INTRACELLULAR MECHANISMS OF COCAINE-MEMORY RECONSOLIDATION IN THE BASOLATERAL AMYGDALA AND DORSAL HIPPOCAMPUS

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ABSTRACT

Audrey Marie Wells: Intracellular Mechanisms of Cocaine-memory Reconsolidation in the Basolateral Amygdala and Dorsal Hippocampus
(Under the direction of Rita A. Fuchs Lokensgard)

The ability of cocaine-associated environmental contexts to promote relapse in abstinent humans and reinstatement of cocaine-seeking behavior in laboratory animals depends on the formation and maintenance of maladaptive context-response-cocaine associative memories, the latter of which can be disrupted by manipulations that interfere with memory reconsolidation. Memory reconsolidation refers to a protein synthesis-dependent phenomenon whereby memory traces are reincorporated back into long-term memory storage following their retrieval and subsequent destabilization. To elucidate the distinctive roles of the basolateral amygdala (BLA) and dorsal hippocampus (DH) in the reconsolidation of context-response-cocaine memories, Experiments 1-3 evaluated novel molecular mechanisms within each structure that control this phenomenon. Experiment 1 tested the hypothesis that activation of the extracellular signal-regulated kinase (ERK) in the BLA and nucleus accumbens core (NACc – a substrate for Pavlovian cocaine-memory reconsolidation) would critically control instrumental cocaine-memory reconsolidation. To determine this, rats were re-exposed to a context that had previously been used for cocaine self-administration (i.e., cocaine memory-reactivation) and immediately thereafter received bilateral intra-BLA or intra–NACc microinfusions of the ERK inhibitor U0126 or vehicle (VEH) and were subsequently tested for drug context-induced cocaine-seeking behavior (non-reinforced lever responding) ~72 h later. Re-exposure to the cocaine-paired

context at test fully reinstated cocaine-seeking behavior, relative to responding in an alternate, extinction context, and post-reactivation U0126 treatment in the BLA, but not the NACc, impaired cocaine-seeking behavior, relative to VEH. This effect was associated with a temporary increase in ERK2, but not ERK1, phosphorylation in the BLA and required explicit reactivation of the target memory trace (i.e., did not similarly manifest when U0126 was administered after exposure to an unpaired context), suggesting that ERK in the BLA plays a critical role in restabilizing contextual cocaine-related memories. Next, Experiment 2 evaluated the hypothesis that the transcription factor (TF) nuclear factor-κB (NF-κB) would also critically mediate instrumental cocaine-memory reconsolidation in the BLA. Remarkably, the NF-κB inhibitor, sulfasalazine (SSZ), administered bilaterally into the BLA following cocaine-memory reactivation, did not significantly alter subsequent cocaine-seeking behavior, relative to VEH, despite producing an observable trend for an *enhancement* in this behavior. Future studies will be needed to further examine this relationship, but the present findings may suggest that NF-κB TFs acts as negative regulators of cocaine-memory reconsolidation. Finally, Experiment 3 tested the hypothesis that members of the Src family of tyrosine kinases (SFKs) are obligatory for instrumental cocaine-memory reconsolidation. Consistent with our hypothesis, PP2, a nonspecific inhibitor of SFKs, administered bilaterally into the DH after cocaine-memory reactivation, attenuated subsequent drug-context induced motivation for cocaine, relative to VEH, in a memory reactivation-dependent manner. This effect was associated with a preferential disruption of SFK-mediated phosphorylation of the NR2a N-methyl-D-aspartate receptor (NMDAR) subunit. Together, these findings begin to illuminate how the BLA and DH may subserve the long-term stability of maladaptive cocaine-related memories that underlie contextual stimulus control over cocaine-seeking behavior.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	anterior-posterior
BLA	basolateral amygdala
B+M	baclofen+muscimol
cAMP	cyclic AMP
COC CTX	cocaine-paired context
CS	conditioned stimuli
DH	dorsal hippocampus
DV	dorsal-ventral
EC	entorhinal cortex
ERK	extracellular signal-regulated kinase
EXT CTX	extinction context
FR1	fixed ratio 1
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
MAPK	mitogen activated protein kinase
MEK	MAPK kinase
mGluR1	metabotropic glutamate receptor – Group I
ML	medial-lateral

NACc	nucleus accumbens core
NF-κB	nuclear factor- κB
NMDAR	N-methyl-D-aspartate receptor
pCPu	posterior caudate putamen
PKA	protein kinase A
SEM	standard error of the mean
SFK	src family tyrosine kinase
SStr	trunk region of the somatosensory cortex
VEH	vehicle

CHAPTER 1

GENERAL INTRODUCTION

Significance of the Problem

Drug addiction remains a serious and inadequately addressed public health concern in the United States, with 22.5 million current illicit drug abusers reported (Substance Abuse and Mental Health Service Administration 2011) and an associated annual cost of *$193 billion dollars* (National Drug Intelligence Center 2011)! Cocaine ranks third among the most abused illicit substances, with 821,000 individuals meeting criteria for cocaine abuse and/or dependence in 2011, a statistic surpassed only by marijuana and prescription pain-killers (Substance Abuse and Mental Health Service Administration 2011). However, emergency room visits related to cocaine use – an estimated 488,101 in 2010 - far exceeded those associated with other illicit substance use, including marijuana and heroin (Substance Abuse and Mental Health Administration 2011).

Negative health consequences and economic costs related to cocaine use are sustained in part by unsuccessful rehabilitation agendas. This is reflected by the small percentage (10.6%) of drug abusers requiring treatment who have endorsed taking part in a treatment/rehabilitation strategy (Substance Abuse and Mental Health Service Administration 2011). Lamentably, in addition to the small proportion of treatment-seeking addicts, efforts to eradicate cocaine addiction are further complicated by a high occurrence of relapse even after extended drug-free

periods (Gawin and Kleber 1986). The prototypical behavioral pattern of cocaine addiction involves alternating bouts of abstinence and binge-like, often escalating use (Gawin and Kleber 1986), which occurs in spite of diminished drug reinforcement (Gawin and Kleber 1986; Koob and Bloom 1988), negative consequences (Everitt *et al,* 2008), and even volition to discontinue drug use (Volkow and Fowler 2000).

Individuals who seek treatment outside of their typical drug-taking environment and in the absence of drug-associated stimuli (i.e., rehabilitation clinics) are particularly vulnerable to relapse following completion of treatment, in that exposure to discrete drug-related stimuli (e.g., paraphanelia) and/or environmental contexts (e.g., crackhouse, bar) are powerful promoters of craving and relapse in humans (O'Brien *et al,* 1992; Childress *et al*, 1988; Foltin and Haney 2000; Franklin *et al*, 2009) and reinstatement of cocaine-seeking behavior in laboratory rats (Alleweireldt *et al,* 2001; Crombag *et al*, 2002, 2008; Fuchs *et al*, 2005; Fuchs *et al*, 2008b). Over the course of addiction, otherwise innocuous environmental stimuli become endowed with conditioned reinforcing and incentive motivational properties by virtue of having been repeatedly paired with the unconditioned motivational effects of the drug of abuse, and this "transfer" of reinforcement and incentive value requires associative learning processes (Milton and Everitt 2012). Environmentally-triggered relapse, per se, is thought to require the initial acquisition and stabilization (i.e., cellular consolidation; Goelet *et al,* 1986), but importantly, also the subsequent maintenance and availability of, a highly complex context-response-cocaine associative memory trace (Fuchs *et al,* 2005; Crombag *et al,* 2008). Research into the mechanisms of drug-memory acquisition and consolidation will likely advance addiction *prevention* initiatives, whereas investigation into the processes that *maintain* long-term drug-related memories may be more beneficial in the development of addiction *treatment*. In support

of this, the average drug user seeks treatment *15.6 years* following initial drug use and consolidation of drug memories (Substance Abuse and Mental Health Administration 2011). As such, the prototypical addict may benefit from treatments designed to weaken, or disrupt the maintenance of, maladaptive drug-related memories (Taylor *et al* 2009; Milton and Everitt 2010). Hence, identification of the neurobiological processes required for memory maintenance, including the *reconsolidation* (see below) of retrieved memories, will be paramount from an addiction treatment perspective.

Memory Reconsolidation: History and Therapeutic Application

Recently, promising evidence supporting the *memory reconsolidation hypothesis* has suggested that drug-associated memory traces are not rigidly maintained and invulnerable to disruption. Instead, associative memories can be destabilized following their retrieval (i.e., memory reactivation; Ben Mamou *et al,* 2006; Finnie and Nader 2012) and require an obligatory protein synthesis dependent memory reconsolidation process to be reincorporated back into long-term memory stores (Nader *et al,* 2000b). Memory destabilization is thought to involve a reversal of synaptic plasticity (Finnie and Nader 2012) and requires protein degradation (Lee *et al,* 2008) and synaptic depotentiation (Clarke *et al,* 2010). Memory destabilization is both necessary and sufficient for subsequent memory reconsolidation (Ben Mamou *et al,* 2006; Maren 2011; Milton *et al,* 2013). The functional consequence of memory destabilization is the availability of an "active," usable memory trace (Lewis 1979). Depotentiation is suggested to allow for the incorporation of new information (i.e., memory "updating,"; Rodriguez-Ortiz *et al,* 2005; Lee 2010; Sevenster *et al,* 2012, 2013) into, or the strengthening or weakening of, the memory trace to accommodate modifications to the CS-US relationship (Forcato *et al,* 2011; Inda *et al,* 2011).

Following memory destabilization, the transient (i.e., 0-4 hours; Nader *et al,* 2000a) maintenance of the labile engram putatively depends on cellular events akin to those involved in the maintenance of recently acquired short-term memory, including neuronal firing and exocytosis of the readily releasable pool of neurotransmitter (Tarnow 2008) and post-translational modification of existing cytoplasmic proteins (Goelet *et al,* 1986).

