-retrieval administration of low dose MK-801 (0.1mg/kg) disrupted Fix-C memory reconsolidation while a higher dose (0.3mg/kg) of the NMDAR antagonist was required to disrupt Esc-C memory reconsolidation. The increased expression of NR2B subunit in the Esc-C group, relative to the Fix-C group, may be associated with facilitated calcium entry (Sobcyk et al., 2005) and therefore a higher dose of the NMDAR antagonist was required to inhibit downstream signaling molecules involved in memory reconsolidation. Second, pre-retrieval administration of MK-801 a) prevented extinction of Fix-C CPP since saline-treated mice showed reduced place preference while CPP was maintained in MK-801-treated mice and b) had no effect on Esc-C CPP (Fig. 3). This finding implies that the Fix-C group, but not the Esc-C group, was undergoing extinction learning (reduction in preference for the cocaine-paired compartment), which was prevented by MK-801. This premise is support by our previous observations that Fix-C memory is more susceptible to extinction compared to Esc-C memory (Itzhak & Anderson, 2012). Similar to the current observations, a) post-retrieval antagonism of the NMDAR disrupted reconsolidation of object recognition (Akirav & Mamoun, 2006) and odor-reward memory (Torras-Garcia et al., 2005) and b) pre-retrieval antagonism of NMDAR prevented extinction of conditioned freezing response (Ben Mamou et al., 2006). Other studies demonstrated that pre- but not post-retrieval systemic administration of MK-801
prevented memory reconsolidation in a conditioned reinforcement behavioral task (Milton et al., 2008). This discrepancy in temporal-dependence of MK-801 may be due to differences in behavioral paradigms which investigate instrumental (self-administration) versus non-operant memory tasks.

Further differences between Fix-C and Esc-C memory are associated with NMDAR downstream signaling molecules. Since the activity of nNOS is coupled to calcium influx through the NMDAR (Christopherson et al., 1999; Sattler et al., 1999) we investigated whether the observed differences in behavioral phenotype between Fix-C and Esc-C was a function of nNOS involvement. First, the nNOS inhibitor 7-NI prevented the formation of Fix-C memory but not Esc-C memory (Fig 2). Second, reconsolidation of Fix-C but not Esc-C memory was disrupted by the nNOS inhibitor. The latter confirm our previous studies on the dependency of Fix-C memory on NO signaling (Itzhak & Anderson, 2007) but reveals now a NO-independent signaling mechanism for Esc-C memory. This differential effect of nNOS inhibition may be due to the recruitment of additional signaling pathways in response to the more salient conditioning schedule (Esc-C). Hence, although nNOS could potentially be activated in response to training by Esc-C, the activation of other signaling pathways may overshadow the involvement of nNOS.

Calcium influx through NMDARs has the potential to activate several calcium-dependent signaling pathways; for instance, the NMDAR-RasGRF1-MEK-ERK pathway. ERK is an important regulator of neuronal plasticity, long-term potentiation (LTP) and LTM formation (Krapivinsky et al., 2003). Since RasGRF1 specifically binds the NR2B subunit of the NMDAR, it couples the activity of ERK with NR2B-containing
NMDARs (Krapvinisky et al., 2003). Studies have shown that associative learning leads to an up-regulation of ERK in the hippocampus and inhibition of the ERK kinase MEK, disrupted the formation of fear memory (Atkins et al., 1998). Furthermore, it has been suggested that NR2A- and NR2B-containing NMDARs are coupled to different signaling pathways; activation of NR2A increased brain derived neurotrophic factor (BDNF) expression while activation of NR2B-containing NMDARs led to phosphorylation of ERK (Chen et al., 2007).

Because the CPP paradigm involves associative learning and we observed differential expression of NR2B subunits, we investigated whether reduced ERK activation via MEK inhibition would disrupt memory reconsolidation of Fix-C and Esc-C. We found that MEK inhibition had differential effects on Fix-C and Esc-C memory where reconsolidation of Esc-C but not Fix-C memory was disrupted by SL327. Because NMDAR-dependent ERK phosphorylation is coupled to NR2B, inhibition of MEK is expected to have a greater effect on NMDAR-dependent ERK signaling where levels of NR2B are substantially elevated; that is, in Esc-C group. The modest increase in NR2B in the Fix-C group may have been sufficient to allow sensitivity to ifenprodil treatment but insufficient to promote a robust NMDAR-dependent activation of ERK. Contrary to our findings where MEK inhibition had no effect on Fix-C memory reconsolidation, bilateral injections of U0126, another MEK inhibitor, into nucleus accumbens core disrupted expression and reconsolidation of Fix-C CPP (Miller & Marshall, 2005). This apparent discrepancy may be due to differences in the route of inhibitor administration (intracerebral vs intraperitoneal) and duration of conditioning (9 days vs 4 days). Others have reported that systemic administration of SL327 in a fixed-dose cocaine CPP
paradigm effectively disrupted memory reconsolidation only when memory retrieval occurred by pairing the context with cocaine (Valjent et al., 2006). This finding suggests that the conditioned stimulus alone (CPP context) was insufficient to engage MEK signaling in Fix-C but instead it required the much stronger unconditioned stimulus (cocaine). Thus in the present study, SL327 may have been ineffective at disrupting Fix-C memory reconsolidation because subjects were exposed to the CPP context alone. However, the MEK inhibitor was effective in disrupting reconsolidation of Esc-C memory. This finding suggests that context re-exposure of Esc-C (but not Fix-C) mice was sufficient to invoke ‘strong memory’ of drug-context association which engaged MEK-signaling.

While the nNOS signaling pathway may also be activated in response to training by Esc-C, it appears that other signaling pathways including NMDAR-MEK-ERK signaling plays a more behaviorally significant role in the development of Esc-C CPP. Additionally, though both NO-cGMP-PKG and MEK signaling pathways converge at the level of ERK (Ota et al., 2008) we posit that the contribution of each pathway to drug memory is dependent on cocaine conditioning schedule.

We focused on identifying molecular changes in the hippocampus because of its role in contextual memory. While our results lend credence to the involvement of the hippocampus with respect to changes in NR2B receptor subunit expression, downstream NMDAR signaling molecules in other brain such as nucleus accumbens, amygdala and prefrontal cortex may also be involved in the behavioral effects we observed.

In summary, we show that the salience of cocaine reward influences memory strength by engaging different neural pathways. The NR2B subunit of NMDARs and
MEK-associated signaling appears to have a major role in drug memory acquired by escalating dose of cocaine, while NMDAR and NO-associated signaling appear to be involved in drug memory encoded by the Fix-C schedule. Given that drug addiction is associated with escalation in drug use, we posit that different schedules of drug exposure may result in differential strength in drug memory which could be relevant to the severity of addiction. Our data opens the door to further investigations into the effects of drug memory strength and its differential susceptibility to pharmacological manipulation.
Figure 2.1. The NR2B subunit of NMDA receptor contributes to the development of Esc-C memory (A) Mice that received cocaine paired to the training context develops CPP. Data are represented as mean ± SEM of difference in time (sec) spent on cocaine- vs. saline-paired compartment. (*p<0.001), difference between Pre-CPP and Post-CPP (CPP Test); (**p<0.05) difference between the paired groups of Fix-C and Esc-C. For Fix-C: saline (n=13); unpaired (n=4); paired (n=10). For Esc-C: saline (n=13); unpaired (n=8); paired (n=9). Panel B pertains to paired and unpaired groups while panels C, D and E pertain only to paired groups. (B) RT-qPCR analysis showed that Grin2b mRNA (which codes for NR2B protein) was significantly up-regulated in Esc-C compared to all other groups. (C) Mice conditioned by Esc-C had significantly elevated levels of NR2B protein compared to Fix-C and saline-treated mice. (D) Expression of NR1 protein was unchanged across conditioning groups. (E) Changes in NR2A subunit expression were not statistically significant. *p<0.05 and **p<0.01. For western blots n=3-4/group and for qPCR n=4/group.
Figure 2.2. Acquisition of cocaine memory: antagonism of NR2B-containing NMDA receptors attenuates the acquisition of both Fix-C and Esc-C memory while nNOS inhibition attenuates Fix-C but not Esc-C memory acquisition. (A) Schematic of experimental procedure. (B, C) The NR2B-containing NMDAR antagonist ifenprodil (10mg/kg) significantly reduces the development of place preference following conditioning by Fix-C and Esc-C. (D, E) The nNOS inhibitor 7-NI (25mg/kg) significantly reduces the development of CPP following conditioning by Fix-C but not Esc-C schedule. Data are presented as mean ± SEM of difference in time spent on cocaine- vs saline-paired compartment (sec). (n=6-10/group; *p<0.05).
Figure 2.3. Reconsolidation of cocaine memory: temporal- and dose-dependence of NMDAR channel blockade. (A) Schematic of experimental procedure. (B) Administration of MK-801 prior to memory retrieval inhibits extinction learning in mice conditioned by Fix-C. (C) Pre-retrieval administration of MK-801 had no effect on Esc-C memory. (D) Post-retrieval administration of low dose MK-801 (0.1mg/kg) disrupts Fix-C memory reconsolidation. (E) Post-retrieval administration of high (0.3mg/kg; n=10) but not low (0.1mg/kg; n=7) dose of MK-801 disrupts subsequent expression of CPP in the Esc-C group compared to saline (n=8). Data are presented as mean ± SEM of difference in time spent on cocaine-vs saline-paired compartment (sec). *p<0.05.
Figure 2.4. Reconsolidation of cocaine memory: antagonism of NR2B-containing NMDARs disrupts Fix-C and Esc-C memory reconsolidation. (A) Schematic of experimental procedure. (B, C) Immediate post-retrieval administration of ifenprodil (10mg/kg) significantly attenuates Fix-C and Esc-C place preference expression. (D) Administration of ifenprodil (10mg/kg) to Esc-C conditioned mice either in the home cage or 6 hr after memory retrieval (outside reconsolidation window) did not reduce subsequent CPP expression. (E, F) The NR2B-containing NMDA receptors antagonist traxoprodil (10mg/kg) disrupts both Fix-C and Esc-C memory reconsolidation. Data are presented as mean ± SEM of difference in time spent on cocaine-vs saline-paired compartment. *p<0.05.
Figure 2.5. Effect of inhibition of NMDAR downstream signaling on reconsolidation of cocaine memory. Fix-C memory reconsolidation is NO-dependent but MEK-independent while Esc-C memory reconsolidation is MEK-dependent and NO-independent. (A) Schematic of experimental procedure. (B, C) Administration of the nNOS inhibitor 7-NI (25mg/kg) immediately following memory retrieval attenuates subsequent place preference acquired by Fix-C but not Esc-C schedule. (D, E) Post-retrieval administration of the MEK inhibitor SL327 (30mg/kg) attenuates subsequent place preference acquired by Esc-C but not Fix-C schedule. Data are presented as mean ± SEM of difference in time spent on cocaine-vs saline-paired compartment. *p<0.05.
Chapter 3

The effect of phosphodiesterase inhibitors on the extinction of cocaine-induced conditioned place preference in mice

Summary

Several phosphodiesterase inhibitors (PDEi) improve cognition, suggesting that an increase in brain cAMP and cGMP facilitates learning and memory. Since extinction of drug seeking behavior requires associative learning, consolidation and formation of new memory, the present study investigated the efficacy of three different PDEi in extinction of cocaine-induced conditioned place preference (CPP) in B6129S mice. Mice were conditioned by escalating doses of cocaine which was resistant to extinction by free exploration. Immediately following each extinction session mice received a) saline/vehicle, b) rolipram (PDE4 inhibitor), c) BAY-73-6691 (PDE9 inhibitor) or d) papaverine (PDE10A inhibitor). Mice that received saline/vehicle during extinction training showed no reduction in CPP for >10 days. BAY-73-6691 a) dose-dependently increased cGMP in hippocampus and amygdala b) significantly facilitated extinction and c) diminished the reinstatement of cocaine CPP. Rolipram, which selectively increased brain cAMP levels, and papaverine which caused increases in both cAMP and cGMP levels, had no significant effect on extinction of cocaine CPP. Results suggest that increase in hippocampal and amygdalar cGMP levels via blockade of PDE9 has a prominent role in the consolidation of extinction learning.