The conceptual and physiological inverse process to memory destabilization, *memory reconsolidation,* is associated with synaptic potentiation (Clarke *et al,* 2010) and the synthesis of new proteins (Nader *et al,* 2000a; Debiec *et al,* 2002; Fuchs *et al,* 2009). Memory reconsolidation is expected to begin immediately following the *offset/termination* of the retrieval-eliciting CS (Perez-Cuesta and Maldonado 2009) and is complete within 6 h (Nader *et al,* 2000b; Tronson and Taylor 2007). Although many of the physiological correlates of memory reconsolidation remain poorly understood, at a minimum, this phenomenon requires one or a combination of processes that are sensitive to anisomycin (ANI) - gene transcription, protein synthesis, and/or post-translational modification of existing proteins (Gold 2008).

The memory reconsolidation hypothesis has challenged existing memory consolidation dogma. The traditional view postulated that, following initial cellular consolidation, memories are structurally integrated into hippocampally-dependent, and later rearranged into cortically-dependent neuronal ensembles where associative information is rigidly guarded and maintained indefinitely (Davis and Squire 1984; Squire 1992; McClelland *et al,* 1995; McGaugh 2000; Frankland and Bontempi 2005; McKenzie and Eichenbaum 2011; but see review on multiple trace theory for alternate account, Nadel and Moscovitch 1997). Hence, amnesia, according to this view, reflects either interference at encoding (Fernandes and Moscovitch 2000) during consolidation (McGaugh 2000; Davis and Squire 1984; Goelet *et al,* 1986) or at the time of

retrieval. However, amnesia is not considered the manifestation of an altered memory trace once consolidation is complete, with the exception of following overt brain damage.

The view that following cellular consolidation, memories are invulnerable to disruption (Frankland and Bontempi 2005) predominated memory research until it was challenged by a report in 1968 (Misanin *et al,* 1968). This study explored whether the amnesic effects of electroconvulsive shock therapy (ECS) were limited to recently consolidated memories (Misanin *et al,* 1968). To investigate this, rats were re-exposed to a footshock-predictive CS (i.e., fear-memory reactivation), and suppression of licking behavior was measured as an index of conditioned fear. Following CS re-exposure, a subset of the rats received ECS therapy. All rats were tested in the presence of the fear-conditioned CS 24 h later. Post-reactivation ECS treatment significantly attenuated CS-elicited suppression of licking behavior during this final test session, relative to control conditions (Misanin *et al,* 1968). A novel finding for its time, this study provided the first evidence for a post-retrieval period of memory lability and consequent memory restabilization process (later coined "reconsolidation"; Nader *et al,* 2000a). This initial evidence for memory reconsolidation, while noteworthy, was not further explored until the year 2000, when Nader and colleagues demonstrated that microinfusions of ANI into the basolateral amygdala (BLA) following re-exposure to an auditory fear-associated CS abolish CS-evoked conditioned freezing behavior 24 h later, relative to vehicle (VEH) (Nader *et al,* 2000a). Importantly, intra-BLA ANI was without effect if administered in the absence of CS re-exposure, demonstrating that the effects of ANI depended on the explicit reactivation of the target memory trace and establishing a precedent for the use of this "no reactivation control" experiment in future reconsolidation studies (Nader *et al,* 2000a; Nader and Einarsson 2010).

Since publication of the aforementioned findings (Nader *et al,* 2000a), the existence of the memory reconsolidation phenomenon has been demonstrated for a variety of distinguishable forms of memory, including, but not limited to, fear memory in auditory (Nader *et al,* 2000a; Debiec and LeDoux 2004; Duvarci *et al,* 2005; Mac Callum *et al,* 2013) and contextual fear conditioning (Debiec *et al,* 2002; Lee *et al,* 2004; Einarsson and Nader 2012; Lee and Hynds 2013), as well as in inhibitory (Amaral *et al,* 2007; Milekic *et al,* 2007; Nikzad *et al,* 2011; Arguello *et al,* 2013a) and passive avoidance (Litvin and Anokhin 2000; Gieros *et al,* 2012; Flint *et al,* 2013) paradigms, general declarative memory in the object recognition model (Kelly *et al,* 2003; Silingardi *et al,* 2011; Clarke *et al,* 2010; Jobim *et al,* 2012), spatial memory in the Morris Water maze task (Przybyslawski and Sara 2000; Rossato *et al,* 2006; Da Silva *et al,* 2013), and natural and drug-related reward memories in conditioned place preference (Miller and Marshall 2005; Valjent *et al,* 2006; Bernardi *et al,* 2006; Robinson and Frankland 2007; Brown *et al,* 2008; Wang *et al,* 2008; Li *et al,* 2010; Theberge *et al,* 2010; Crespo *et al,* 2012) and instrumental drug self-administration models (Lee *et al,* 2005, 2006; Milton *et al,* 2008a, b; Fuchs *et al,* 2009; Ramirez *et al,* 2009; Sanchez *et al,* 2010; Theberge *et al,* 2010; Wells *et al,* 2011, 2013; Crespo *et al,* 2012). Additionally, the capacity for memory reconsolidation appears to be evolutionarily conserved and is reliably demonstrated in a number of species, including the crab Crasthamaus, the 1-day old chick, rodents, nonhuman primates, and – encouraging from a translational perspective – in humans (reviewed in Nader and Einarsson 2010).

Disruption of Drug Memory Reconsolidation

The implication of much of the existing research into the mechanisms that support memory reconsolidation is that targeting memories, specifically to impair or weaken maladaptive

ones, is feasible. Furthermore, this approach may be effective for the treatment of neuropsychiatric disorders characterized by pathological memories, such as post-traumatic stress disorder, phobias, and addiction (reviewed in Taylor *et al,* 2009). In particular, the growing body of literature investigating mechanisms of *drug memory reconsolidation, per se,* suggests that labile drug memories are an accessible and exploitable target for pharmacotherapies aimed at relapse prevention (Taylor *et al,* 2009; Sorg 2012; Milton and Everitt 2012).

The majority of research aiming to better understand the neuroanatomical and cellular mechanisms of drug-memory maintenance, including retrieval-induced drug-memory reconsolidation, have utilized animal models of addiction (for a topical review on this subject, see Sorg 2012). In preclinical models, disruption of drug-memory reconsolidation manifests as enduring suppression of drug-conditioned behaviors that were previously elicited by the target memory trace (Miller and Marshall 2005; Lee *et al* 2005, 2006; Milekic *et al* 2006; Fuchs *et al* 2009). This is paralleled in clinical studies, where in drug users, β-adrenergic receptor antagonist treatment at the putative time of memory reconsolidation preferentially disrupts target memories for heroin-related, but not neutral, words and phrases (Zhao *et al* 2011). Hence, disruption of memory reconsolidation may be a promising tool for disabling drug-associated memories (Diergaarde et al 2008; Taylor et al 2009; Milton and Everitt 2012; Sorg 2012), and it may be a viable method by which relapse propensity is therapeutically regulated.

Selection of Animal Models: Consideration of Boundary Conditions

According to the memory reconsolidation hypothesis, following memory retrieval, a memory trace is permissive to modification and can be disrupted with a variety of amnesic agents (Tronson and Taylor 2007). However, the existence of boundary conditions (i.e.,

circumstances that impinge on the ability of a memory trace to undergo reconsolidation; Besnard *et al,* 2012), including asymptotic learning (i.e., the need for memory updating; Rodriguez-Ortiz *et al,* 2005; Lee 2010; Sevenster *et al,* 2012, 2013), extinction interference (Pedreira and Moldonado 2003; Eisenberg *et al,* 2003; Suzuki *et al,* 2004; Duvarci *et al,* 2006; de la Fuentes *et al,* 2012; Auber *et al,* 2013), and memory age and strength (Milekic and Alberini 2002; Suzuki *et al,* 2004; Eisenberg and Dudai 2004; Finnie and Nader 2012), challenge this existing dogma (Besnard *et al,* 2012) and underscore the importance of using animal models with exceptional face, construct, and experimental validity. Memory strength and age are particularly relevant to the reconsolidation of drug-related memories. Addicts typically engage in extensive drug use followed by prolonged periods of abstinence (Gawin and Kleber 1986). Therefore, over the course of drug use, context-drug associations undergo *explicit* retrieval and reconsolidation repeatedly and this may likely lead to memory strengthening (Inda *et al,* 2011). Additionally, during abstinence, context-drug memories can undergo *implicit* reactivation and reconsolidation, which also reinforces the strength of the trace. A phenomenon that is consistent with this interpretation is the so-called *incubation effect* – a time-dependent increase in drug stimuli-induced drug-seeking behaviors (Tran-Nguyen *et al,* 1998; Grimm *et al,* 2001; Wells *et al,* 2011) in preclinical models and a positive correlation between rumination and likelihood of relapse in human addicts (Nolen-Hoeksema *et al,* 2007). Therefore, the ideal animal model for studying the neuroanatomical and intracellular underpinnings of drug memory reconsolidation is one that uses at least a subchronic drug administration training regimen (Fuchs *et al,* 2008b) and consequently engenders remote yet strong contextual-drug associations, closely paralleling the human condition (Lu *et al,* 2006; Kalivas and O'Brien 2008; Finnie and Nader 2012).

Selection of Animal Models: Pavlovian versus Instrumental Models of Drug Relapse

The two most commonly used paradigms in cocaine-memory reconsolidation research are the conditioned place preference (CPP) and extinction-reinstatement paradigms (Fuchs *et al,* 2008b; Sorg 2012; Milton and Everitt 2013). The former is used to measure the conditioned rewarding properties of drugs of abuse, like cocaine (Tzschentke 2007; Sorg 2012). To this end, animals receive passive, experimenter-administered, injections of cocaine and are subsequently confined in one of two distinctive contexts. On alternating days, animals receive saline plus confinement in another, distinctly dissimilar context (Tzschentke 2007; Tronson and Taylor 2007). Pavlovian conditioning, as an index of the conditioned rewarding properties of cocaine, is ascertained following a brief conditioning regimen (i.e., typically 8 alternating days of cocaine/saline conditioning) by measuring preference for the cocaine-associated environment in a drug-free state (i.e., post-conditioning test; Tzschentke 2007). Conversely, in the extinction-reinstatement paradigm, animals are trained to perform an operant response for delivery of cocaine reinforcement that is either time-locked to the presentation of a discrete stimulus or is independent of CS exposure in the CS- and context-based models, respectively (Everitt *et al,* 2008; Fuchs *et al,* 2008b). To determine the ability of cocaine-paired CSs or environmental contexts to promote cocaine-seeking behavior (i.e., non-reinforced operant responses), responding is first extinguished in the cocaine-paired or an alternate context and later reinstated, or restored, upon response-contingent presentation of the cocaine-associated CS or passive re-exposure to the cocaine-paired context (Fuchs *et al,* 2008b), respectively.