Background

The administration of a drug that changes the affective state of the organism in a specific context triggers an associative learning process and the formation of long-term memory (LTM). The expression of conditioned place preference (CPP) is viewed as a test
for reactivity to drug-associated conditioned stimulus (CS); this test has validity for cue-reactivity in human drug users. The CPP paradigm has also been used to investigate extinction of “drug-seeking behavior” and reinstatement of conditioned response (Aguilar et al., 2009; Itzhak & Martin, 2002; Parker & McDonald, 2000; Mueller & Stewart, 2000; Mueller et al., 2002). Interestingly, reinstatement of cocaine CPP following extinction is a drug-specific phenomenon that can be triggered only by drugs that share a similar mechanism of action with that of cocaine (Itzhak & Martin, 2002). Therefore, the reinstatement of place preference, like the reinstatement of drug self-administration presents a meaningful resource for the study of relapse. Animal and human studies suggest that re-exposure to a low dose of psychostimulants, opiates or alcohol, following abstinence or extinction of drug use, may cause relapse (Shaham et al., 2003). It is therefore critical to develop pharmacotherapies and behavioral practices by which extinction of drug seeking behavior will ultimately result in resistance to both drug-associated cues and drug-priming.

Extinction learning by “exposure therapy” is thought to be essential for the management of drug addiction (Carter & Tiffany, 1999; Powell et al., 1993; Siegel & Ramos, 2002). Extinction typically requires long or multiple re-exposures to a CS (Nader, 2003; Power et al., 2006). Results from fear conditioning studies suggest that the extinction process does not eliminate or cause ‘unlearning’ of the initial conditioned response; rather, the organism learns that the CS no longer elicits the previous stimulus (Bouton, 2002; 2004; Havermans & Jansens, 2003). Thus, extinction requires associative learning, consolidation and formation of new memory (Milad & Quirk, 2002; Santini et al., 2001).
Through the activation of their respective kinases (PKA and PKG), cyclic nucleotide (cAMP and cGMP) signaling pathways are important regulators of neural function and synaptic homeostasis (Bales et al., 2010). While adenylyl cyclase (AC) and guanylyl cyclase (GC) generate the second messengers cAMP and cGMP respectively, phosphodiesterases (PDEs) hydrolyze these cyclic nucleotides into their inactive monophosphates, 5’-AMP and 5’-GMP, and thereby contribute to the regulation of their intracellular levels (Essayan, 2001). Eleven different families of mammalian PDE’s have been identified in the CNS and periphery. All neurons express multiple PDEs which differ in cyclic nucleotide specificity, affinity, regulatory control and subcellular distribution (Bender & Beavo, 2006; Blokland et al., 2006; Boswell-Smith et al., 2006; Menniti et al., 2006).

The differential localization of PDEs in the CNS and periphery determine how effective phosphodiesterase inhibitors (PDEi) are at regulating different processes. Brain PDEs include PDE1, PDE2, PDE4, PDE5, PDE9, PDE10 and PDE11. PDE4 is widely distributed throughout the brain (Bender & Beavo, 2006). The PDE4 inhibitor rolipram has been used as an anti-inflammatory and has shown antipsychotic-like therapeutic effects (Kelly et al., 2007). Rolipram enhances learning and memory in various paradigms (Cheng et al., 2010; Monti et al., 2006; Rose et al., 2005; Tully et al., 2003; Zhang & O’Donnell, 2000), but unexpectedly it disturbed expression and extinction of conditioned fear in mice (Mueller et al., 2010). PDE9 is highly localized in all sub-areas of the hippocampus (van Staveren et al., 2002; 2004; Reyes-Irisarri et al., 2007) and the specific PDE9 inhibitor BAY-73-6691 improves learning and memory in rodents (van der Staay et al., 2008). PDE10A is densely localized in the striatum but less in the
hippocampus (Seeger et al., 2003). Papaverine is a specific inhibitor of PDE10A that increased levels of cAMP and cGMP (Siuciak et al., 2006) and improved phencyclidine-induced cognitive deficits in rats (Rodefer et al., 2005).

The use of selective PDEi as potential cognitive enhancers is suggested by studies in which PDEi facilitated learning and memory in animal models with experimentally induced learning and memory deficits (Bender & Beavo, 2006; Blokland et al., 2006; Boswell-Smith et al., 2006; Menniti et al., 2006). However, it is unclear whether selective PDEi facilitate learning and memory in subjects with no cognitive impairments. Therefore, the effects of the PDEi on extinction learning may be different than their effects on improving learning following cognitive deficits.

The hippocampus and amygdala are implicated in spatial/contextual and emotional/cued memory, respectively. We hypothesized that increases in hippocampal and amygdalar cyclic nucleotide levels through the action of PDE inhibitors will facilitate extinction learning of cocaine-induced place preference. We sought to investigate PDE inhibitors with different specificities to cAMP and cGMP in order to determine which if any group of PDE inhibitors has a more prominent role in the consolidation of extinction learning. We first determined how cyclic nucleotide levels were affected in the hippocampus and amygdala in response to a PDE4 (cAMP specific) inhibitor rolipram, a PDE9 (cGMP specific) inhibitor BAY-73-6691 and a PDE10A (dual specificity) inhibitor papaverine. Then, the efficacies of these PDE inhibitors to extinguish and prevent reinstatement of cocaine CPP were investigated. We report that of the three PDE inhibitors only BAY-73-6691, which increased hippocampal and amygdalar cGMP
levels, induced the extinction and attenuated the reinstatement of cocaine place preference.

Materials and Methods

Animals

Male B6129S adult mice (8-10 weeks old; weighing 25-35g) were supplied from breeding colonies in our facilities at the University of Miami, Miller School of Medicine, Miami FL as previously described in detail (Balda et al., 2006). Following weaning (postnatal day 21), mice were housed in single-sex groups; males were used for the current study. ‘Litter effect’ was negated by grouping mice from 4-5 different litters into each cage. Animals were housed in a temperature (22 ± 0.5 °C) and humidity (50%) controlled room and maintained on a 12-hour light/dark schedule with food and water ad lib except during training and testing. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and approved by the University of Miami Animal Care and Use Committee.

Measurement of cyclic nucleotide levels

Our studies focused on the effects of the PDEi on cyclic nucleotides levels particularly in hippocampus and amygdala because these brain regions are involved in associative learning (Robbins et al., 2008). Papaverine (Sigma, St. Louis, MO) were dissolved in saline (0.9% NaCl); rolipram (Sigma, St. Louis, MO) was dissolved in 2% DMSO (Sigma, St. Louis, MO); BAY-73-6691 (Sigma, St. Louis, MO) was dissolved in 10% Solutol® HS 15 (BASF, Ludwigshafen, Germany) solution (vehicle). To determine the dose effects of the PDE inhibitors, mice received single intraperitoneal (IP) injections of (a) rolipram (0.05, 0.25, 1mg/kg; n=3/group) (b) BAY-73-6691 (0.03, 0.3 and 3mg/kg;
n=3/group), (c) papaverine (10, 20, 40mg/kg; n=3-4/group) or (d) saline/vehicle (n=3/group). Thirty minutes later, mice were sacrificed by cervical dislocation and brains were immediately removed and placed in ice-cold saline. The hippocampus and amygdala were dissected on an ice-cold surface according to The Mouse Brain atlas (Paxinos & Franklin, 2001), snap-frozen on dry ice and stored at -80°C. Levels of cGMP (sensitivity, 25fmol/mL) and cAMP (sensitivity, 0.39pmol/mL) were quantified with direct EIA kits (Enzo Life Sciences International, Inc., PA) as described in the manufacturer’s protocols and determined by spectrophotometry (OD450nm). The optimal doses of the PDE inhibitors for the behavioral studies were determined from these experiments.

**Place conditioning apparatus**

An Opto-Max Activity Meter (Colombus Instruments, Columbus, Ohio, USA) was used to monitor place preference. The training context consisted of two compartments separated by a removable divider. One compartment had black walls and smooth black floor while the other compartment had white walls with a floor covered with sandpaper (fine grit 150C, Norton; Stephenville, TX) thus providing distinct visual and tactile cues. Each compartment was scanned by seven infrared beams at a rate of 10Hz (2.54 cm intervals). The horizontal sensors were mounted alongside opposing lengths of each compartment. A null zone of 8 cm was assigned at the interface of the two compartments to ensure that only full entry into one compartment or the other was registered as distinct time spent on each side. Time spent in each compartment and locomotor activity were recorded and analyzed by the Opto-max interface and software.
**Conditioning**

Mice were trained and tested in a room separate from the housing room as described previously (Itzhak & Anderson, 2012). The testing room was equipped with a fluorescent lamp strategically positioned to create a dimmed lighting environment. On the first day of each experiment, between 12:00-14:00 hours, mice were habituated for 20 minutes to the training context; time spent in each compartment was recorded. Extremely biased mice (about 10%) were eliminated from the study. About 50% of the remaining animals had slight preference for the black compartment and the other 50% had slight preference for the white compartment. Accordingly, the assignment criterion was such that mice were conditioned by cocaine in their least preferred compartment. Thus, the training procedure was counterbalanced where half the mice were trained with cocaine in the black compartment while the other half was trained with cocaine in the white compartment. Mice were trained with IP injections of saline during the morning (10:00-12:00 hours) session and cocaine during the afternoon (14:00-16:00 hours) session. Each conditioning session lasted 30 minutes.

Mice were conditioned with ascending dosages (3, 6, 12, 24mg/kg) of cocaine over four days (Table 3.1). This regimen of escalating dosage caused a CPP that was of higher magnitude and longer-lasting than the CPP that resulted from a fixed daily dose; this enhanced CPP was also resistant to extinction by free exploration (Itzhak & Anderson, 2012). Hence we hypothesized that investigating extinction in a model of robust conditioning is more significant to the real-life human situation of escalating drug use than the typical model of conditioning (fixed daily dose of cocaine) which affords relatively quick extinction.
Cocaine was administered immediately before the animal was placed into its respective compartment. To maintain a consistent environment for each mouse, the sandpaper was removed and the cages thoroughly cleaned with dilute laboratory-grade detergent followed by water and then dried, following each training session. The locomotor activity in response to the different doses of cocaine was recorded daily.

**CPP Test**

The expression of place preference was tested 3 days after the final training session in order to eliminate possible contributions from residual cocaine administration on the last training session and also to allow the consolidation of long-term memory of drug reward. Similar to habituation, CPP testing was carried out between 12:00 and 14:00 hours.

**Extinction and reinstatement**

Conditioned mice were allowed free exploration for 20 minutes in the training context 24 hours after the CPP test. Since we aimed to facilitate the consolidation of extinction, immediately following each extinction session mice received single IP injections of either a) vehicle (n=5; n=8)/saline (n=5), b) rolipram (0.25mg/kg, n=6), c) BAY-73-6691 (3mg/kg, n=10), or d) Papaverine (20mg/kg, n=6) and were returned to their home cages. Rates of extinction, herein defined as a significant reduction in the magnitude of CPP compared to CPP test, were recorded daily. Mice received a two-day break from extinction training after 4 days. Training resumed the following day and continued for the next 3 days (Table 3.1).

Twenty-four hours after the last extinction session, mice received a priming dose of cocaine (12mg/kg; the average daily dose during conditioning) in the absence of the
PDE inhibitor. Time spent in each compartment as well as locomotor activity was recorded for 20 minutes.

**Data Analysis**

Changes in levels of cyclic nucleotides in response to different doses of a PDE inhibitor were analyzed by one-way ANOVA supplemented by Holm-Sidak method for post-hoc. Results of CPP are presented as mean ± SEM of the difference in time spent in cocaine- and saline-paired compartments. The overall effect on acquisition, extinction and reinstatement of cocaine-induced place preference was analyzed by two-way ANOVA (group X time) general linear model supplemented with post-hoc multiple pairwise comparisons using the Tukey Test. Further, unpaired Student t-test or Mann-Whitney rank sum test where appropriate were used to compare differences in the magnitude of CPP upon cocaine priming versus the final day of extinction training. For all measures, significance was considered from a value of p<0.05.