Instrumental models of cocaine seeking, like the extinction-reinstatement paradigm, may be better suited for investigating the mechanisms of drug memory reconsolidation than the Pavlovian CPP model. The type of associative memories formed following passive drug

administration and undergo reconsolidation in the CPP paradigm (i.e., context-reward associations) may be less relevant to relapse that context-response-reward associations. Furthermore, the brief experimental timeline and small number of stimulus-drug pairings result in memories that significantly differ both in age and strength from cocaine memories characteristic of human addiction (Fuchs *et al* 2008b). As memory age and strength impact the ease at which long-term memories are destabilized and subsequently reconsolidated (Milekic and Alberini 2002; Suzuki *et al* 2004; Finnie and Nader 2012), all experiments in this dissertation have employed the instrumental extinction-reinstatement paradigm, in order to maximize the translational capacity of our findings to human addiction research.

Selection of Animal Models: Cue- Versus Context-based Models

Our understanding of stimulus-triggered relapse to cocaine seeking has been substantially furthered by the development of the discrete CS- and context-based extinction-reinstatement models. These models possess remarkable predictive validity (Fuchs *et al,* 2008b). Drug stimulus-evoked relapse to cocaine seeking in humans typically involves passive, inadvertent exposure to cocaine-related stimuli (i.e., attending a party and unwittingly experiencing conditions associated with previous drug use) rather than response-contingent presentation of these cues. Therefore, the contextual model has arguably better face validity for capturing the *initiation* of drug stimulus-elicited relapse to cocaine seeking, an index of incentive motivation (i.e., desire to take drug; Robinson and Berridge 1993; Koob and Volkow 2010). Conversely, the CS-based paradigm appears to assume that cue-induced motivation to seek drug precedes re-exposure to drug-related stimuli. This likely provides a measure of conditioned reinforcement and of the *maintenance* of drug stimulus-evoked relapse to cocaine seeking, (Cardinal *et al,*

2002; Everitt *et al,* 2008). Importantly, the initiation, per se, of cocaine seeking requires the retrieval and utilization of cocaine-related memories (Sorg 2012) and is therefore a more appropriate behavioral output for memory reconsolidation studies. Furthermore, the contextual variant offers increased experimental control over the CS-based model, as exposure to contextual stimuli during acquisition training is independent of responding and therefore invariable across animals (Fuchs *et al,* 2008b). Based on these considerations, the contextual extinction/reinstatement model is the preferred model for studying cocaine-memory reconsolidation and has been utilized in the dissertation experiments.

The classic contextual reinstatement model (described in detail in Fuchs *et al,* 2009; Wells *et al,* 2011, 2013 and in **Methods, Chapters II**) has been modified to permit investigation into cocaine-memory reconsolidation processes *per se*. Briefly, following self-administration training in one distinct operant context and extinction training in a different context, rats are re-exposed to the cocaine-paired context (i.e., cocaine-related memory reactivation) for 15-min to destabilize, and promote the reconsolidation of, cocaine memories (Fuchs et al. 2009). Thus, site-directed microinfusions delivered after this session are temporally suited to alter memory reconsolidation (Fuchs *et al,* 2009). The effects of these manipulations on the ability of the cocaine-paired context to reinstate extinguished cocaine-seeking behavior are tested after additional extinction training (i.e., 72 h later). In VEH-treated rats, placement into the cocaine-paired context during testing reliably reinstates cocaine-seeking behavior, operationalized for subsequent analysis as *non-reinforced* lever responding (Fuchs *et al,* 2009). A memory reconsolidation deficit is expected to manifest as a *reactivation-dependent* alteration of this behavior. Specifically, it is indicated by a lack of an effect of the manipulation in rats that do not receive explicit cocaine-memory reactivation (Tronson and Taylor 2007).

While the contextual extinction-reinstatement model of environmentally-triggered relapse is exceptional in many ways, its limitations are noted. First, similar to the CS-based model, the contextual extinction-reinstatement paradigm uses explicit extinction training to reduce cocaine-seeking behavior prior to reinstatement. With the exception of drug seeking in the presence of pharmacological antagonism (e.g., heroin-seeking following naltrexone treatment), human addicts rarely receive explicit extinction training (Katz and Higgins, 2003). In animal models, extensive extinction learning produces pronounced neuroadaptations in relapse circuitry (Sutton *et al,* 2003; Self and Choi 2004; Fuchs *et al,* 2006; Ghasemzadeh *et al,* 2009). Thus, both face and construct validity of the extinction-reinstatement paradigm are somewhat compromised by the inclusion of explicit extinction. However, in our model, extinction training is required to produce low level of cocaine seeking behavior, compared to which statistically significant, *drug context-elicited increases in cocaine-seeking behavior* can be detected. Moreover, evaluating responding in the extinction context ~48 h after cocaine memory reactivation allows for the assessment of nonspecific amnesic effects of reconsolidation inhibitor manipulations – for instance the possible disruption of context discrimination (Winocur *et al,* 2007; Winocur *et al,* 2013).

Second, in both the CS- and context-based extinction-reinstatement paradigms, rats are typically food-restricted prior to, and often during, self-administration (Lee *et al,* 2006; Fuchs *et al,* 2009). Food restriction increases the rewarding effects of psychostimulants (Carr *et al,* 2000), and thus facilitates the acquisition of cocaine-reinforced instrumental behavior, especially in the context-based model where the absence of a discrete, drug-associated CS increases attrition rates (Fuchs *et al,* 2008b). While some drug-dependent humans experience reduced caloric intake due to poverty, the anorectic effects of psychostimulants, etc., this typically occurs as a consequence,

as opposed to an antecedent, of long-term drug use (Cooper and Vanderhoek 1993; Ersche *et al,* 2010; Vanbuskirk and Potenza 2010) and is not required for a DSM V diagnosis of stimulant dependence (American Psychiatric Association 2013). Thus, forced food restriction reduces the face validity of the extinction-reinstatement paradigm and might engender a stronger, more resilient cocaine memory trace, owing to its ability to augment cocaine reinforcement during self-administration (Carr *et al,* 2000; Fuchs *et al,* 2008b). Importantly, however, the inhibition of protein kinase A (PKA), in particular, in the BLA disrupts the reconsolidation of cocaine memories and instrumental cocaine-seeking behavior following both food restriction or ad libitum food access (Arguello *et al,* 2013b and Sanchez *et al,* 2010, respectively).

Finally, lever responding during the memory reactivation session is not reinforced, and this may represent a limitation. It has been argued that extinction learning, following the non-reinforced presentations of drug-related stimuli, produces a new memory trace (Myers and Davis 2002; Eisenberg *et al,* 2003; Fischer *et al,* 2004). Thus, it is difficult to rule out that the memory reconsolidation manipulation strengthens the extinction trace instead of inhibiting the target drug memory trace. Consistent with this possibility, in some paradigms, reinforced reactivation sessions are required for full reactivation of the drug memory and subsequent disruption of conditioned behavior by memory reconsolidation inhibitors (Valjent *et al,* 2006; Milekic *et al,* 2006; Brown *et al,* 2008). However, electrophysiological analysis of amygdala neurons during fear memory reconsolidation has suggested that various elements of an associative memory are inextricably bound such that reactivation of one element of a conditioned fear memory (i.e., either the CS or US) is sufficient to induce lability and subsequent reconsolidation of the full memory trace (Diaz-Mataix *et al,* 2011). An unrelated argument in support of the use of non-reinforced memory reactivation sessions is that exclusive presentation of the CS, in the absence

of the US, necessitates memory updating, thus increasing the probability of memory destabilization and reconsolidation (Rodriguez-Ortiz *et al,* 2005; Lee 2010; Sevenster *et al,* 2012, 2013). Perhaps most important, drugs of abuse acutely activate synaptic and intracellular processes involved in memory reconsolidation, thus having the drug on board during the memory reactivation session reduces the interpretability of findings. Perhaps for this reason, non-reinforced memory reactivation sessions are relatively conventional in this line of research (Sorg 2012). Furthermore, in our hands, a 15-min non-reinforced memory reactivation session is optimal to elicit cocaine-memory destabilization/reconsolidation, while minimizing new extinction learning (Fuchs *et al,* 2009; Ramirez *et al,* 2009; Wells *et al,* 2011, 2013; Arguello *et al,* 2013b).

Neuroanatomical Substrates of Cocaine-Memory Reconsolidation

Converging lines of evidence suggest that the BLA is a critical site for memory reconsolidation (for review see Nader and Einarsson 2010), including the reconsolidation of drug-related associative memories in several models of stimulus-evoked drug-seeking behavior (Lee *et al* 2005, 2006; Milton and Everitt 2008a, 2008b; Fuchs *et al* 2009; Li *et al* 2010; Sanchez *et al* 2010; Wells *et al* 2011). Most relevant to this dissertation, our laboratory has shown that microinfusions of anisomycin, a protein synthesis and post-translational modification inhibitor (Gold 2008), into the BLA at the time of memory reconsolidation (i.e., following memory reactivation produced by re-exposure to a previously cocaine-paired context) abolishes the subsequent ability of the drug-paired context to trigger cocaine seeking (Fuchs *et al* 2009). Remarkably, this effect is *dependent on memory reactivation*, in that the same treatment is without effect when administered in the absence of explicit memory reactivation (i.e., following

exposure to a novel, unpaired context), highlighting that disruption of memory reconsolidation only affects the reactivated target memory as opposed to producing general amnesia or a protracted nonspecific deficit in the expression of cocaine seeking. These studies verify the involvement of the BLA in cocaine-memory reconsolidation; however, little is known about the molecular mechanisms of this phenomenon.

Interestingly, while the DH is an unequivocal locus of memory consolidation and the storage of newly acquired memories (Frankland and Bontempi 2005; Squire 2009), its role in memory reconsolidation remains ambiguous. Reconsolidation inhibitors used in some learning and memory paradigms have been effective in disrupting memory reconsolidation in the DH (Debiec *et al* 2002; Rossato *et al* 2006; Jobim *et al* 2012); however, evidence also exists to the contrary (Cammarota *et al* 2004). One possible explanation for this is that DH-dependent memory reconsolidation seems to be highly sensitive to boundary conditions, like memory age and strength (Nader *et al* 2000b; Finnie and Nader 2012). Experimentally, the influence of boundary conditions on memory reconsolidation in the DH has manifested as an overall failure to destabilize a memory trace (Cammarota *et al* 2004), spontaneous recovery of conditioned behaviors (Amaral *et al* 2008), and/or parametric constraints on the effectiveness of reconsolidation inhibitors, including the requirement for memory updating or extended memory reactivation sessions (Rodriguez-Ortiz *et al* 2005).

Interestingly, we have demonstrated that, unlike the BLA, the DH does not support ANI-sensitive cocaine-memory reconsolidation (Ramirez *et al* 2009), and yet the functional integrity of the DH appears to be a requirement for successful reconsolidation within our experimental parameters (Ramirez *et al* 2009). Extending these findings, we proposed that functionally significant intrahemispheric connections exist between the DH and the BLA that critically

regulate the reconsolidation of instrumental cocaine memories (Wells *et al* 2011). Using a disconnection manipulation, we demonstrated that unilateral microinfusions of ANI into the BLA plus contralateral, but not ipsilateral, microinfusions of the $GABA_{A/B}$ agonist cocktail baclofen + muscimol (B+M) into the DH (i.e., functional disconnection of the BLA and DH) robustly attenuated subsequent drug-context induced cocaine-seeking behavior in a memory reactivation-dependent manner (Wells *et al* 2011). Overall, identification of intracellular mechanisms that contribute to cocaine-memory reconsolidation in the BLA and DH will contribute to a greater understanding of the molecular mechanisms involved in the DH that facilitate BLA-dependent cocaine-memory reconsolidation and have the potential to facilitate the optimization of reconsolidation inhibition as a viable therapeutic tool and the development of highly specific candidate compounds for this purpose.

Cellular signaling during memory reconsolidation

Investigation into intracellular correlates of drug-memory reconsolidation is a relatively new area of research. Most studies so far have focused on Pavlovian drug memories underlying the expression of drug CPP (Miller and Marshall 2005; Bernardi *et al* 2006; Valjent *et al* 2006; Wang *et al* 2008; Li *et al* 2010; Wu *et al* 2011). As noted above, in the CPP paradigm, drug-memory reconsolidation is typically manipulated after a limited experimental time-line with relatively few stimulus-drug pairings (discussed in Fuchs *et al,* 2008b and above). In comparison, in instrumental models (i.e., the extinction-reinstatement paradigm), drug memory reconsolidation is targeted after a more extensive training regimen, consisting of ~weeks-months of active administration of cocaine and consequently, relatively unlimited stimulus-drug pairings (Fuchs *et al,* 2008b). Therefore, the memory trace to be manipulated in each of these models is

presumably fundamentally different due to differences in the extent of drug-induced metaplasticity in overlapping reward (Everitt *et al,* 2008) and memory circuitries (Finnie and Nader 2012). Importantly, memory reactivation durations capable of promoting the destabilization and reconsolidation of weakly trained or recently acquired conditioned fear memories are insufficient when these associations are over-trained or remote, respectively (Suzuki *et al,* 2004; Milekic and Alberini 2002). These, and other reports, lend credence to the idea that there are inextricable interactions between memory age, strength, and requirements for successful destabilization/reconsolidation (Finnie and Nader 2012). Thus, potential cellular mechanisms of drug-memory reconsolidation may differ across paradigms. Mechanisms assessed in CPP models must also be evaluated in instrumental models of cocaine seeking (e.g., the extinction-reinstatement paradigm; Fuchs *et al* 2008). Therefore, investigation of the cellular mechanisms of instrumental drug-memory reconsolidation is necessary in order to fill a critical gap in our understanding of memory reconsolidation as it pertains to addiction.

Extracellular Signal-Regulated Kinase (ERK) Signaling in Cocaine-Memory Reconsolidation

Core elements of the ERK signaling pathway, including upstream activators and downstream substrates of ERK, appear to be critical for the restabilization of retrieved memories, in general. For instance, activation of the NMDAR or β-adrenergic receptor-mediated signaling pathways, both of which converge on ERK (Adams and Sweatt 2002), have been indicated in other forms of memory reconsolidation (Bozon *et al* 2003; Kelly *et al* 2003; Duvarci *et al* 2005; Miller and Marshall 2005; Lee *et al* 2005, 2006; Milton *et al* 2008a, 2008b*;* Tronson and Taylor 2007; Valjent *et al* 2000, 2006). Specifically, ERK itself and the immediate early gene

zincfinger268 (zif268), which is activated downstream from ERK activation (Bozon *et al,* 2003), in the nucleus accumbens (NAC) have been implicated in the reconsolidation of Pavlovian memories that support cocaine-CPP (Miller and Marshall 2005; Theberge *et al* 2010). However, NAC zif268 is not required for the reconsolidation of cocaine-memories that maintain instrumental cocaine seeking (Theberge *et al* 2010). NMDAR or β-adrenergic receptor stimulation, PKA activation, and zif268 expression in the BLA have been implicated in the reconsolidation of CS-drug memories that support explicit CS-induced instrumental cocaine seeking (Lee *et al* 2005; Milton *et al* 2008a, 2008b; Sanchez *et al* 2010). However, no study to date has evaluated the role of ERK signaling or elements of the MEK/ERK signaling pathway in the reconsolidation of contextual memories that control instrumental cocaine-seeking behavior.

Nuclear Factor-κB Signaling in Cocaine-Memory Reconsolidation

The nuclear factor κB (NF-κB) is a transcription factor whose role in synaptic plasticity and memory acquisition and consolidation has been well documented (Barger *et al,* 2005; Romano *et al,* 2006; Oikawa *et al,* 2012), while its role in memory reconsolidation is gaining appreciation (Boccia *et al,* 2007; de la Fuentes *et al,* 2011; Lee and Hynds 2012; Si *et al,* 2012). In the cytoplasm of neurons, release of NF-κB from IκB, the inhibitory protein to which it is constitutively bound, requires the phosphorylation of IκB by IκB kinase (IKK; Romano *et al,* 2006a). Recently, a NMDAR-IKK-NF-κB signal transduction cascade was identified as being an important regulator of contextual fear memory reconsolidation, but not consolidation, in the DH (Lee and Hynds 2013). NMDAR stimulation is required for the reconsolidation of cocaine-related memories in both CPP (Brown *et al,* 2008) and instrumental models (Milton *et al,* 2008b). Therefore, NF-κB activation is at least in part, implicated in this phenomenon.

Consistent with this, transcriptional activity of NF-κB in the BLA is necessary for the reconsolidation of a morphine-associated memory underlying the expression of morphine CPP (Yang *et al,* 2011), but, to date, the role of NF-κB activation in BLA-mediated instrumental contextual cocaine-memory reconsolidation is unknown.

Src Family of Tyrosine Kinases in Cocaine-Memory Reconsolidation

The Src family of tyrosine kinases (SFKs), including Src and Fyn, are thought to play an important role in synaptic plasticity, likely related to their ability to regulate the activity of neurotransmitter receptors (Ohnishi *et al* 2011). Specifically, SFKs can enhance NMDAR-mediated currents (Wang and Salter 1994; Yu *et al* 1997) and promote the induction of LTP in hippocampal slice preparations (Yu *et al* 1997). The SFK-mediated enhancement of NMDAR function is likely related to the ability of SFKs to phosphorylate the NR2b NMDA receptor subunit at tyrosine residue 1472 (Tyr-1472) or the NR2a NMDAR subunit at tyrosine residue 1325 (Tyr-1325; Ohnishi *et al,* 2011). The former is necessary for the successful translocation of NMDARs to the synaptic membrane (Nakazawa *et al* 2001; Zhang *et al* 2008) and for interfering with adaptor protein 2 complex (AP2) binding to the same residue and thus the clatharin-mediated endocytosis of NMDARs (Prybylowski *et al* 2005). As such, SFKs tightly regulate the surface expression of NR2b-containing NMDARs (Salter and Kalia 2004). With respect to NR2a function, SFK-mediated phosphorylation of Tyr-1325 enhances NMDAR-mediated currents in the striatum (Taniguchi *et al,* 2009) and in slices from the CA1 subregion of the DH (Yang *et al,* 2012). Additionally, SFKs phosphorylate the $GABA_A$ receptor (Vithlani and Moss 2009) and the GluR2 subunit of the AMPA receptor (Hayashi *et al* 1999; Hayashi and Huganir 2004), although the functional relevance of these latter interactions is less understood (Ohnishi *et al,* 2011).

Despite ample data outlining SFK-dependent interactions in cell cultures and slice preparations, the functional role of SFKs *in vivo* has not been well characterized. Our laboratory has recently demonstrated that SFK activation in the DH is critical for the expression of drug context-induced cocaine-seeking behavior, via an NR2b-dependent mechanism (Xie *et al,* 2013). However, whether SFK activation in the DH similarly regulates the reconsolidation of contextual cocaine memories remains to be determined, and the downstream substrates of putative SFK involvement in cocaine-memory reconsolidation is unknown.