**Results**

**Effects of PDE inhibitors on cAMP and cGMP levels**

The PDE4 inhibitor rolipram dose dependently increased levels of cAMP in both the hippocampus and amygdala (Figure 3.1A) but had no effect on cGMP levels (data not shown). Rolipram treatment resulted in an overall significant increase in cAMP in both the hippocampus ($F_{3,8}$=18.463; $p<0.001$) and amygdala ($F_{3,8}$=17.824; $P<0.001$). At doses of 0.25 and 1mg/kg rolipram administration resulted in about 2-fold increase in cAMP in the amygdala ($t=6.102; p<0.001$ and $t=6.166; p<0.001$, respectively) and hippocampus ($t=6.072; p<0.001$ and $t=4.858; p<0.001$, respectively) compared to vehicle controls; a dose of 0.05mg/kg rolipram also significantly increased levels of cAMP in the
amygdala (t=2.606; p=0.031). A dose of 0.25mg/kg rolipram was used for the subsequent behavioral experiment. The PDE9 inhibitor BAY-73-6691 dose dependently increased levels of cGMP in both the hippocampus ($F_{[3,8]}=27.052; p<0.001$) and amygdala ($F_{[3,8]}=7.941; p<0.001$) (Fig 3.1C) but had no effect on levels of cAMP (data not shown). A dose of 3mg/kg of BAY-73-6691 was used in the extinction experiments because this dose resulted in the highest increase in levels of cGMP (t=4.208; p=0.003 and t=8.549; p<0.001) for amygdala and hippocampus, respectively. Significant increases in cGMP were also observed in the hippocampus for dosages of 0.03mg/kg (t=3.157; p=0.013) and 0.3mg/kg (t=5.969; p<0.001) and amygdala at 0.3mg/kg (t=3.272; p=0.011). An overall significant effect of papaverine on levels of cAMP was observed in the hippocampus ($F_{[3,8]}=6.531; p=0.015$) but not in the amygdala ($F_{[3,9]}=1.225; p=0.356$). Significant increases in cGMP in both the hippocampus ($F_{[3,10]}=7.613; p=0.006$) and amygdala ($F_{[3,10]}=4.929; p=0.024$) were observed. Holm-Sidak post-hoc analysis showed that the PDE10A inhibitor papaverine at 10, 20 and 40mg/kg significantly increased levels of cAMP in the hippocampus (t=3.997; p=0.004, t=3.640; p=0.007, and t=2.674; p=0.028, respectively) but not in the amygdala (Fig. 3.1B). However, post-hoc comparisons showed that papaverine increased cGMP in both the hippocampus at 10, 20 and 40mg/kg (t=2.984; p=0.014, t=4.286; p=0.002 and t=4.077; p=0.002, respectively) and the amygdala at 40mg/kg (t=3.715; p=0.004) (Fig. 3.1D). A dose of 20mg/kg was chosen for further experiments because a) unpaired Student t-test showed a significant difference between controls and mice that received 20mg/kg papaverine ($t_{[5]}=-9.542; p<0.001$) and b) the high dose of 40mg/kg resulted in ataxia in mice. Differences in control levels of cAMP in Fig. 3.1A compared to Fig. 3.1B may have arisen because two different
vehicles were used, 2% DMSO for rolipram experiment versus saline for papaverine experiment. Similarly, in Fig. 3.1C, the vehicle for BAY-73-6691 was Solutol HS 15 while that for papaverine in Fig. 3.1D was saline.

_Extinction of cocaine-induced place preference_

Mice that received saline or vehicle during extinction training did not show “extinction” (Fig. 3.2). These results are consistent with a previous study showing that conditioning by escalating doses of cocaine resulted in extinction-resistant CPP (Itzhak & Anderson, 2011). Administration of rolipram immediately after each extinction session for a total of 8 days had no effect on the magnitude of place preference compared to the vehicle group (Figure 3.2A). In a separate experiment, prolonged extinction training (12 days) followed by rolipram administration had also no effect (data not shown). Since there was no evidence of extinction in both groups, a test for reinstatement was not performed.

Administration of BAY-73-6691 immediately after each extinction session resulted in significant reduction in the magnitude of CPP compared to controls \(F_{[1,176]}=12.168; p<0.001\), two-way ANOVA) (Fig. 3.2B). Post-hoc comparison by Tukey test showed significant differences between the two groups on days 10 (q=3.307; p=0.019), 11 (q=3.402; p=0.016) and 12 (q=3.932; p=0.005). On day 11, the difference between times spent in drug- and saline-paired compartments was 95±162 seconds (Fig. 3.2B), suggesting extinction of CPP since this was significantly different from CPP test (T=133; p=0.038, Mann-Whitney rank sum test). BAY-73-6691 also attenuated the reinstatement of CPP; the difference between the magnitude of CPP on the final day of extinction (day 11) and the day of cocaine priming (day 12) was not significant (T=90;
p=0.273, Mann-Whitney Rank Sum test). However, the vehicle treated group showed a significant increase in the magnitude of CPP following cocaine priming compared to the final day of extinction ($t_{[14]}=-2.314; p=0.038$) (Fig. 3.2B). This finding suggests that BAY-73-6691 may have provided partial resistance against reinstatement of cocaine CPP. The differences in extinction rate and resistance to reinstatement in mice treated with BAY-73-6691 versus vehicle control mice were not due to differences in locomotor behavior. Results of locomotor activity, which was recorded during each session, showed that during the reinstatement test, ambulatory counts in control and BAY-73-6691 groups were 1560±143 and 1642±139 counts/20min, respectively.

Finally, administration of papaverine immediately after each extinction session had no significant effect on extinction ($F_{[1,99]}=0.539; p>0.05$) (Fig. 3.2C). The magnitude of CPP in the papaverine group fluctuated over time. Although a reduction in CPP was observed on days 8-11 (Fig. 3.2C), the differences between the control and the papaverine group did not reach statistical significance. However, because there was a trend of reduction in the magnitude of CPP in both groups, challenge cocaine (12mg/kg) was given on day 12. A significant increase in CPP was observed in the papaverine treated group (T=98; p<0.003, Mann-Whitney rank sum test) upon cocaine priming. However, the increase in CPP following a priming injection to the control group was not statistically significant (Fig. 3.2C).

**Discussion**

The current study employed an escalating dose schedule for cocaine-induced CPP as opposed to a typical fixed schedule used in other studies. Mice conditioned by ascending doses of cocaine maintain a higher magnitude of CPP and show greater
resistance to extinction compared to mice conditioned by a fixed daily dose of cocaine (Itzhak & Anderson, 2012). Furthermore, an increasing dose schedule more closely resembles human drug use, in which the quantity of drug increases over time. Indeed results depicted in Fig. 3.2 indicate that cocaine CPP was long-lasting in control groups that received saline/vehicle during post-extinction training. Along this vein, a PDEi that successfully extinguishes place preference at its highest degree, and provides resistance to cocaine reinstatement, may be particularly valuable relative to its effect on extinction of a weaker conditioned response that results from conditioning by a fixed dose of cocaine.

The purpose of this study was to determine how different PDEi with differential specificity for elevating levels of cAMP and cGMP influence extinction learning. Our studies focused on the effects of the PDEi on the levels of cyclic nucleotides in the hippocampus and amygdala because these brain regions have a role in spatial and emotional learning, respectively, associated with the CPP paradigm.

Since PDE4 is highly expressed in the hippocampus, investigation of a specific PDE4 inhibitor seemed feasible to study consolidation of extinction. Here we show that although acute injection of rolipram increased levels of cAMP in both the hippocampus and amygdala, it did not influence the extinction of cocaine-induced place preference. Treatment with rolipram during or 30-min prior to but not following cocaine administration attenuated CPP (Thompson et al., 2004) and reduced cocaine-induced behavioral sensitization (Janes et al., 2009). This suggests that rolipram is not effective in reducing cocaine effects if given after drug administration. Therefore, it appears that an increase in cAMP may not facilitate extinction of cocaine CPP. Although the present
study was not focused on determination of compensatory mechanism associated with repeated rolipram administration, it has been reported that two weeks administration of rolipram resulted in a 17-fold increase in PDE4D3 and a down-regulation of PDE4A1 (30%) and PDE4A5 (37%) in rat hippocampus (Dlaboga et al., 2006). The latter may also explain why rolipram did not induce the extinction of place preference.

We next investigated whether selective increases in brain cGMP facilitate extinction learning. BAY-73-6691 is a selective inhibitor of PDE9 and may be a potential therapeutic for Alzheimer’s disease (Bender & Beavo, 2006). PDE9 is one of the most recently discovered PDE families with the highest affinity for cGMP. In rat and mouse brain, high densities of PDE9 were found in regions containing soluble guanyl cyclase (sGC) and neuronal nitric oxide synthase (nNOS) including the hippocampus, amygdala, olfactory bulb, allocortex and basal ganglia (Andreeva et al., 2001; van Staveren et al., 2002). Additionally, BAY-73-6691 has been shown to improve learning and memory in rodents (van der Staay et al., 2008).

In the present study, acute injection of BAY-73-6691 increased levels of cGMP in the hippocampus and amygdala, induced extinction of cocaine-induced place preference and attenuated place preference reinstatement upon cocaine priming. A plausible explanation for BAY-73-6691 accelerating extinction learning is the involvement of cGMP in the NO/sGC/PKG/CREB signaling pathway. It has been reported that elevated cGMP levels in the hippocampus and amygdala, brain regions known to be important for learning and memory, enhances synaptic plasticity through NO which acts both pre- and post-synaptically to promote LTP (Arancio et al., 2001; Kleppisch & Feil, 2009; Lu et al., 1999; Ota et al., 2010; Son et al., 1998).
The PDE5 inhibitor sildenafil (Viagra), which also increases cGMP in the CNS and periphery (Rutten et al., 2005), reversed memory deficits due to nNOS inhibition in rats (Devan et al., 2006). In the present study, BAY-73-6691 was chosen because of its high selectivity for PDE9. Also, PDE9 is distributed in brain regions involved in learning and memory whereas PDE5 is expressed in the cerebellum and to a lesser degree the hippocampus (Menniti et al., 2006).

The Km of PDE9 is in the range of 170nM for cGMP and the Vmax is about 4.9nM/min/µg of recombinant protein which is about twice as fast as the Vmax of PDE4 for cAMP (Fisher et al., 1998). This accelerated rate of cGMP hydrolysis may partially explain why BAY-73-6691 was more successful at facilitating extinction than rolipram. By blocking the catalytic activity of PDE9, the rapid increase in cGMP may have more significant effects on downstream molecules that are involved in memory consolidation (e.g. CREB). The impact of other PDEs that degrade cGMP may be less significant because the pharmacokinetics of PDE9 may overshadow the kinetics of the other PDEs.

After considering PDE inhibitors that specifically increases cAMP (rolipram) or cGMP (BAY-73-6691) we investigated whether a dual specificity PDE inhibitor which elevates levels of both cAMP and cGMP would induce extinction of place preference. Papaverine is a specific inhibitor of PDE10A that increased levels of cAMP and cGMP (Siuciak et al., 2006) and improved phencyclidine-induced cognitive deficits in rats (Rodefer et al., 2005). We found that acute injection of papaverine significantly elevated cGMP levels in both the hippocampus and amygdala, but it only increased levels of cAMP in the hippocampus. However, papaverine had no significant effect on extinction of CPP (Fig. 3.2C).
Studies have shown that PDE10A is densely localized in the striatum but less so in the hippocampus (Seeger et al., 2003), while PDE9 is highly localized in all sub-areas of the hippocampus (van Staveren et al., 2002; 2004; Reyes-Irisarri et al., 2007). Given the role of hippocampus in extinction learning, it is likely that inhibiting PDE9 has a more profound effect on hippocampal functions than inhibiting PDE10A. This was manifested through the behavioral experiment where BAY-73-6691 but not papaverine, facilitated new learning and thereby induced extinction of cocaine-induced place preference. Another possible explanation for the failure of papaverine to induce extinction is that PDE9 metabolizes cGMP at a very high rate. Because of this, while papaverine blocks hydrolysis of cGMP by PDE10A, PDE9 activity may increase and quickly reduce levels of cGMP. In addition, PDE10A has a much higher affinity for cAMP than cGMP (Boswell-Smith et al., 2006; Francis et al., 2010) and it does not hydrolyze cGMP as efficiently as PDE9. This may also explain the finding that blockade of PDE9 (BAY-73-6691) afforded extinction while blockade of PDE10A (papaverine) did not (Fig. 3.2B, C). However, further studies are required to determine the long-term effects of repeated administration of PDEi; this will help to elucidate whether a compensatory action of PDEs could explain differential efficacies of the different PDEi.