Overview of the Experiments

The overarching goal of this dissertation was to increase our understanding of the cellular and molecular mechanisms by which the BLA and DH contribute to the reconsolidation of contextual-cocaine memories that drive cocaine-seeking behavior in the contextual extinction-reinstatement paradigm (Fuchs *et al,* 2008b). A secondary objective was to draw comparisons between our findings and existing literature that has explored the significance of these mechanisms in a Pavlovian model of cocaine-seeking, where possible. To accomplish these objectives, rats were allowed to self-administer cocaine in one context and then underwent extinction training in a different context. Rats were re-exposed to the cocaine-associated context for 15-min to allow for memory destabilization and subsequent reconsolidation. Manipulations were adminstered during the period of putative memory reconsolidation and subsequent drug context-induced motivation for cocaine was measured in the cocaine-paired context after 2 or more days of additional extinction training. Experiments in Chapter 2 evaluated whether activation of the MEK/ERK signaling cascade in the BLA would be necessary for successful memory reconsolidation and consequently, preserved drug context-induced cocaine-seeking

behavior. An additional exploration into the role of ERK signaling in the NACc in this phenomenon was also performed, to follow up on and provide comparison to an earlier study using the CPP paradigm (Miller and Marshall 2005). Experiments in Chapter 3 explored whether IκK-dependent activation of NF-κB would regulate cocaine-memory reconsolidation. Finally, experiments in Chapter 4 examined whether, similar to their role in regulating the expression of drug context-induced cocaine-seeking behavior (Xie *et al,* 2013), SFKs in the DH would critically control the reconsolidation of memories subserving this phenomenon.

CHAPTER 2

CONTRIBUTION OF THE MEK/ERK SIGNALING CASCADE TO THE RECONSOLIDATION OF INSTRUMENTAL CONTEXTUAL COCAINE MEMORIES IN THE BASOLATERAL AMYGDALA

INTRODUCTION

Extracellular signal regulated kinase (ERK) is one member of a larger mitogen-activated protein kinase (MAPK)-signaling cascade, whose role in synaptic plasticity underlying both associative learning/memory and addiction has been well characterized (for review, see Davis and Laroche 2006 and Lu *et al,* 2006, respectively). Activation of the canonical MAPK/ERK pathway begins with receptor stimulation by growth factors at the cell surface and the successive activation of Ras, Raf, and MAPK kinase (MEK). In turn, MEK-induced dual phosphorylation of specific threonine and tyrosine residues in the activation loop of both ERK 1 (44 kDA) and ERK 2 (42 kDA) isoforms triggers the full activation of kinase activity of these molecules (Davis and Laroche 2006). However, ERK activation can also be achieved via stimulation of certain non-growth factor extracellular and upstream intracellular signals (Sweatt 200), including known regulators of drug-memory reconsolidation - β-adrenergic (Milton *et al,* 2008b; Fricks-Gleason and Marshall 2008) and N-methyl aspartate receptors (Brown *et al,* 2008; Milton *et al*, 2008a), as well as the activation of protein kinase A (Sanchez *et al,* 2010; Arguello *et al,* 2013b).

Several lines of evidence suggest that ERK plays a fundamental role in memory reconsolidation. In turn, ERK phosphorylates several transcription factors (Goodman 1990; Impey *et al,* 1999; Sgambato *et al,* 1998) that have been implicated in drug-memory reconsolidation, including the cAMP response element binding protein (CREB) and E twenty-six (ETS)-like transcription factor 1 (Miller and Marshall 2005). Furthermore, MEK/ERK inhibition at the putative time of memory reconsolidation disrupts subsequent object recognition memories (Kelly *et al,* 2003; Silingardi *et al,* 2011), conditioned-fear associations (Duvarci *et al,* 2005), and importantly, Pavlovian cocaine-related memories that underlie the expression of cocaine conditioned place preference (CPP; Miller and Marshall 2005; Valjent *et al,* 2006).

As noted in the Introduction (**Chapter 1**), there are several fundamental differences between cocaine CPP and the instrumental extinction-reinstatement paradigm, and this necessitates further investigation into the role of the MAPK/ERK signal transduction cascade in *instrumental* drug-memory reconsolidation *per se*. For instance, relative to instrumental extinction-reinstatement paradigm, cocaine CPP has a shorter experimental timeline and fewer stimulus-drug pairings. Moreover, the use of passive versus active administration of drug in CPP and instrumental models, respectively, likely confers metaplasticity in some mutually exclusive, albeit partially overlapping, memory circuits (Everitt *et al* 2008). This may impact the ease with which drug-related memories are destabilized and reconsolidated (Finnie and Nader 2012) and the neural mechanisms that are recruited for these phenomena. Hence, Experiment 1 utilized the extinction-reinstatement model of environmentally-triggered drug relapse in order to determine whether ERK signaling would be required for the reconsolidation of *context-response-cocaine* memories in the basolateral amygdala (BLA) and the nucleus accumbens core (NACc), two brain regions that critically regulate drug-memory reconsolidation in instrumental (Sanchez *et al*,

2010; Li *et al*, 2010; Fuchs *et al*, 2009) and Pavlovian models of cocaine seeking (Miller and Marshall, 2005; Théberge *et al*, 2010), respectively.

METHODS

Animals

Male Sprague-Dawley rats (N = 98; 275-300 g; Charles River Laboratories, Wilmington, MA) were individually housed in a temperature- and humidity-controlled vivarium on a reversed light-dark cycle. Rats were fed 20-25 g of rat chow per day with water available *ad libitum*. Protocols detailing the housing and treatment of rats were consistent with specifications in the *Guide for the Care and Use of Laboratory Rats* (Institute of Laboratory Animal Resources on Life Sciences, 1996) and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Food training

To facilitate subsequent cocaine self-administration, rats were initially trained to lever press under a continuous schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH, USA) in sound-attenuated operant conditioning chambers (26 x 27 x 27 cm high; Coulbourn Instruments, Allentown, PA, USA) during a 16-h overnight session. Responses on a designated active lever produced one food pellet, whereas responses on an alternate, inactive lever had no scheduled consequences. None of the contextual stimuli that would be subsequently used for self-administration and extinction training were present during food training.

Surgery

Forty-eight h after food training, rats were fully anesthetized with a cocktail containing ketamine hydrochloride (75 mg/kg, i.p.) and xylazine (5 mg/kg i.p.). Rats were implanted with chronic indwelling jugular catheters that had been constructed in house, as described previously (Fuchs *et al,* 2009). Following jugular catheter insertion, rats were implanted with 26-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA), aimed bilaterally at the BLA (-2.7 mm AP, ± 5.1 mm ML, -6.7 mm DV, relative to bregma) or at the NACc (angled laterally by 10°;+1.4 mm AP, ±3.1 ML, -4.8 mm DV, relative to bregma), based on Paxinos and Watson (1997), using standard stereotaxic procedures. Stainless steel screws and cranioplastic cement were used to secure the guide cannulae to the skull. Stylets (Plastics One) were cut to size so they did not extend beyond the guide cannulae and were inserted into the guide cannulae to prevent occlusion. Tygon caps and crystalline applicators (Plastics One) were used to seal the catheter.

To extend catheter patency, catheters were flushed daily with an antibiotic solution of cefazolin (10.0 mg/ml; Schein Pharmaceuticals, Albuquerque, NM, USA) and heparinized saline (70 U/ml; Baxter Health Care Corp, Deerfield, IL, USA), as described previously (Fuchs *et al,* 2009). Rats received 5 days of post-operative recovery before the start of self-administration training. Catheter patency was assessed several times during the experiment by administering propofol (1mg/0.1ml, i.v. Eli Abbott Lab, North Chicago, IL, USA) intravenously and confirming a rapid loss of muscle tone.

Cocaine self-administration training

All behavioral training (self-administration, extinction, memory reactivation, reinstatement testing) took place in sound-attenuated operant conditioning chambers configured to one of two distinctly different contexts, as described previously (**Table 1:** Contexts A, B). Context A contained a continuous red house light (0.4 fc brightness) on the wall opposing the active lever, an intermittent pure tone (80 dB, 1 kHz, 2 sec on, 2 sec off), a pine-scented air freshener strip (4.5 x 2 cm, Car Freshener Corp, Watertown, NY, USA), and wire mesh flooring (26 X 27 cm). Context B contained an intermittent white stimulus light located above the inactive lever (1.2 fc brightness, 2 sec on, 4 sec off), a continuous pure tone (75 dB, 2.5 kHz), a vanilla-scented air freshener strip (4.5 x 2 cm, Sopus Products, Moorpark, CA, USA), and a slanted ceramic tile that bisected the bar floor (19 cm X 27 cm)

Rats were randomly assigned to Context A or Context B for self-administration training. During daily, 2-h self-administration training sessions during the dark cycle, rats were allowed to press the active lever for infusions of cocaine (cocaine hydrochloride; 0.15 mg/0.05 mL per infusion, i.v.; NIDA, Research Triangle Park, NC, USA) under a fixed ratio-1 (FR-1_ schedule of cocaine reinforcement. The rats' catheters were connected to an infusion apparatus (Coulbourn Instruments) via polyethylene 20 tubing and liquid swivels (Instech). Specifically, one active lever press activated an infusion pump for 2 s, followed immediately by a 20-s time-out period, during which active lever responses had no consequences. Throughout the sessions, responses on the other (inactive) lever were recorded but had no programmed consequences. Training continued until rats reached a criterion of ≥10 cocaine infusions per session on at least 10 sessions (i.e., acquisition criterion). Data collection and reinforcer delivery were controlled using Graphic State Notation software version 2.102 (Coulbourn).

Table 1. Contextual Stimuli

CTX	Stimulus Components			
	Auditory	**Visual**	**Tactile**	**Olfactory**
A	Intermittent pure tone (80 dB, 1 kHz; 2s on, 2s off)	Continuous red houselight	Wire mesh floor (26 cm x 27 cm)	Pine-scented air freshener strip (4.5 cm x 2 cm)
B	Continuous pure tone (75 dB, 2.5 kHz)	Intermittent white stimulus light (2s on, 4s off)	Bar floor (19 cm x 27 cm) and slanted ceramic tile	Vanilla-scented air freshener strip (4.5 cm x 2 cm)
No reactivation control context	Continuous complex tone (80 dB, alternating between 1, 1.5, and 2.5 kHz at 1 s intervals)	2 continuous white stimulus lights and continuous red houselight	Ceramic tile flooring (26 cm x 27 cm)	Citrus-scented air freshener strip (4.5 cm x 2 cm)

Table 1. Multi-modal stimuli that were used in operant conditioning chambers for all experiments. Context (CTX) A and context B were used for self-administration and extinction training. The no reactivation control context was used exclusively as a novel, unpaired environment in Experiments 1b and 1c, to model the absence of explicit cocaine-memory reactivation.