In summary, the present study suggests that inhibitor of PDE9 has a prominent role in the consolidation of extinction learning. It also appears that targeting a specific PDE is more critical than targeting any PDE which metabolizes cGMP.
Table 3.1. Schematic presentation of the timeline and different phases of the extinction experiment. On day 1, mice were allowed free access to both compartments to assess pre-conditioning preference. For the next 4 days (Days 2-5) mice were conditioned with saline in one compartment during the morning and with cocaine paired to the other compartment during the afternoon session. Following a 2 days break, mice were tested for the acquisition of place preference (Day 8). For the next 4 days (Days 9-12) mice were allowed free access to both compartments after which they were injected with different PDE inhibitors or vehicle. This continued for another 4 days (Days 15-18) after a 2-day break (Days 13-14). On day 19, mice were injected with cocaine to assess recovery of place preference.

<table>
<thead>
<tr>
<th>Habituation</th>
<th>Conditioning (cocaine)</th>
<th>CPP test</th>
<th>Extinction training (PDE inhibitor)</th>
<th>Break</th>
<th>Extinction training (PDE inhibitor)</th>
<th>Reinstatement (cocaine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Days 2–5</td>
<td>Day 8</td>
<td>Days 9–12</td>
<td>Days 13–14</td>
<td>Days 15–18</td>
<td>Day 19</td>
</tr>
</tbody>
</table>

CPP: conditioned place preference; PDE, phosphodiesterase
Figure 3.1. Effect of different doses of PDE inhibitors on levels of cAMP and cGMP in the hippocampus and amygdala. A. Rolipram (n=3/group) significantly increased levels of cAMP in the hippocampus (F[3,8]=18.463; p<0.001) and amygdala (F[3,8]=17.824; p<0.001). B. BAY-73-6691 (n=3/group) dose dependently increased levels of cGMP in the hippocampus (F[3,8]=27.052; p<0.001) and amygdala (F[3,8]=7.941; p<0.001). C. Papaverine (n=3-4/group) significantly increased levels of cAMP in the hippocampus (F[3,8]=6.531; p=0.015) but not in the amygdala (F[3,9]=1.225; P=0.356). D. Papaverine significantly increased levels of cGMP in the hippocampus (F[3,10]=7.613; p=0.006) and amygdala (F[3,10]=4.929; p=0.024). Data are presented as mean ± SEM. (*p<0.05 compared to control groups, Holm-Sidak method post-hoc).
Figure 3.2. Effect of different PDE inhibitors on extinction of cocaine-induced CPP. The y-axes represent the mean ±SEM of difference in time spent on the drug-paired versus the saline-paired compartment. The x-axis shows the timeline for the extinction experiment. Day 1 represents the CPP test. The test drugs and vehicles were administered from the second day (first extinction training session) and for 7 more days with a 2-day break as illustrated between days 5 and 8. A. Rolipram (n=6) had no significant effect on extinction of cocaine induced place preference. B. BAY-73-6691 (n=10) significantly reduced the magnitude of CPP over time ($F_{[1,176]} = 12.168; p < 0.001$ two-way ANOVA (group X time) general linear model). Post hoc comparisons by Tukey test showed significant differences between the two groups on days 10 (q=3.307; *p=0.019), 11 (q=3.402; *p=0.016) and 12 (q=3.932; *p=0.005). The vehicle-treated group showed a significant increase in place preference upon cocaine priming (t$[14]= -2.314; #p=0.036$) C. There was no significant difference in the magnitude of CPP between the papaverine (n=6) and the saline (n=5) treated groups over time ($F_{[1,99]} = 0.380; p=0.539$, two-way ANOVA). The papaverine treated group showed a significant increase in place preference upon cocaine priming (T=98; #p<0.003, Mann-Whitney rank sum test) compared to day 11 which was the final day of extinction training.
Chapter 4

The pattern of cocaine conditioning differentially influences stress-induced reinstatement of conditioned place preference

Summary

Vulnerability to relapse following prolonged periods of abstinence presents a major challenge to combating drug addiction. It is therefore essential that much attention be placed on understanding the neural mechanisms that sub-serve relapse to drug use. We have previously shown that the acquisition and reconsolidation of cocaine-context memory associated with conditioning by a fixed daily dose (Fix-C) and escalating daily doses (Esc-C) of cocaine engage different signaling pathways downstream of the N-methyl-D-aspartate receptor (NMDAR). The present study was undertaken to elucidate the signaling molecules in stress-induced reinstatement of previously extinguished Fix-C and Esc-C CPP in C57 BL6/J mice. Blockade of NMDAR signaling had differential effects on forced swim-induced reinstatement of Fix-C and Esc-C CPP; MK-801 attenuated the reinstatement of Fix-C but not Esc-C CPP. Likewise, the neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI) attenuated stress-induced reinstatement of Fix-C CPP but it had no effect on Esc-C CPP. The corticotrophin releasing hormone receptor subtype 1 (CRH-R1) antagonist antalarmin also attenuated stress-induced reinstatement of Fix-C but not Esc-C CPP. Antagonism of NR2B-containing NMDARs had no effect on stress-induced reinstatement of neither Fix-C nor Esc-C CPP. The suppressing effects of MK-801, 7-NI and antalarmin were not due to inhibition of plasma stress hormone corticosterone levels. These results suggest differential signaling pathways for stress-induced reinstatement of Fix-C and Esc-C CPP.
where Fix-C CPP is CRH-R1-, NMDAR- and NO-dependent, while stress-induced reinstatement of Esc-C CPP is likely mediated by different signaling molecules.

**Background**

An important target for combating drug addiction is to understand the neurobiological mechanisms that sub-serve relapse to drug use. Drug addiction is thought to usurp the neural mechanisms of learning and memory (Hyman, 2005) and induce long term plasticity as a result of changes in gene expression through chromatin remodeling (McClung & Nestler, 2008). Memory of the rewarding effects of drugs of abuse is stored in the brain even after prolonged periods of abstinence. This memory can resurface when an individual is re-exposed to priming doses of the drug itself, drug-related cues or to certain stressors (Shaham *et al.*, 2003). To date, an effective treatment strategy for preventing relapse to drug use following extended periods of abstinence remains a challenge.

Stress is an unavoidable part of life and it represents a major risk factor for relapse susceptibility in abstaining addicts (Sinha, 2008). As such, understanding the molecular factors that contribute to the re-establishment of drug seeking behavior following exposure to stressful stimuli will aid in the development of effective pharmacological therapies for the treatment of drug addiction. The physiological response to stress involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Harbuz & Lightman, 1992). In response to stressful stimuli, corticotrophin-releasing hormone (CRH) is secreted by the hypothalamus. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary which subsequently induces the release of corticosteroids from the adrenal cortex (Knol, 2011).
Corticosteroids such as glucocorticoids play an important role in metabolism, immune response and behavior (Sapolsky et al., 2000) as well as in regulation of the HPA axis through negative feedback (Dallman, 2005). Along with the HPA axis, the extra-hypothalamic CRH system is also activated in response to stressful stimuli. Indeed, the CRH receptors subtype 1 (CRH-R1) of extra-hypothalamic CRH system has been shown to mediate stress-induced changes in cocaine reward (Goeders, 2002).

The conditioned place preference (CPP) paradigm, which is based on the principles of Pavlovian conditioning, is used to model different features of addictive behavior (Sanchis-Segura & Spanagel, 2006). Traditionally, in animal models of stress-induced reinstatement in self-administration studies, the stressor (e.g., footshock) must be given in the same context as the conditioning drug (Shalev et al., 2000). However, stressors outside the drug-taking environment are able to precipitate relapse in humans (Shiffman, 1982). Studies have shown that stressors such as mild foot-shock and the forced swimming test (FST) are capable of reinstating previously extinguished place preference for a variety of drugs of abuse including cocaine (Wang et al., 2000), morphine (Ma et al., 2007) and alcohol (Bhutada et al., 2012). Additionally, the CRH receptor has been shown to play a critical role in the effects of stress on reinstatement of CPP (McReynolds et al., 2014).

We have previously shown that cocaine-associated memory acquired through different schedules of cocaine administration show differences in the magnitude and persistence of CPP. Mice conditioned by escalating daily doses of cocaine (Esc-C) display higher magnitudes of CPP and were resistant to extinction by non-reinforced exposure to the training context compared to mice conditioned by fixed daily doses of
cocaine (Fix-C) where CPP is readily extinguished (Itzhak & Anderson, 2012).
Furthermore, we found that mice conditioned by Esc-C showed higher levels of NR2B-containing NMDARs compared to Fix-C. Additionally, while antagonism of NR2B-containing NMDARs was equally effective at attenuating the acquisition and disrupting the reconsolidation of both Fix-C and Esc-C memory, inhibition of nNOS signaling was effective against Fix-C but not Esc-C memory (Liddie & Itzhak, 2014, Chapter 2). Given these observations, our goal was to determine whether differences observed in the acquisition and extinction of CPP between Fix-C and Esc-C would also hold true when assessing stress-induced reinstatement. The present study was undertaken to elucidate the contribution of different signaling pathways to the re-establishment of previously extinguished Fix-C and Esc-C CPP evoked by exposure to a stressful stimulus, the forced swim test (FST).

Materials and Methods

Subjects

Male C57BL/6J mice (8-10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in groups of 5/cage with food and water ad-libitum and were acclimatized to the vivarium for one week before experiments began. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and was approved by the University of Miami Animal Care and Use Committee.

Drugs

Cocaine HCl and (+)-MK801 hydrogen maleate (dizocilpine) were dissolved in saline (0.9% NaCl). The NR2B antagonist ifenprodil tartrate salt (Williams, 1993) was
dissolved in distilled water. The CRH-receptor subtype 1 (CRH-R1) antagonist antalarmin (Webster et al., 1996) was dissolved in 15% DMSO, 25% polyethylene glycol and 60% distilled water and the nNOS inhibitor 7-nitroindazole (7-NI) was dissolved in a 1:3:6 mixture of DMSO, polyethylene glycol and distilled water, respectively. All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Drugs and vehicles were administered intraperitoneally (i.p.) in a volume of 0.1ml/10g.

**Conditioned place preference apparatus**

Place preference was monitored using custom-designed Plexiglas cages (Opto-Max Activity Meter v2.16; Columbus Instruments, Columbus, OH) as previously described (Liddie et al., 2012). The training context consists of two compartments separated by an opaque removable divider. One compartment has black and white striped walls and a white floor covered with stainless steel grid; the other compartment has black walls and smooth black floor, thus providing distinct visual and tactile cues. Time spent in each compartment was recorded and analyzed by the Opto-max interface and software.

**Conditioning procedure**

On the first day, mice were habituated (20 min) to the training context; preconditioning compartment-preference/aversion was determined. Half the subjects showed slight preference for one side or the other. Accordingly, mice were paired with cocaine in the less preferred compartment; half were paired with cocaine in the black compartment and the other half were paired with cocaine in the black and white-striped compartment. Following habituation, mice were conditioned by saline in the morning and by cocaine a) 11.25mg/kg X 4 days (Fix-C) or b) 3, 6, 12 and 24mg/kg; one dose per day (Esc-C) in the afternoon. Doses were chosen to ensure that the total amount of cocaine
administered during the 4 days was equal between Fix-C and Esc-C mice. Hence, the Fix-C schedule for each day represents the average dose of the Esc-C schedule. All experiments comprised four training days (days 2-5), two sessions per day (30 min each), separated by 2-3h interval.

**Extinction**

Seventy-two hours after the final cocaine administration, mice were tested for the acquisition of CPP (day 8). Following the acquisition of Fix-C and Esc-C memory, for the next 4 days mice were subjected to extinction sessions to eliminate the acquired cocaine-associated memory. For extinction training, mice were confined to the previously saline-paired compartment during the morning session and to the previously cocaine-paired compartment during the afternoon session following administration of a saline injection. A test for extinction was carried out 72h after the final saline reconditioning session (day 15).

**Stress-induced reinstatement of Fix-C and Esc-C CPP**

Stress-induced reinstatement was assessed using the forced swim test (FST). The FST is a common behavioral paradigm used to model stress-induced relapse to drug use (Kreibich & Blendy, 2004). Twenty-four hours after a test for CPP extinction, mice were exposed to FST. Thirty minutes prior to FST mice were injected with either a) MK-801 (0.3mg/kg), b) ifenprodil (10mg/kg), c) 7-NI (25mg/kg), d) antalarmin (10mg/kg) or e) vehicle. Doses for MK-801, ifenprodil and 7-NI were chosen based on their effectiveness at disrupting reconsolidation of Fix-C and Esc-C memory (Liddie & Itzhak, 2014, Chapter 2). A dose of 10mg/kg antalarmin was chosen because this dose was effective at attenuating swim-induced reinstatement of place preference developed using a fixed dose
schedule of cocaine (McReynolds et al., 2014). For the FST, mice were placed in a beaker (15cm wide x 19 cm deep) filled to 11cm with water (26±1°C) for 6 min. The beaker was filled with enough water to avoid tails touching the bottom. After 4min recovery and drying under a heat lamp in their home cage, CPP was measured for 20min.

Because we found a significant effect of 7-NI on suppressing stress-induced reinstatement, we also investigated the amount of time mice spent floating/immobile in each of five separate FST trials during a period of one month following a single administration of 7-NI or vehicle. Immobility during FST is often interpreted as depression-like behavior. Thus drugs that reduce immobility during FST are considered to possess antidepressant properties (Petit-Demouliere et al., 2005).

### Measurement of corticosterone levels

Plasma corticosterone levels were determined before and after FST. One hundred microliters whole blood was collected from mouse superficial temporal vein. The vein was punctured using a 26G needle and blood was collected in heparin coated capillary tubes. Upon completion, the wound was gently patted with cotton to stop bleeding. Mice were then returned to their home cage. Samples were centrifuged (10min; 2000xg) and plasma corticosterone levels were quantified using a corticosterone enzymeimmunoassay kit (immunodiagnostic systems, Scottsdale AZ). The pre-FST blood was collected at the end of extinction test (Day 15) while the post-FST blood was collected following CPP test on Day 16. Pre- and post-FST blood samples were collected from opposite sides of the face. Since plasma corticosterone levels follow a circadian pattern of secretion (Malisch et al., 2008), blood samples were collected at the same time each day.
**Statistical Analysis**

Data for CPP testing and immobility during forced swim were analyzed using two-way repeated measures ANOVA (group x time) followed by Student-Newman-Keuls post hoc test. Two-way ANOVA (drug treatment x session) was used to compare pre- and post-FST plasma corticosterone levels. One-way repeated measures ANOVA followed by Holm-Sidak post hoc test was used to compare differences in CPP magnitude for no-stress controls. Data analysis was done using Sigma Stat version 3.1 (Systat Software Inc.).

**Results**

**Stress-induced reinstatement of Fix-C and Esc-C CPP**

*a) Effect of MK-801*

A test of place preference following extinction training (day 15) showed that mice displayed a reduction in preference for the previously cocaine-paired compartment (Fig. 4.1A, B). We then investigated the effect of an acute injection of MK-801 on forced swim-induced reinstatement of Fix-C and Esc-C CPP. While vehicle-treated mice subjected to FST showed significant recovery of CPP, administration of MK-801 prior to FST-1 significantly attenuated stress-induced reinstatement in Fix-C but not Esc-C mice (day 16). Additionally, exposure to a subsequent FST trial where mice were not under the influence of MK-801 failed to reinstate CPP to vehicle control levels (day 18) in Fix-C but not Esc-C. Two-way repeated measures ANOVA showed a significant time effect [for Fix-C: $F_{(5,65)}=8.768; p<0.001$; for Esc-C: $F_{(5,65)}=9.959; p<0.001$] but no significant group effect [for Fix-C: $F_{(1,65)}=4.187; p=0.062$; for Esc-C: $F_{(1,65)}=1.169; p=0.299$] and no significant group x time interaction (p>0.05). Post hoc analysis showed that for Fix-C,
the magnitude of place preference in MK-801-treated mice was significantly attenuated compared to vehicle-treated mice on days 16 (p=0.024) and 18 (p=0.016). Importantly, mice not subjected to the FST did not show spontaneous recovery of place preference on days 16 and 18 (Fig. 4.5). One-way repeated measures ANOVA showed a significant time effect (F(5,40)=55.824; p<0.001). Post hoc analysis showed that the magnitude of place preference after conditioning (day 8) was significantly higher compared to all other test days (in all cases p<0.001). Together, these results suggest differential effects of MK-801 on stress-induced reinstatement of Fix-C and Esc-C CPP; MK-801 attenuated reinstatement of Fix-C CPP but had no effect on reinstatement of Esc-C CPP.

b) *Effect of the NR2B antagonist ifenprodil*

Systemic administration of the NR2B-containing NMDAR antagonist ifenprodil had no effect on stress-induced reinstatement of Fix-C and Esc-C CPP (Fig. 4.1C, D). Two-way repeated measures ANOVA showed a significant time effect [for Fix-C: F(5,80)=15.546; p<0.001; for Esc-C: F(5,85)=13.668; p<0.001] but no significant group effects (p>0.05) and no significant group x time interaction (p>0.05). Results suggest that there is no specific requirement for the NR2B subunit of NMDAR in stress-induced reinstatement of Fix-C and Esc-C CPP.

c) *Effect of the nNOS inhibitor 7-NI*

We next investigated whether inhibition of signaling molecules downstream of the NMDAR would attenuate stress-induced reinstatement of Fix-C and Esc-C CPP. The nNOS inhibitor 7-NI disrupted reinstatement of Fix-C but not Esc-C CPP (Fig. 4.2). For Fix-C, two-way repeated measures ANOVA showed a significant overall group effect (F(1,110)=11.597; p=0.003), a significant time effect (F(5,110)=10.851; p<0.001) and a
significant group x time interaction \( (F_{(5,110)}=3.928; \ p=0.003) \). Post hoc analysis showed that 7-NI treated animals displayed significantly lower place preference compared to vehicle-treated animals when subjected to FST on days 16 \( (p<0.001) \) and 18 \( (p=0.005) \). On the other hand, inhibition of nNOS had no effect on stress-induced reinstatement of Esc-C CPP. Two-way repeated measures ANOVA showed there was a significant time effect \( (F_{(5,85)}=18.588; \ p<0.001) \) but no significant group effect \( (p>0.05) \) and no significant group x time interaction \( (p>0.05) \). Results suggest that while Fix-C CPP engages NO-signaling, stress-induced reinstatement of Esc-C CPP is insensitive to manipulation of NO-signaling.

Additionally, 7-NI administration prior to FST did not affect mobility in subsequent FST trials (Fig. 4.3). A two-way repeated measures ANOVA shows a significant time effect \( [F_{(4,44)}=8.789; \ p<0.001] \) but no significant group effect \( [F_{(1,44)}=0.00147; \ p=0.97] \) and no significant group x time interaction \( (p>0.05) \).

\textbf{d) Effect of the CRH-R1 antagonist antalarmin}

To determine the contribution of CRH receptor subtype 1 to stress-induced reinstatement of Fix-C and Esc-C CPP, we administered antalarmin 30 min prior to exposure to FST-1. Antalarmin attenuated stress-induced reinstatement of Fix-C but not Esc-C CPP (Fig. 4.4). With respect to Fix-C, two-way repeated measures ANOVA showed a significant time effect \( [F_{(5,50)}=2.635; \ p=0.034] \) but no significant group effect \( [F_{(1,50)}=3.416; \ p=0.094] \) and no significant group x time effect \( [F_{(5,50)}=1.285; \ p=0.285] \). However, post hoc analysis showed that antalarmin-treated mice showed significantly reduced magnitude of place preference compared to vehicle-treated animals on day 16. There was a non-significant trend toward a reduction in CPP magnitude for antalarmin-
treated animals following the second forced swim test on day 18 (Fig. 4.4A). With respect to Esc-C CPP, two-way repeated measures ANOVA showed a significant time effect \[F(5,65)=29.951; p<0.001\] but no significant group effect \(p>0.05\) and no significant group x time interaction \(p>0.05\). Antalarmin-treated mice showed similar levels of place preference compared to vehicle-treated mice following exposure to two FST trials (days 16 and 18; Fig. 4.4B).

**Plasma corticosterone levels**

Plasma corticosterone levels measured following FST were approximately 7-10 fold higher compared to corticosterone levels prior to exposure to FST (Fig. 4.6). Two-way ANOVA (treatment x session) analysis of levels of corticosterone before and after FST showed there was a significant effect of test session \(F(1,26)=273.242; p<0.001\). However, there was no significant treatment effect as pre-FST administration of antalarmin, MK-801 or 7-NI showed similar levels of plasma corticosterone as vehicle-treated animals \(F(3,26)=0.306; p=0.821\). Additionally, there was no significant treatment x test session effect \(p>0.05\).

**Discussion**

**Stress-induced reinstatement of Fix-C and Esc-C CPP**

Stress is known to precipitate relapse to drug use (Sinha, 2008) but the exact signaling molecules that govern these behavioral responses are unclear. The FST is one stressor that can be used to model stress-induced reinstatement outside the conditioning context in animal models (Kreibich & Blendy, 2004). The present study shows that exposure to FST successfully reinstates previously extinguished Fix-C and Esc-C CPP (Fig. 4.1, 4.2 and 4.4). The reinstatement of CPP was not a spontaneous event since mice
that were re-exposed to the testing context without being subjected to the FST did not show recovery of place preference (Fig. 4.5). Thus, there appear to be a requirement for stress hormones and other signaling molecules working in concert to remind the animal of the associations learned during conditioning and thus promote reinstatement of CPP.

Studies from our laboratory (Itzhak & Anderson, 2012) and others (Conrad et al., 2013) have shown that mice trained by daily increases in the dose of cocaine (Esc-C) produced higher magnitudes and more persistent CPP than mice trained by daily fixed dose of cocaine (Fix-C). These findings suggest that different neural mechanisms may contribute to the development of cocaine-associated memory. Consequently, this study was undertaken to elucidate the contribution of signaling molecules to stress-induced reinstatement of extinguished Fix-C and Esc-C CPP.

Because the NMDAR has a role in synaptic plasticity and the effects of cocaine (Collingridge, 1987; Kelley et al., 2007; Alaghband & Marshall, 2013), we investigated the effects of the non-competitive NMDAR antagonist MK-801 on stress-induced reinstatement of Fix-C and Esc-C CPP. We found that NMDAR antagonism attenuated stress-induced reinstatement of Fix-C but not Esc-C CPP (Fig 4.1A, B). This suggests a more central role for an interaction between stress and the NMDAR in Fix-C CPP but not in Esc-C CPP. With respect to Fix-C CPP, MK-801 may have interfered with the ability of stress to remind the animal of the associations learned during conditioning and as such prevented reinstatement (Ma et al., 2007). Studies have shown that CRH, the primary regulator of the neuroendocrine response to stress, facilitates pre-synaptic glutamate release (Hollrigel et al., 1998). Thus MK-801 may have attenuated stress-induced reinstatement of Fix-C CPP by blocking the stimulating effect of increased synaptic
glutamate. However, because Esc-C memory is more stable and more resistant to pharmacological manipulation (Itzhak & Anderson, 2012; Liddie et al., 2012; Liddie & Itzhak, 2014, Chapter 2) it is likely that a dose of 0.3mg/kg MK-801 was ineffective at preventing stress-induced NMDAR activation and as such could not attenuate reinstatement of Esc-C CPP. Higher doses of MK-801 were not tested because of marked effects on motor behavior. Previous work from our laboratory has shown that higher doses of MK-801 were required to disrupt Esc-C memory reconsolidation when administered post-memory retrieval (Liddie & Itzhak, 2014, Chapter 2). Thus, the conditioning schedule determines the degree to which NMDAR channel blockade influences cocaine-associated contextual memory.

We have previously shown that a) mice conditioned by Esc-C showed marked increases in hippocampal expression levels of the NR2B subunit of the NMDAR compared to mice conditioned by Fix-C and b) NR2B-containing NMDARs contribute significantly to the acquisition and reconsolidation of Fix-C and Esc-C memory (Liddie & Itzhak, 2014, Chapter 2). However, the present study found that there is no specific requirement for NR2B-containing NMDARs in stress-induced reinstatement of Fix-C and Esc-C memory (Fig. 4.1C, D). These results suggest that NR2B-mediated mechanisms underlying the acquisition and reconsolidation of cocaine-associated memory are dissociable from those required for stress-induced reinstatement of CPP. Our results are in accordance with others who found that while ifenprodil attenuated the reinstating effect of morphine priming, it had no effect on stress-induced reinstatement of extinguished morphine CPP (Ma et al., 2007). Taken together, it appears that while the
NMDAR is involved in stress-induced reinstatement, albeit with differential effects on Fix-C and Esc-C CPP, the NR2B subunit is not critical for this response.