Extinction Training

After meeting the acquisition criterion, rats received seven daily 2h extinction training sessions in the context that had not been used for cocaine self-administration training. Active and inactive lever responses were recorded but had no programmed consequences. Following the fourth extinction-training session, rats were adapted to the intracranial microinfusion procedure (i.e., sham infusions). To this end, 33-gauge injection cannulae (Plastics One) were inserted into the rats' guide cannulae to a depth 2 mm below the tip of the guide cannulae and were left in place for 4 minutes. No liquid was infused. For rats in experiment 1c, whose brains would be subsequently collected at the time of microinfusions for western blot analysis of ERK1/2 activation, the sham infusion procedure was repeated following the sixth and seventh

extinction training sessions, to maximize acclimation and to fully circumvent the possible effects of infusion stress on protein expression on the day of the infusion. However, the injection cannulae were only fully lowered once, to minimize cell loss/damage.

Experiment 1a

Experiment 1a evaluated whether intra-BLA administration of the MEK/ERK inhibitor, U0126, which prevents the phosphorylation-dependent activation of ERK (London and Clayton 2008), would impair instrumental, context-response-cocaine memory reconsolidation. On the day after the last extinction training session, rats were re-exposed to the cocaine-paired context for 15 min to initiate the destabilization and reconsolidation of cocaine memories (i.e., cocaine-memory reactivation; Fuchs *et al,* 2009; see **Fig. 1.1A**). The destabilization and subsequent reconsolidation of a memory trace is highly sensitive to interactions between memory reactivation duration and certain boundary conditions, such as the age and strength of the memory (Suzuki *et al,* 2004, Inda *et al,* 2011, Finnie and Nader 2012). As such, 15-min was selected in the present study based on an earlier parametric analysis, which demonstrated that under our experimental conditions; a 15-min re-exposure session produces optimal memory reconsolidation with minimal observable extinction learning (Fuchs *et al,* 2009). During the 15-min cocaine-memory reactivation session, levers were extended but cocaine was not infused upon lever pressing given that cocaine itself stimulates ERK phosphorylation (Zhai et al. 2008). Immediately after the session, rats received bilateral microinfusions of 5% DMSO/6% TWEEN vehicle (VEH) or U0126 (1.0 μg/ 0.5 μl/hemisphere) into the BLA. The dose of U0126 was chosen based on a previous report, in which this concentration of U0126, when microinfused into the NACc, robustly impaired Pavlovian cocaine-memory reconsolidation (Miller and Marshall,

2005). Treatment assignment was counterbalanced based on previous cocaine intake. During the microinfusion procedure, injection cannulae were lowered to a depth of 2 mm below the tip of the guide cannulae and were connected to Hamilton syringes (Hamilton Co.) that were mounted on a microdrive pump (KD Scientific). The microinfusion was delivered over 2 min, and the injection cannulae were left in place for 1 min before and 1 min after the infusion, in order to minimize drug diffusion out of the BLA.

Post-reactivation Extinction and Test of Drug Context-induced Cocaine Seeking

Beginning the day following cocaine-memory reactivation, rats received additional daily 2-h extinction-training sessions (2.28±0.20 days) until they reached the extinction criterion (i.e., ≤25 active lever responses per session on a minimum of 2 consecutive days). Twenty-four hours later, rats were returned to the cocaine-paired context for a 2-h test of drug context-induced cocaine-seeking behavior. Cocaine-seeking behavior was operationally defined as non-reinforced active lever presses and is considered to be a reliable index of context-elicited motivation for cocaine. During the test session, both active and inactive lever responses were recorded but had no programmed consequences.

Experiment 1b

A genuine memory reconsolidation impairment can only be inferred when a reconsolidation inhibitor manipulation impairs the target memory trace without impacting non-specific memories or general motor performance (Nader *et al,* 2000b; Alberini 2006; Tronson and Taylor 2007; Sorg 2012). This can be confirmed experimentally by demonstrating that memory reconsolidation inhibitor treatments are only effective when administered in conjunction

with, but not in the absence of, explicit memory reactivation, conditions expected to destabilize versus preserve the stability of, the memory trace, respectively (Tronson and Taylor 2007). Hence, Experiment 1b was designed to evaluate whether the effects of U0126 in the BLA would require explicit memory reactivation. To this end, experimental protocols mirrored those in Experiment 1a (see **Fig. 1.1A**), except that no-reactivation control groups were placed into a novel, unpaired context (i.e., No-reactivation control context; **Table 1**) for 15 min. The control context distinctly differed from Contexts A and B. The no-reactivation control context contained continuous white stimulus lights, located above each lever, a continuous red house light on the wall opposing the active lever, a continuous complex tone (80 dB, alternating between 1, 1.5, and 2.5 kHz at 1 sec intervals), a citrus-scented air freshener strip (4.5 cm x 2 cm, Locasmarts LLC), and ceramic tile flooring (26 cm x 27 cm). Exposure to this alternate context was expected to circumvent explicit reactivation of the context-response-cocaine memory trace while producing similar behavioral history to that in Experiment 1a – for instance, transportation to the testing room; novelty akin to that experienced by the groups in Experiment 1a that were re-exposed to the cocaine-paired context ~7 days after self-administration training, and access to levers. Immediately after the end of the session in the control context, rats received bilateral microinfusions of VEH or U0126 into the BLA.

Experiment 1c

The MEK/ERK signaling pathway contains several points of convergence for upstream extracellular and intracellular signals, in particular at the level of Ras/Raf activation and at the level of gene transcription (Davis and Laroche 2006). As such, ERK activation is a principal component in a variety of cellular processes underlying a diverse repertoire of behaviors and

processes. For instance, ERK is activated in the hippocampus during exposure to novel stimuli and is required for novelty-induced enhancement of memory consolidation (Izquierdo *et al,* 2002). ERK is also hyperphosphorylated in the nucleus accumbens core and dorsal striatum during the expression of cocaine (Miller and Marshall 2005) and methamphetamine CPP (Mizoguchi *et al,* 2004), respectively, and this increased activation was demonstrated to be required for CPP expression in both studies (Miller and Marshall 2005 and Mizoguchi *et al,* 2004).

In the present study, re-exposure to the cocaine-paired context occurred ~7 days or more after self-administration and therefore was likely associated with some perceived novelty. Because both novelty and cocaine-seeking behavior during the memory reactivation session can impinge on ERK phosphorylation, Experiment 1c was designed to validate that the temporal parameters used in Experiment 1a are optimal for U0126-induced transient and selective suppression of ERK1/2 activation related to memory reconsolidation processing *per se*. Specifically, Experiment 1c assessed changes in phosphorylated ERK1/2 using western blotting. Experimental parameters were identical to those in Experiment 1a/b (see **Fig. 1.1A, 1.3A**) except that rats were exposed to the cocaine-paired or the novel, unpaired context for 15 min or they stayed in their home cages, prior to receiving bilateral microinfusions of VEH or U0126 into the BLA (see **Fig. 1.3A)**.

The rats were euthanized 30 min later based on the phosphorylation kinetics of ERK1/2 (Valjent *et al,* 2000; Choe and McGinty, 2001; Zhang *et al*, 2004; Miller and Marshall, 2005) in order to capture maximal ERK phosphorylation related to the onset of memory reconsolidation (Pedreira and Maldonado 2003). These rats were compared to rats euthanized after the final test session (i.e., ~72 h after treatment) in Experiment 1a. Following euthanasia by rapid

decapitation, brains were removed, flash frozen in isopentane, and stored at -80°C. Punches were taken from the BLA or the dorsally adjacent posterior caudate putamen (pCPu; i.e., anatomical control) with 19Ga neuropunches (Fine Science Tools) from 40μm tissue sections, which were also collected to verify cannula placement. Punched tissue was stored at -80°C in lysis buffer containing 10 mM HEPES, 1% SDS, and 1x protease and phosphatase inhibitor cocktails (Sigma Aldrich, St. Louis, MO).The pCPu was chosen as an anatomical control region, as previous reports suggest that the likelihood of drug diffusion following intracranial manipulations is greatest in the dorsal direction, relative to the injection site (Baker *et al,* 1996; Neisewander *et al,* 1998).

Western Blotting

Samples were thawed on ice, manually homogenized, and boiled for 10 min at 100 °C. Protein concentrations were determined using the Biorad DC protein assay. For each sample, 15 μg of protein were electrophoresed on a 12% Tris-HCL polyacrylamide gel and transferred to a polyvinylidene difluoride membrane for 1 h at 100 V. Membranes were then blocked in 5% milk for 1 h and incubated in rabbit anti-phosphorylated ERK1/2 (pERK1/2; 44/42 kDA, respectively) (1:2000, Cell Signaling, Beverly, MA) overnight (16-20 h) at 4 °C. Membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (1:10,000, GE Healthcare, Piscataway, NJ), for 1 h followed by development with an enhanced chemiluminescence (ECL) system (Pierce Biotech, Rockford, IL). Membranes were subsequently incubated with stripping buffer (62.5 mM Tris-HCL at pH 6.7, 2% SDS, 100 mM beta-mercaptoethanol) to permit re-probing with antibodies to total (phosphorylated plus unphosphorylated) ERK1/2 (1:2000, Cell Signaling, Beverly, MA) and later actin (i.e., loading control; 1:50,000, Santa Cruz

Biotechnology, Santa Cruz, CA). Protein levels of pERK1/2, total ERK1/2, and actin were quantified by densitometry, using NIH Image J software. To evaluate changes in ERK activation, pERK1/2 was normalized to total ERK1/2 and actin. Subsequent discussion of ERK1/2 activation refers to normalized levels.