Given the disparities in the effect of MK-801 on stress-induced reinstatement of Fix-C and Esc-C CPP (Fig. 4.1), we aimed to elucidate the contribution of signaling molecules downstream of the NMDAR. nNOS is functionally coupled to NMDAR activity (Christopherson *et al.*, 1999; Sattler *et al.*, 1999) and we have previously found differential effects of nNOS inhibition on Fix-C and Esc-C memory acquisition and reconsolidation (Liddie & Itzhak, 2014, Chapter 2). Therefore, we investigated whether nNOS inhibition would have differential effects on stress-induced reinstatement of Fix-C and Esc-C CPP. We found that the nNOS inhibitor 7-NI prevented stress-induced reinstatement for Fix-C but not Esc-C CPP (Fig. 4.2). An additional FST trial carried out 3 weeks later showed that the acute administration of 7-NI prior to FST-1 provided long lasting protection since stress-induced reinstatement was significantly attenuated (data not shown). This suggests that along with acquisition and reconsolidation, stress-induced reinstatement of Fix-C CPP is NO-dependent while Esc-C CPP bypasses the dependence on NO activity and is mediated by alternative signaling molecules. Previous studies suggest that nNOS inhibitors possess anxiolytic and antidepressant properties (Harkin *et al.*, 1999; Yildiz *et al.*, 2000). In the current study, there were no overt behavioral anxiolytic or antidepressant effects of 7-NI with respect to immobility during FST and locomotor activity; a result possibly due to the use of a relatively low dose of 7-NI. However, there is still the possibility that 7-NI’s effect on reinstatement of Fix-C CPP was due to a) antidepressant activity that was not manifested in motor behavior or b) a reduction in the influence of NO at modulating the neuroendocrine response to stress.
(Gulati et al., 2006). Indeed it has been suggested that NO may play a physiological role in regulating the secretion of hypothalamic and pituitary hormones (Kim & Rivier, 1998; Rivier, 1995). Because Esc-C CPP is potentially NO-independent (Liddie & Itzhak, 2014, Chapter 2), it is not surprising that inhibition of nNOS was ineffective at disrupting stress-induced reinstatement of Esc-C CPP. Thus, future experiments should be geared toward identifying molecular factors which contribute to stress-induced reinstatement of Esc-C CPP.

Although the FST is often used to measure depression-like behavior in experimental animals, an alternative interpretation of behavior is that the immobility observed reflects effects of learning and memory, a reflection of learned habituation, rather than despair (West, 1990). This idea is supported by the observation that administration of an amnesic agent (anisomycin) during the first phase of FST increased mobility in the second FST (De Pablo et al., 1989). Thus anisomycin may have interfered with the memory consolidation process rather than attention or activity. Based on the latter interpretation, results demonstrate that 7-NI does not interfere/disrupt memory associated with the FST since mice treated with 7-NI prior to FST-1 show similar immobility as vehicle-treated mice (Fig. 4.3).

We next investigated the role of CRH-R1 in swim-induced reinstatement of Fix-C and Esc-C CPP. The CRH-R1 plays a role in mediating stress responsivity (Smith et al., 1998); thus highlighting an anxiogenic role for this receptor. Additionally, oral administration of the CRH-R1 antagonist antalarmin has been shown to attenuate behavioral, neuroendocrine, and autonomic responses to stress in primates (Habib et al., 2000). We found that pretreatment with antalarmin attenuated stress-induced
reinstatement in Fix-C but not Esc-C CPP (Fig. 4.4). The effect of antalarmin on Fix-C CPP may be due to its anxiolytic nature which may have attenuated the influence of stress on reinstatement of extinguished Fix-C CPP. Our finding that CRH-R1 is involved in stress-induced reinstatement of Fix-C CPP is in accordance with others who showed that systemic administration of antalarmin blocked swim-induced reinstatement of CPP acquired by a 4 day 15mg/kg fixed cocaine administration schedule (McReynolds et al., 2014). The observation that antalarmin had no effect on Esc-C CPP is unclear but it suggests that while stress-induced reinstatement of Fix-C CPP is mediated by CRH-R1, Esc-C CPP engages additional or distinct molecular factors that contribute to the re-establishment of the extinguished cocaine-associated memory. As such further research is necessary to elucidate the disparate contribution of CRH-R1 in stress-induced reinstatement of Fix-C and Esc-C CPP.

**Corticosterone levels and stress-induced reinstatement**

One possible explanation for attenuation of stress-induced reinstatement of Fix-C CPP is that pre-treatment with MK-801, 7-NI or antalarmin may have suppressed HPA activity and thereby prevented activation of the stress response. This explanation is unlikely since we found that none of the abovementioned pharmacological modulators attenuated swim-induced increases in corticosterone levels indicating an activation of the HPA axis comparable to vehicle-treated animals (Fig. 4.6). The fact that antalarmin did not block corticosterone increase is somewhat surprising considering CRH activation is upstream of glucocorticoid release. However, conflicting lines of evidence have shown that acute administration of antalarmin both blocks (Kreibich et al., 2009) and has no effect (Deak et al., 1999) on plasma corticosterone levels following exposure to stressful
stimuli. The discrepancy between our work and that of Kreibich and colleagues (2009) may result from a) differences in mouse strain used based on innate variations in response to stress and b) plasma corticosterone assay kits which may have inherent procedural variations for detecting corticosterone levels. Taken together, although MK-801, 7-NI and antalarmin had no effect on HPA axis hormones they still attenuated stress-induced reinstatement of Fix-C CPP suggesting a greater influence of these pharmacological modulators at extrahypothalamic brain targets. Indeed, it has been shown that the activity of CRH at extrahypothalamic targets, but not on the HPA axis, mediate stress-induced relapse in rats (Erb et al., 1998).

In summary, we show that the pattern of cocaine administration during conditioning engages different signaling molecules that differentially contribute to stress-induced reinstatement of place preference. Stress-induced reinstatement of Fix-C CPP is NMDAR-NO-CRH-R1-dependent while stress-induced reinstatement of Esc-C CPP is likely dependent on other signaling molecules. Since drug addiction is associated with escalation in drug use, future studies should be aimed at identifying an effective pharmacotherapeutic that attenuates stress-induced reinstatement of Esc-C CPP as this will be of valuable utility for the management of relapse. The behavioral differences between Fix-C and Esc-C CPP in response to pharmacological manipulations may be relevant to a) the severity of addiction and b) the inconsistencies in treatment outcomes in human drug-users.
Figure 4.1. The NMDAR antagonist MK-801 attenuates stress-induced reinstatement of Fix-C but not Esc-C CPP whereas the NR2B antagonist ifenprodil has no effect on stress-induced reinstatement of Fix-C and Esc-C CPP. A and B: Following extinction (Day 15) of previously acquired place preference (Day 8), an acute injection of the noncompetitive NMDAR antagonist MK-801 attenuated the reinstatement of Fix-C but not Esc-C CPP in response to two FST trials (Days 16 and 18). When not exposed to a stressful stimulus, the magnitude of place preference was significantly diminished (Day 17). C and D: Stress-induced reinstatement of Fix-C and Esc-C CPP was not attenuated by selective antagonism of NR2B-containing NMDARs. Data are presented as mean ± SEM of difference in time spent on the cocaine paired side versus the saline-paired saline. *p<0.05. Arrow indicates time of injection of test drug or vehicle control solution.
Abbreviations: CPP, conditioned place preference; Ext, extinction; FST, forced swim test; Test, free exploration; 7-NI, nNOS inhibitor 7-nitroindazole; Veh, vehicle solution.

Figure 4.2. Inhibition of nNOS signaling disrupts stress-induced reinstatement of Fix-C but not Esc-C CPP. A: An acute injection of 7-NI administered prior to FST-1 attenuated stress-induced reinstatement of Fix-C CPP tested on days 16 and 18. B: 7-NI administration had no effect on stress-induced reinstatement of Esc-C CPP. Data are presented as mean ± SEM of difference in time spent on the cocaine paired side versus the saline-paired saline. *p<0.05. Arrow indicates time of injection of test drug or vehicle control solution.
Figure 4.3. The nNOS inhibitor 7-NI does not interfere with mobility during the FST. Data are presented as mean ± SEM % of time spent immobile in the water. There was an overall significant increase in the time spent floating over time. However, there was no significant difference between 7-NI and vehicle treated groups. Arrow indicates time of injection of 7-NI or vehicle. *p<0.05; n=8/group.
Figure 4.4. The CRH-R1 antagonist antalarmin attenuates stress-induced reinstatement of Fix-C but not Esc-C CPP. A: An acute systemic injection of antalarmin 30 min prior to FST-1 (day 16), significantly diminishes forced swim-induced re-establishment of Fix-C CPP. There was a non significant trend toward a reduction following a second FST trial on day 18. B: Antalarmin administration had no effect on stress-induced reinstatement of Esc-C CPP. Mice treated with antalarmin showed similar magnitudes of place preference to saline-treated animals following exposure to two FST trials (days 16 and 18). Data are presented as mean ± SEM of difference in time spent on the cocaine paired side versus the saline-paired saline. *p<0.05. Arrow indicates time of injection of test drug or vehicle control solution.
Figure 4.5. Mice not exposed to FST do not spontaneously reinstate place preference. Following extinction of place preference (Day 15), exposure to the conditioning context in the absence of stressful stimulus (FST) did not reinstate place preference on days 16 and 18. Data are presented as mean ± SEM of difference in time spent on the cocaine paired side versus the saline-paired saline. *p<0.05; n=9.
Figure 4.6. Plasma corticosterone increases following exposure to FST despite attenuation of stress-induced reinstatement of Fix-C CPP. Administration of the CRH-R1 antagonist antalarmin, the NMDAR antagonist MK-801 or the nNOS inhibitor 7-NI had no effect on forced swim induced increases in plasma corticosterone levels. FST, forced swim test; n=4-5/group; *p<0.05.
Chapter 5

DISCUSSION

Summary

My studies have shown that the pattern of cocaine administration influences the strength of cocaine-associated contextual memory by engaging different signaling molecules. Additionally, my studies highlight the importance of experiments geared toward identifying molecular mechanisms underlying differences in cocaine-associated memory strength with the goal of developing potent pharmacotherapies for the management of addictive behaviors. First, it was determined that cocaine-associated memory developed using an escalating conditioning regimen results from marked elevations in expression of the NR2B subunit of the NMDAR compared to memory developed from fixed regimen of cocaine (Liddie & Itzhak, 2014). Second, it was shown that while the acquisition, reconsolidation and stress-induced reinstatement of the ‘weak’ Fix-C memory was NO-dependent, the ‘strong’ Esc-C memory is mediated through alternative signaling molecules including ERK (Liddie & Itzhak, 2014, Chapter 2; and Chapter 4). Third, it was shown that inhibition of phosphodiesterase 9 (PDE9) which selectively increases cGMP in the hippocampus and amygdala, induced extinction learning and attenuated cocaine-primed reinstatement in mice conditioned by Esc-C (Liddie et al., 2012, Chapter 3). Given the role of classical Pavlovian conditioning in the formation of drug-associated LTM, the CPP paradigm represents a good model for investigating and understanding learning and memory mechanisms associated with drug addiction. Thus, these studies have identified novel NO-dependent and NO-independent
pathways relevant to the formation, reconsolidation and reinstatement of cocaine-associated memory (Table 5.1).

**NO-dependent and NO-independent cocaine-associated memory**

**Acquisition**

The CPP paradigm which employs the principles of Pavlovian learning is often used to investigate the incentive value of drugs of abuse. One caveat to conditioned reward studies is the use of a fixed daily dose of the addictive drug during training. However, the transition from drug use to addiction in human addicts involves an escalation in drug intake (Gawin, 1991). Thus, a paradigm that can effectively simulate increases in drug intake will better model the human drug use pattern.

Research investigating differences in the pattern of cocaine dosing schedule in a CPP paradigm have shown that the schedule of cocaine administration, rather than the dose of cocaine, has a significant impact on the development of drug associated memory (Itzhak & Anderson, 2012; Conrad et al., 2013). This suggests that different mechanisms may govern the formation of cocaine-associated memory developed by different schedules of cocaine administration during conditioning.

Evidence from studies by Rescorla and Wagner (1972) on natural reinforcement suggests that learning is dependent on the discrepancy between expected and obtained reward. Schultz’s work extended this theory to say that if background reward is unchanged or elevated and the conditioned stimulus is unchanged then “reward prediction” is reduced and thus learning is diminished (Bermudez & Schultz, 2010). Accordingly, if reward outcome is greater than expected, a positive prediction error is encoded; when reward is lower than expected a negative prediction error occurs and
extinction ensues; when reward is as expected then no prediction error occurs and no further behavioral changes will occur (Schultz, 1998; Schultz & Dickinson, 2000). Along this vein, it is plausible that conditioning by Esc-C where the reward is increased during conditioning may produce positive prediction error and thereby create a strong/stable memory. On the other hand, conditioning by Fix-C (which essentially was devoid of prediction error) may create a more labile memory which could be easily manipulated.

In Chapter 2 it was shown that the NR2B subunit of the NMDAR was markedly elevated in hippocampus of the enhanced and persistent ‘strong’ Esc-C memory compared to ‘weak’ Fix-C memory (Fig. 2.1). Given the role of the NR2B subunit in learning and memory (Wang et al., 2009; Zhao et al., 2005), the elevation in NR2B may have contributed to the increased strength of the Esc-C memory. Pharmacological antagonism of NR2B-containing NMDARs effectively attenuated the acquisition of both Fix-C and Esc-C memory. However, administration of the nNOS inhibitor 7-NI attenuated the acquisition of Fix-C memory but it had no effect on Esc-C memory (Fig. 2.2). While the blockade of Fix-C memory was expected since previous work from our lab has shown this effect (Itzhak et al., 1998), the finding that nNOS inhibition was ineffective against Esc-C memory was novel. The current findings therefore highlight for the first time that the pattern of cocaine administration, rather than the dose of cocaine, resulted in the differential recruitment of signaling molecules downstream of NR2B-containing NMDARs that were essential to the formation of Fix-C and Esc-C memory.

**Disruption of cocaine-associated memory**

Increasing lines of evidence suggest that retrieval of previously consolidated memories is a dynamic process that either reinforces or alters memory. Memory retrieval
may initiate two potentially opposing but distinguishable processes: reconsolidation and extinction (Suzuki et al., 2004). Memory reconsolidation acts to stabilize retrieved memories whereas extinction tends to weaken the expression of the original memory. Recently, these two processes have become important targets for reduction of drug-associated memory. My studies have shown that a) reconsolidation of Fix-C and Esc-C memory engage NO-dependent and NO-independent signaling pathways, respectively and b) the extinction of Esc-C memory, which is resistant to extinction by unreinforced re-exposures the testing context, can be induced by the use of cognitive enhancers in the form of PDE inhibitors.

The disruption of drug-memory reconsolidation may represent a powerful tool for the management of addiction. Previously consolidated drug-associated memory, when retrieved, can be disrupted using pharmacological tools. This disruption subsequently attenuates the alluring impact of drug-related cues thereby preventing relapse. However, a complete understanding of the signaling molecules that govern this process is lacking. In Chapter 2, the contribution of different signaling pathways to Fix-C and Esc-C memory reconsolidation was investigated. While pharmacological inhibition of NO signaling disrupted Fix-C memory reconsolidation, it had no effect on Esc-C memory (Fig. 2.5). Thus, similar to acquisition experiments, the ‘weak’ Fix-C memory is mediated by NO-dependent signaling while the ‘strong’ Esc-C memory bypasses the dependence on NO signaling. The lack of effect on Esc-C memory is a novel finding which now identifies a previously unknown ‘NO-independent’ cocaine-memory reconsolidation process. It should be noted, however, that it is likely that Esc-C memory activates nNOS but it appears that conditioning by Esc-C recruits additional signaling
pathways which may over rule the involvement of NO-signaling. Identifying a ‘NO-independent’ cocaine-memory reconsolidation mechanism prompted investigation into other potential molecular contributors to this process. Because the ERK signaling pathway plays a role in the formation of cocaine-associated contextual memory (Miller & Marshall, 2005; Valjent et al., 2006), it represented a possible alternative signaling pathway to assess for its contribution to Esc-C memory reconsolidation. It was shown that while inhibition of the ERK kinase MEK disrupted the reconsolidation of Esc-C memory, it had no effect on the reconsolidation of Fix-C memory (Fig. 2.5). These findings demonstrate for the first time that different signaling molecules are recruited in the reconsolidation of cocaine-associated memory depending on the pattern and salience of cocaine administration. Thus, these findings highlight the importance of understanding the significance of drug memory strength, which could be relevant to the severity of addiction, as it relates to the development of pharmacotherapeutics for the management of addiction.

While one goal of addiction research is to disrupt drug memory reconsolidation, another equally important addiction management strategy is extinction learning by ‘exposure therapy’ (Carter & Tiffany, 1999; Powell et al., 1993; Siegel & Ramos, 2002). Extinction typically requires long or multiple re-exposures to a CS (Nader, 2003; Power et al., 2006). Studies suggest that the extinction process does not eliminate or cause ‘unlearning’ of the initial conditioned response; rather, the organism learns that the CS no longer elicits the previous stimulus (Bouton, 2002; 2004; Havermans & Jansens, 2003). Thus, extinction requires new learning (Milad & Quirk, 2002; Santini et al., 2001).
Mice conditioned by Fix-C readily show extinction with multiple unreinforced exposures to the conditioning context. Alternatively, mice conditioned by Esc-C maintain preference for the cocaine paired side for greater than 10 days. In Chapter 3, it was investigated whether acceleration of extinction learning using different PDE inhibitors (cognitive enhancers) could facilitate extinction learning in mice conditioned by Esc-C. PDE inhibitors have previously been shown to facilitate learning and memory in animal models of experimentally-induced learning and memory deficits (Bender & Beavo, 2006; Blokland et al., 2006; Boswell-Smith et al., 2006; Menniti et al., 2006). It was shown that the inhibition of PDE9 which increases levels of cGMP in the hippocampus and amygdala induced extinction learning and prevented cocaine-primed reinstatement in mice conditioned by Esc-C. However, PDEs that increased levels of cAMP or dual-specific PDEs (increased both cAMP and cGMP) did not induce extinction learning (Fig. 3.2). These data suggest that PDE9 which is highly localized in all sub-areas of the hippocampus (van Staveren et al., 2002; 2004; Reyes-Irisarri et al., 2007) has a prominent role in consolidation of extinction learning. It also appears that targeting a specific PDE is more critical than targeting any PDE which metabolizes cGMP.

**Stress-induced reinstatement of Fix-C and Esc-C memory**

Vulnerability to relapse following prolonged periods of abstinence presents a major challenge to combating drug addiction. Stress is an unavoidable part of life and a major contributor to relapse to drug use. However, a thorough understanding of the neural mechanisms that sub-serve stress-mediated relapse is lacking. In Chapter 4, the contribution of different signaling molecules to stress-induced reinstatement of Fix-C and Esc-C CPP was investigated. While antagonism of NMDAR and inhibition of nNOS
effectively attenuated forced swim-induced reinstatement of Fix-C CPP, these
manipulations had no effect on Esc-C CPP (Fig. 4.1 and 4.3). Thus, like the acquisition
and reconsolidation of Fix-C memory, stress-induced reinstatement of Fix-C memory is
NO-dependent while Esc-C memory is NO-independent. My studies add to the list of
signaling molecules that play a role in stress-induced reinstatement of Fix-C CPP.
However, none of the test drugs investigated that successfully attenuated stress-induced
reinstatement of Fix-C was effective against Esc-C CPP. Therefore, my studies point to
the existence of additional signaling molecules that contribute to stress-induced
reinstatement of Esc-C CPP.

**Proposed model for the development of Fix-C and Esc-C memory**

Figure 5.1 proposes a model for the contribution of different signaling pathways
to the formation of Fix-C and Esc-C memory. Fix-C and Esc-C memory results from
increased protein expression levels of NR2B subunit of the NMDAR. However, NR2B is
markedly elevated in mice conditioned by Esc-C compared to mice conditioned by Fix-C.
NR2B-containing NMDARs allow greater calcium entry thus elevated NR2B levels in
Esc-C conditioned mice allow for increased calcium influx upon NMDAR activation by
glutamate. My findings show that Fix-C memory acquisition, reconsolidation and stress-
induced reinstatement can be blocked by inhibiting nNOS but Esc-C memory remains
unperturbed. With respect to the Fix-C model, calcium influx activates calmodulin which
mediates nNOS-induced increases in NO levels. NO stimulates soluble guanylate cyclase
(sGC) which leads to cGMP-mediated activation of protein kinase G (PKG) which
subsequently contributes to the phosphorylation of ERK. With respect to Esc-C memory,
evidence suggests that the NR2B subunit of NMDAR has potential to carry greater
calcium current per unit charge (Sobeyk et al., 2005) which may confer a greater influence on downstream signaling cascades that affect synaptic plasticity and learning and memory such as the NMDAR-RasGRF1-MEK-ERK pathway (Krapivinsky et al., 2003). Since RasGRF1 specifically binds the NR2B subunit of the NMDAR, it couples the activity of ERK with NR2B-containing NMDARs (Krapivinsky et al., 2003). My studies show that inhibition of MEK, the ERK kinase, disrupted reconsolidation of Esc-C memory but had no effect on Fix-C memory (Fig. 2.5). Thus the MEK-ERK pathway plays a role in Esc-C memory. While the nNOS signaling pathway may also be activated in response to training by Esc-C, it appears that other signaling pathways including NMDAR-MEK-ERK signaling plays a more behaviorally significant role in the development of Esc-C CPP. Additionally, though both NO-cGMP-PKG and MEK signaling pathways converge at the level of ERK (Ota et al., 2008) it is conceivable that the contribution of each pathway to drug memory is dependent on cocaine conditioning schedule. The differential activation of ERK could result in different degrees of activation of molecules downstream of ERK including cAMP response element binding protein (CREB). CREB is a known mediator of synaptic plasticity and the generation of new synapses through upregulation of gene expression which subsequently contribute to memory strength. Thus increased phosphorylation of CREB (pCREB) provides greater propensity for rapid metaplasticity to strengthen drug-associated synapses associated with ‘strong’ Esc-C memory.
Future Directions

Overview

My findings opened up several avenues for additional research. First, an investigation of the role of silent synapses (see below) in cocaine-associated memory strength will help to identify a mechanism through which NR2B-containing NMDARs contribute to Fix-C and Esc-C memory. Second, site-specific inhibition of signaling targets in the hippocampus and other brain regions associated with drug addiction will help to further define the roles of different signaling pathway in Fix-C and Esc-C memory acquisition, reconsolidation and reinstatement. Third, since NR2B antagonism was effective against acquisition and reconsolidation of Fix-C and Esc-C memory but ineffective against stress-induced reinstatement, future investigations into the physiological changes that occur during withdrawal with respect to NR2B expression could shed light on inherent differences between the processes of acquisition and reconsolidation versus stress-induced reinstatement. Fourth, since my work has not identified a signaling pathway that effectively attenuates stress-induced reinstatement of Esc-C CPP, future studies should probe for the contribution of alternate signaling pathways such as metabotropic glutamate receptor signaling. Fifth, my studies have shown that varying the stimulus salience of cocaine reward through changes in the pattern of drug administration results in differential drug-memory strength and the recruitment of different signaling pathways. Future studies should investigate whether varying the stimulus salience of an aversive memory such as fear conditioning will similarly engage different signaling molecules.
**Silent synapses in cocaine-associated memory strength**

Recent reports demonstrate that repeated cocaine exposure generate silent glutamatergic synapses which contribute to metaplasticity (Huang et al, 2009) and the development of cocaine-induced locomotor sensitization (Brown et al, 2011). Silent synapses are glutamatergic synapses which express NMDAR-mediated currents in the absence of AMPAR-mediated currents (Isaac et al, 1995; Liao et al, 1995). Interestingly, in vitro evidence supports the idea that silent glutamatergic synapses contain higher levels of NR2B-containing NMDARs compared to neighboring active synapses. Moreover, the authors showed that elevated levels of NR2B at silenced synapses increased calcium entry into neurons and lowered the threshold for LTP induction (Lee et al, 2010). This suggests that silent synapses are capable of undergoing rapid metaplasticity to strengthen synaptic connections. Given these observations, it is reasonable to assume that my cocaine administration schedules generated silent synapses illustrated by the increases in NR2B protein. Furthermore, it is plausible that the extent to which silent synapses were generated contributed to the differential salience of cocaine reward elicited by Fix-C and Esc-C. Thus, increased numbers of silent synapses through enhanced expression of NR2B provide greater potential for metaplasticity and subsequent memory strengthening. Further research should use electrophysiological tools to investigate the extent to which silent synapses were generated and how they may have contributed to the observed behavioral effects.