Experiment 1d

The nucleus accumbens core (NACc) is an important substrate of ERK-dependent Pavlovian cocaine-memory reconsolidation required for the expression of cocaine CPP (Miller and Marshall 2005). ERK involvement requires activation of elements of the MEK/ERK signaling cascade including EKR1/2 phosphorylation (Miller and Marshall 2005), and it results in the expression of the immediate early gene *zif268* (Theberge *et al,* 2010). Interestingly, a recent study indicated that *zif268* expression in the NACc is *not necessary* for the reconsolidation of an *instrumental* cue-cocaine memory underlying conditioned reinforcement (Theberge *et al,* 2010). Hence, Experiment 1d assessed whether intra-NACc administration of U0126 would impair instrumental contextual cocaine-memory reconsolidation and the subsequent expression of drug context-induced cocaine seeking. The experimental parameters used were identical to those in Experiment 1a, except that rats received bilateral microinfusions of VEH or U0126 into the NACc immediately after cocaine-memory reactivation (see **Fig. 1.4A**).

Experiment 1e

Experiment 1e investigated whether global neural inactivation of the NACc would impede cocaine-memory reconsolidation, in order to evaluate the possibility that the NAC contributes to memory reconsolidation by an ERK-independent mechanism. The procedures used

were identical to those in Experiment 1d, except that rats received bilateral intra-NACc microinfusions of phosphate buffered saline VEH or the GABAA/B agonist cocktail, baclofen+muscimol (B+M; 106.8/5.7 ng/0.5µl/hemisphere), immediately after cocaine memory reactivation (see **Fig. 1.4A**). B+M suppresses neuronal activity while sparing fibers of passage (Martin and Ghez, 1999).

Histology

In Experiments 1a, 1b, 1d, and 1e, rats were overdosed with ketamine hydrochloride and xylazine (66.6 and 1.3 mg/kg, i.v. or 199.8 and 3.9 mg/kg, i.p., respectively, depending on catheter patency) and transcardially perfused with a 1x phosphate buffered saline (Fischer Scientific) plus 10% formaldehyde solution (Sigma). Brains were dissected out and stored in 10% formaldehyde solution until they were sectioned coronally using a vibratome. Cannula placements were verified on 75µm sections stained using cresyl violet (Kodak, Rochester, NY, USA). The most ventral portion of each cannula tract was mapped onto schematics from the rat brain atlas (Paxinos and Watson, 1997).

Data Analysis

Separate t-tests were used to test for possible pre-existing differences in cocaine intake, in active and inactive lever responding during self-administration training (mean of last 3 d), extinction training (days 1 and 7), and during the memory reactivation session, as well as in the number of days required to reach the post-reactivation extinction criterion for rats in Experiments 1a-1e that would subsequently receive either VEH or drug (U0126 or B+M) treatment following cocaine-memory reactivation or no memory reactivation. Separate mixed-

factorial ANOVAs were conducted to examine the effects of post-reactivation manipulations on active and inactive lever responses on the test days in the cocaine-paired and extinction contexts (last extinction session before the test in the cocaine-paired context). In these analyses, treatment (VEH, U0126, B+M) was included as a between-subjects factor and testing context (EXT, COC-paired) was included as a within-subjects factor. Significant main and interaction effects were further examined using post-hoc Tukey tests. In Experiment 1c, a one-way ANOVA was conducted to examine the effects of context (cocaine-paired, novel, home-cage) on pERK1/2 activation (i.e., ratio of phosphorylated to total ERK1/2 protein) in the BLA in VEH-treated rats. Separate t-tests were used to examine the effects of U0126 treatment on pERK1/2 activation in the BLA or posterior caudate putamen (pCPu; anatomical control), relative to VEH. Alpha was set at 0.05.

RESULTS

Histology

Schematics and photomicrographs illustrating cannula placements are included in **Figures 1.1B 1.2,** and **1.4B**. The target brain regions were defined as the lateral and basolateral nuclei of the amygdala (BLA) and the nucleus accumbens core (NACc). High power microscopy did not reveal abnormal tissue damage (i.e., extensive cell loss or gliosis) at injection sites. Only data from rats with correct cannula placements were included in statistical analyses.

Behavioral History

There were no pre-existing differences between the groups in cocaine intake, active or inactive lever responding during cocaine self-administration training, active or inactive lever

responding on extinction training days 1 and 7, or the memory reactivation session; or in the number of days required to reach the extinction criterion (all $Ts \leq 2.07$, $Ps \geq 0.06$) . These descriptive statistics are provided in **Table 2**.

Table 2. Behavioral History

Exp./Group		Cocaine Intake	SA	EXT 1	EXT 2	Reactivation	Days to EXT
1a	VEH (*N*=7)	23.1 ± 2.6	75.9 ± 15.6	77.0 ± 28.1	10.3 ± 4.9	26.9 ± 4.9	2.57 ± 0.30
	U0126 (*N*=8)	30.0 ± 3.8	69.8 ± 11.9	69.8 ± 14.1	12.8 ± 2.9	17.9 ± 7.6	2.13 ± 0.13
1b	VEH/NR (*N*=7)	26.2 ± 3.1	59.4 ± 14.0	74.4 ± 24.0	3.6 ± 0.7	10.5 ± 4.2	2.00 ± 0.00
	U0126/NR (*N*=8)	21.2 ± 1.2	52.8 ± 10.0	60.3 ± 16.2	2.9 ± 0.7	13.0 ± 5.3	2.25 ± 0.25
1c	VEH (*N*=6)	25.9 ± 2.6	68.1 ± 12.5	88.3 ± 27.3	13.0 ± 6.2	37.6 ± 8.9	N/A
	U0126 (*N*=8)	23.8 ± 2.1	62.9 ± 8.7	64.2 ± 12.7	10.0 ± 2.4	30.3 ± 13.8	N/A
	VEH/Nov (*N*=8)	21.4 ± 4.2	59.2 ± 7.9	44.6 ± 14.6	7.6 ± 3.0	9.1 ± 2.3	N/A
	U0126/Nov (*N*=6)	21.4 ± 2.8	59.5 ± 8.6	39.7 ± 13.1	7.2 ± 3.0	11.5 ± 5.8	N/A
	VEH/HC (*N*=6)	26.1 ± 2.3	72.6 ± 26.7	63.3 ± 22.8	9.0 ± 5.3	N/A	N/A
	U0126/HC (*N*=5)	28.5 ± 10.4	74.9 ± 36.1	67.2 ± 17.7	29.3 ± 21.28	N/A	N/A
1d	VEH (*N*=7)	26.2 ± 3.0	57.5 ± 7.2	78.0 ± 22.6	5.3 ± 2.0	24.4 ± 3.5	2.43 ± 0.3
	U0126 (*N*=7)	26.3 ± 1.9	62.8 ± 10.3	39.6 ± 16.1	6.3 ± 1.9	13.1 ± 6.4	2.14 ± 0.14
1e	VEH (*N*=7)	27.1 ± 2.8	72.9 ± 8.7	41.3 ± 10.8	6.7 ± 1.6	34.3 ± 8.7	2.40 ± 0.25
	B+M (*N*=6)	25.2 ± 4.6	61.5 ± 11.5	41.6 ± 13.1	5.1 ± 2.2	29.4 ± 9.6	2.33 ± 0.21

Table 2. Cocaine intake (mean ± SEM infusions/session for the last 3 training sessions) and active lever responses during self-administration (SA; mean ± SEM for the last 3 training sessions), as well as active lever responses (mean ± SEM) during the first (Extinction 1) and last (Extinction 7) extinction training sessions, and during the 15-min memory reactivation (Reactivation) session. The number of days required to meet the extinction criterion (Days to EXT) is also included. Means are provided for rats that received U0126 or 5% DMSO/6% TWEEN VEH into the BLA (Experiment 1a-c) or NACc (Experiment 1d) or B+M or PBS VEH into the NACc (Experiment 1e) after cocaine-memory reactivation, novel context exposure (Nov, no reactivation controls), or confinement to their home cages (HC, no reactivation controls).

Experiment 1a: Effects of U0126 microinfused into the BLA following cocaine-memory reactivation on subsequent drug context-induced cocaine seeking

U0126 administration into the BLA following cocaine-memory reactivation altered subsequent cocaine seeking in a context-dependent fashion (**Fig. 1.1C**; ANOVA context x treatment interaction, $F_{(1,13)}$=5.15, P=0.03; context main effect, $F_{(1,13)}$=33.54, P<0.001; treatment main effect, $F_{(1,13)}$=5.01, P=0.04). In the group that had received VEH into the BLA following cocaine-memory reactivation, active lever responding increased upon exposure to the cocaine-paired context at test, relative to that in the extinction context (Tukey's test, P<0.05). Conversely, the group that had received U0126 responded less on the active lever in the cocaine-paired context, but not the extinction context, relative to the VEH-treated group (Tukey's test, P<0.05). As a result, the U0126-treated group exhibited no difference in active lever responding in the extinction and cocaine-paired contexts.

Experiment 1b: No reactivation control experiment

U0126 administration into the BLA following exposure to a novel, unpaired context failed to alter subsequent drug context-induced cocaine seeking (**Fig. 1.1D**). Active lever responding increased upon exposure to the cocaine-paired context at test, relative to that in the extinction context (ANOVA context main effect only, $F_{(1,13)}$=13.15, P=0.03). Furthermore, there was no difference between the VEH- and U0126-treated groups in active lever responding in either context (treatment main and interaction effects, $F_{(1,13)}$=0.01-0.20, P=0.66-0.91).