**Site-specific inhibition of signaling targets**

The current studies employed systemically administered modulators of various signaling molecules and showed in some instances a similar effect on Fix-C and Esc-C
memory while in other cases differential effects. While the use of pharmacological tools could be the gold standard for the treatment of human addicts, in order to better understand the mechanisms associated with drug-associated behavior, it is necessary to elucidate the contribution of specific brain regions to these effects. As such, brain region-specific injection of modulators of signaling molecules may aid in this endeavor. This will reduce the ‘noise’ associated with systemic administration since there is always the potential for test drugs to have off-target effects that may influence behavior.

Additionally, future studies using region-specific and conditional knockout (KO) models of specific signaling targets will be of great value in specifying the roles of anatomical substrates for cocaine-associated memory. For instance, conditional and inducible manipulation of specific signaling molecules will minimize the potential for compensatory mechanisms that could functionally substitute for the inhibited molecule.

**Effect of extinction and withdrawal on NR2B**

The current studies showed that antagonism of NR2B-containing NMDARs by ifenprodil effectively disrupted the acquisition and reconsolidation of Fix-C and Esc-C memory. However, ifenprodil had no effect on stress-induced reinstatement of Fix-C and Esc-C CPP. One possible explanation for this lack of effect is that NR2B subunit may have been replaced by NR2A during the time of extinction and withdrawal from cocaine. Thus, with reduced expression of NR2B at the time of reinstatement, a selective NR2B antagonist would have no effect on the behavior. My findings are in accordance with others who found that NR2B-containing NMDARs are required for morphine-induced reinstatement but not forced swim-induced reinstatement of morphine CPP (Ma *et al.*, 2006). This suggests that at the time of reinstatement, exposure to the drug (such as
morphine or cocaine) engages NR2B-containing NMDARs to reinstate place preference. However, it appears that exposure to stress induces reinstatement in a manner independent of NR2B-containing NMDARs. Thus, future studies should investigate a) how levels of NR2B change with extinction and withdrawal and b) other possible signaling molecules/pathways that interact with stress-related molecules to induce reinstatement of extinguished place preference.

**Signaling molecules involved in stress-induced reinstatement of Esc-C CPP**

While the current studies identified that the NMDAR and NO-signaling play a role in stress-induced reinstatement of Fix-C CPP, an effective test drug against Esc-C memory was not identified. Since Esc-C memory represents a ‘stronger’ and more ‘resistant’ type of drug memory compared to Fix-C memory, there is a need to identify the signaling pathways that contribute to stress-related re-emergence of extinguished Esc-C CPP. It appears that the interaction between stress-related molecules and those that mediate reinstatement are different between Fix-C and Esc-C. Because modulation of ionotropic glutamate receptors was ineffective at attenuating stress-induced reinstatement of Esc-C CPP, a possible alternative to target is metabotropic glutamate receptors (mGluRs). Specifically, the mGluR2/3 agonist LY379268 has been shown to inhibit cocaine seeking in preclinical animal models and decrease stress-induced relapse due to its anxiolytic effects. Similarly, the mGluR1/5 antagonists, 2-methyl-6-(phenylethynyl)pyridine and 3-[2-methyl-4-thiazolyl]ethynyl]pyridine, have shown to be effective in preclinical models of cocaine addiction (Uys & LaLumiere, 2008). Hence, an investigation of these mGluRs as well as other potential candidates may identify pharmacological agents that could attenuate stress-induced reinstatement of the ‘strong’
Esc-C memory. Successful suppression of stress-induced reinstatement of Esc-C memory should yield effective pharmacotherapies against stress-induced relapse to drug use.

**Fear Conditioning**

Maladaptive behaviors such as anxiety disorders are associated with learning and memory processes. Fear conditioning is often used as a model for understanding anxiety disorders including post-traumatic stress disorder (PTSD). Like CPP experiments, fear conditioning is based on Pavlovian conditioning in which an organism learns to predict aversive events based on associative learning. The expression of learned fear functions to prepare an organism for “fight or flight” responding.

An investigation of how memory strength influences the recruitment of different signaling molecules will be of immense clinical value that could be applicable to the treatment of many debilitating learning and memory diseases. I had shown that altering the stimulus salience of cocaine reward engages different neural substrates. However, it is unclear whether this effect is specific to appetitive learning and memory or if this phenomenon is applicable to other paradigms of learning and memory. My preliminary work used the fear conditioning model to assess how changing the stimulus salience affects the acquisition of fear memory. Mice were divided into two groups: a) *Fixed shock*: mice given 4 shocks, each at 1.1mA intensity and b) *Escalating shock*: mice given 4 shocks at increasing intensities (0.6, 0.8, 1.2 and 1.8mA). The intensity for the fixed shock group represents the average shock intensity over 4 shocks of the escalating shock group. Thus, I controlled for the total shock intensity to which mice were exposed. Figure 5.2 shows the effect of the different training schedules on conditioned contextual freezing response. Mice conditioned by escalating shock intensities show higher freezing
which was generally resistant to unreinforced exposures to the training context. However, mice conditioned by fixed shock intensities showed a significantly lower magnitude of freezing and freezing levels at the second test (re-test 1; day 6) and subsequent tests were not statistically different from basal freezing levels. Results suggest that an escalating regimen of conditioning, be it appetitive or aversive, results in ‘stronger’ memory. Future studies should investigate whether these behavioral differences between conditioning by fixed and escalating shock intensities engage different signaling pathways in the formation of fear memory. Additionally, an investigation of the contribution of NR2B-containing NMDARs in determining memory strength would solidify the involvement of the NR2B subunit as an important regulator of memory strength. This would therefore identify NR2B-containing NMDARs as potential targets for the treatment of myriad maladaptive behaviors that arise from Pavlovian conditioning.
<table>
<thead>
<tr>
<th>Conditioning Paradigm</th>
<th>Treatment</th>
<th>Cocaine Memory acquisition</th>
<th>Cocaine Memory Reconsolidation</th>
<th>Extinction Learning</th>
<th>Stress-induced Reinstatement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Effect</td>
<td>Effect</td>
<td>Effect</td>
<td>Effect</td>
</tr>
<tr>
<td>Fix-C</td>
<td>MK-801</td>
<td>N/A</td>
<td>disrupt</td>
<td>N/A</td>
<td>attenuate</td>
</tr>
<tr>
<td></td>
<td>Ifenprodil</td>
<td>attenuate</td>
<td>no effect</td>
<td>N/A</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>SL327</td>
<td>N/A</td>
<td>disrupt</td>
<td>N/A</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>7-Ni</td>
<td>attenuate</td>
<td>disrupt</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Antalarmin</td>
<td>N/A</td>
<td>disrupt</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>no effect</td>
<td>no effect</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Esc-C</td>
<td>MK-801</td>
<td>N/A</td>
<td>disrupt</td>
<td>N/A</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>Ifenprodil</td>
<td>attenuate</td>
<td>no effect</td>
<td>N/A</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>SL237</td>
<td>N/A</td>
<td>disrupt</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>7-Ni</td>
<td>no effect</td>
<td>no effect</td>
<td>N/A</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>Antalarmin</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>BAY-73691</td>
<td>N/A</td>
<td>N/A</td>
<td>accelerate</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Papaverine</td>
<td>N/A</td>
<td>N/A</td>
<td>no effect</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Rolipram</td>
<td>N/A</td>
<td>N/A</td>
<td>no effect</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
</tr>
</tbody>
</table>

Abbreviations: N/A, not assessed

**Table 5.1. Effect of different pharmacological agents on Fix-C and Esc-C memory acquisition, reconsolidation and stress-induced reinstatement.** The acquisition of Fix-C memory is NR2B and NO-dependent whereas the acquisition of Esc-C memory is NR2B-dependent but NO-independent. Reconsolidation of Fix-C memory was disrupted by antagonism of NMDARs and inhibition of nNOS while reconsolidation of Esc-C memory was disrupted by antagonism of NMDARs but not inhibition of nNOS. Reconsolidation of Esc-C memory but not Fix-C memory engages MEK-ERK signaling. BAY-73691, but not papaverine nor rolipram, induced extinction learning of Esc-C memory. Stress-induced reinstatement of Fix-C memory is NMDAR-, NO- and CRH-R1-dependent but there is no specific requirement for NR2B-containing NMDARs. Stress-induced reinstatement of Esc-C memory was not attenuated by any of the test drugs suggesting NMDAR and NO-independent mechanisms associated with stress-induced reinstatement of Esc-C CPP. MK-801: noncompetitive NMDAR antagonist; Ifenprodil: NR2B-containing NMDAR antagonist; SL327: MEK inhibitor; 7-Ni: nNOS inhibitor.
Table 5.2. Effect of conditioning schedule on expression of NR2B subunit of the NMDAR at the mRNA (Grin2b) and protein (NR2B) levels. Quantitative RT-PCR showed an upregulation of Grin2b only in mice conditioned by Esc-C; levels of Grin2b in mice conditioned by Fix-C were unchanged. Western blot analysis of protein expression showed an upregulation of NR2B in both Fix-C and Esc-C compared to saline. Additionally, levels of NR2B were elevated in mice conditioned by Esc-C compared to mice conditioned by Fix-C.

<table>
<thead>
<tr>
<th>Conditioning Paradigm</th>
<th>Hippocampal Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>qRT-PCR (Grin2b)</td>
</tr>
<tr>
<td>Saline</td>
<td>Baseline</td>
</tr>
<tr>
<td>Fix-C</td>
<td>=</td>
</tr>
<tr>
<td>Esc-C</td>
<td>↑</td>
</tr>
</tbody>
</table>

Symbols: =, no change; single upward arrow, upregulation; double upward arrow, greater upregulation
Abbreviations: Esc-C, escalating dose cocaine schedule; Fix-C, fixed dose cocaine schedule; NMDAR, N-methyl-D-aspartate ionotropic glutamate receptor; NR2B, subunit of NMDAR; Ca\(^{2+}\), calcium; nNOS, neuronal nitric oxide synthase; sGC, soluble guanylyl cyclase; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; Ras-GRF1, Ras protein-specific guanine nucleotide-releasing factor 1; MEK, mitogen-activated protein kinase kinase; pERK, phosphorylated extracellular signal-regulated kinase; pCREB, phosphorylated cAMP response element binding protein.

**Figure 5.1. Proposed model for the development of Fix-C and Esc-C memory.**
Conditioning by Fix-C results in modest upregulation of the NR2B subunit of the NMDAR. Calcium entry through NMDAR activates nNOS which produces NO. NO then stimulates sGC which leads to cGMP-mediated activation of PKG. PKG then phosphorylates ERK. ERK phosphorylates CREB which facilitates upregulation in gene expression and subsequent formation of new synapses. Conditioning by Esc-C results in marked increases in NR2B that facilitate greater calcium influx upon NMDAR activation. While calcium entry may simulate nNOS, it also activates additional signaling molecules including Ras-GRF1. Ras-GRF1 mediates MEK-induced phosphorylation of ERK. Since conditioning by Esc-C could potentially recruit both nNOS and Ras-GRF1-MEK signaling, this suggests increased activation of ERK and CREB leading to exaggerated gene expression and synapse formation.
Figure 5.2. Repeated shocks at increasing intensities elicit higher magnitude and more persistent freezing response than repeated shocks at a fixed intensity. Mice were conditioned by escalating (0.6, 0.8, 1.2 and 1.8mA) or fixed (4 X 1.1mA) foot shocks following habituation to the training context for 2 min (basal). Each shock was preceded by a buzzer tone and mice were removed from the shock apparatus for varying intervals between 6 and 24 min following each shock. Mice on the escalating shock schedule displayed higher freezing response when tested for contextual long-term memory (LTM) 24 hr after the final shock compared to mice on the fixed shock schedule. Mice on the fixed shock schedule showed a faster freezing extinction rate than mice conditioned on the escalating shock schedule. (*) represent difference between the groups on each test day. (#) represents difference from Basal freezing.
REFERENCES