Experiment 1c: Effects of U0126 in the BLA on ERK1/2 activation

Quantitative Western blot analyses indicated that U0126 administration into the BLA significantly reduced ERK1 and ERK2 phosphorylation in the BLA in a transient and memory reactivation-dependent manner. Specifically, bilateral microinfusions of U0126 following explicit cocaine-memory reactivation significantly attenuated ERK1 (*t(13)*=3.095, *P*=0.009) and ERK2 (*t(13)*=3.062, *P*=0.009) activation in the BLA, relative to VEH (**Fig. 1.3B**) when assessed 30 min post treatment. Conversely, when U0126 treatment was administered following exposure to the novel, unpaired context (**Fig. 1.3C**) or the home cage (**Fig. 1.3D),** it did not alter ERK1 (*t(12)*=0.476, *P*=0.64; *t(9)*=0.505, *P*=0.63, respectively) or ERK2 (*t(12)*=0.165, *P*=0.87; *t(9)*=0.960, *P*=0.36, respectively) activation in the BLA, relative to VEH. Separate one-way ANOVAs indicated a marginal difference in ERK2 ($F(2,17) = 3.25$ $P=0.06$), but not in ERK1 ($F(2,17) = 1.29$, $P = 0.30$), activation between the groups that received VEH after exposure to the cocaine-paired context, the novel context, or the home cage. Furthermore, planned pairwise comparisons revealed that there was an increase in ERK2, but not ERK1, activation in these groups following exposure to the cocaine-paired context (one-tail $t(10) = 2.042$, $P = 0.03$), but not the novel context (one-tail $t(12) = 0.675$, $P = 0.26$), relative to the home cage.

The effects of intra-BLA U0126 administration on ERK1/2 activation were anatomically selective and transient. Intra-BLA infusions of U0126 following cocaine-memory reactivation did not alter ERK1 (*t(12)*=0.673, *P*=0.51) or ERK2 (*t(12)*=0.196, *P*=0.84) activation in the pCPu, relative to VEH (**Fig. 1.3E**). Furthermore, U0126 administered following cocaine-memory reactivation did not alter ERK1 (*t(12)*=0.073, *P*=0.94) or ERK2 (*t(12)*=0.160, *P*=0.88) activation in the BLA, relative to VEH when assessed ~72 h post treatment, immediately after the test of cocaine seeking (**Fig. 1.3F**).

Experiment 1d: Effects of U0126 microinfused into the NACc following cocaine-memory reactivation on subsequent drug context-induced cocaine seeking

U0126 administration into the NACc after cocaine-memory reactivation did not alter subsequent drug context-induced cocaine seeking (**Fig. 1.4C**). Active lever responding increased upon exposure to the cocaine-paired context at test, relative to that in the extinction context (ANOVA context main effect only, $F_{(1,12)}$=35.88, P<0.001). Furthermore, there was no difference between the previously VEH- and U0126-treated groups in active lever responding in either context (treatment main and interaction effects, $F_{(1,12)}$=0.67-1.64, P=0.22-0.43).

Experiment 1e: Effects of NACc neural inactivation following cocaine-memory reactivation on subsequent drug context-induced cocaine seeking

B+M administration into the NACc following cocaine-memory reactivation failed to alter subsequent drug context-induced cocaine seeking (**Fig. 1.4**). Active lever responding increased upon exposure to the cocaine-paired context at test, relative to that in the extinction context (context main effect only, $F_{(1,11)}$=20.43, P=0.001). Moreover, there was no difference between the previously VEH- and B+M-treated groups in active lever responding in either context (treatment main and interaction effects, $F_{(1,11)}$=0.10-0.30, P=0.59-0.75).

Inactive Lever Responding

Inactive lever responding was low in all experiments and did not significantly differ as a function of treatment group or testing context (all Fs<3.017, Ps>0.108; **Figs. 1.1 & 1.4 C', D').**

DISCUSSION

Results from Experiments 1a-1e suggest that the BLA, but not the NACc, critically regulates the reconsolidation of contextual memories that trigger instrumental cocaine-seeking behavior. Consistent with this, ERK inhibition in the BLA following brief re-exposure to the cocaine-paired context – a manipulation designed to reactivate context-response-cocaine associative memories and prompt their reconsolidation – disrupted subsequent drug context-induced cocaine-seeking behavior, presumably by inhibiting memory reconsolidation (**Fig. 1.1C**). Conversely, ERK inhibition in, or GABA agonist-induced neural inactivation of, the NACc was without effect under the same experimental conditions (**Figs. 1.4C, D)**.

ERK signaling in the BLA critically regulates the reconsolidation of contextual cocaine memories

Administration of the MEK/ERK inhibitor, U0126, into the BLA following cocaine-memory reactivation decreased ERK1/2 phosphorylation in the BLA during the putative time of memory re-stabilization (**Fig. 1.3B**) and attenuated drug context-induced cocaine seeking approximately 72 hours later (**Fig**. **1.1B**), relative to VEH. As expected from a genuine memory reconsolidation deficit, these effects depended on explicit cocaine-memory reactivation in that U0126 administration in the absence of cocaine-paired context re-exposure (i.e., following novel context exposure) failed to alter subsequent drug context-induced cocaine-seeking behavior (**Fig. 1.1D**).

There was an increase in ERK2, but not ERK1, phosphorylation in the BLA following exposure to the cocaine-paired context, relative to the home cage (**Figs. 1.3B, D**). This differential response in ERK1 and ERK 2 activation might reflect functional differences between these ERK isoforms in the effects of drugs of abuse (Mazzucchelli *et al,* 2002; Girault *et al,* 2007). Conversely, there was no increase in ERK1/2 phosphorylation following exposure to the novel context, relative to the home cage (**Figs. 1.3C, D**), despite a previous report to the contrary in drug-naïve rats (Izquierdo *et al,* 2002). Because novelty was most robust at the start of novel context exposure and ERK1/2 phosphorylation typically peaks ~15-30 min following induction (Valjent *et al,* 2000), novelty-induced ERK activation possibly dissipated by the time of tissue collection. Remarkably, U0126 selectively attenuated ERK1/2 phosphorylation after exposure to the cocaine-paired context (**Fig. 1.3B**) without an overt effect on basal ERK1/2 phosphorylation (**Fig. 1.3 D**). While the latter may have been due to a floor effect or alteration in ERK1/2 phosphorylation kinetics following cocaine experience (Berhow *et al,* 1996), these findings support the importance of ERK1/2 activation in the re-stabilization of contextual cocaine memories in an instrumental setting.

Importantly, U0126 administration did not exert a protracted inhibitory effect on instrumental motor performance or on ERK1/2 activation. Specifically, it did not alter active lever responding in the extinction context (**Fig. 1.1C**) or inactive lever responding in either context (**Fig. 1.1C'**) at test**.** Furthermore, it failed to alter ERK1/2 activation in the BLA ~72 h later at test (**Fig. 1.3F**), consistent with the short half-life of U0126 (~2h; London and Clayton, 2008). These findings support that U0126 transiently interfered with ERK activation during memory reconsolidation.

Finally, the effects of U0126 were anatomically selective to the BLA, given that intra-BLA administration of U0126 failed to reduce ERK1/2 phosphorylation in the posterior caudate-putamen (pCPu), a brain region that is dorsally adjacent to the BLA thus the most probable recipient of inadvertent drug diffusion (**Figs. 1.3E**). While we did not similarly examine the possible role of the central nucleus (CeA), a brain region dorsal-medially adjacent to the BLA, in the effects of U0126 in the present study, a role for the CeA in drug-memory reconsolidation has been examined repeatedly and was not supported (Wang *et al,* 2008; Li *et al,* 2010; Wu *et al,* 2011).

ERK1/2 activation in the CeA, but not the BLA, is necessary for the *incubation* phenomenon, a reliable enhancement in cue-induced drug seeking following an extended drug-free period (Lu *et al* 2005; Li *et al* 2008). The development of incubation may be facilitated by the repeated retrieval and reconsolidation of drug-related associations during abstinence (Nolen-Hoeksema *et al* 2007; Wells *et al* 2012). However, in light of the above incubation studies, the present findings suggest that ERK1/2 activation is differentially involved in drug-memory reconsolidation and in the retrieval/utilization of repeatedly reconsolidated drug memories. Thus, overall, the most parsimonious interpretation of the present findings is that U0126 in the BLA interfered with ERK phosphorylation related to context-response-cocaine memory reconsolidation *per se*, and thereby disrupted the subsequent influence of this associative memory on cocaine-seeking behavior.

The present findings significantly contribute to prior research, indicating that the BLA is a site for memory reconsolidation (Tronson and Taylor, 2007; Milton and Everitt, 2010; but see Cammarota *et al*, 2004) and expand upon previous findings from our laboratory, demonstrating that the BLA is a locus for the protein synthesis-dependent restabilization of instrumental,

context-response-cocaine memories (Fuchs *et al*, 2009). ERK signaling may be key to memory reconsolidation as a regulator of gene transcription and protein synthesis via the posttranslational modification of transcription factors, including Elk1 and/or CREB (Miller and Marshall, 2005). Because ERK is a fundamental component of several intracellular signaling cascades (Adams and Sweatt, 2002), future research will be necessary to characterize the molecular pathways through which it mediates cocaine-memory reconsolidation.

The NACc and the reconsolidation of cocaine memories

ERK inhibition in, or global neural inactivation of, the NACc following cocaine-memory reactivation failed to impair subsequent drug context-induced cocaine seeking (**Fig. 1.4C, D**). These findings were unanticipated because ERK signaling and *zif268* immediate-early gene expression within the NACc play critical roles in the reconsolidation of Pavlovian context-cocaine memories required for cocaine-CPP (Miller and Marshall, 2005 and Théberge *et al*, 2010, respectively). The negative findings in the present study cannot be attributed to insufficient dosing, as the doses of U0126 and B+M used impair the reconsolidation of Pavlovian cocaine memories (Miller and Marshall, 2005) and the expression of drug context-induced cocaine seeking (Fuchs *et al*, 2008a), respectively. Instead, these null effects could reflect NACc-specific boundary conditions in memory reconsolidation. CPP studies typically involve a shorter timeline and fewer CS-US pairings than those in the present study (Miller and Marshall, 2005; Valjent *et al*, 2006; Théberge *et al*, 2010; Brown *et al*, 2008). Memory age and strength are inversely related to the ease of memory destabilization and/or disruption (Milekic and Alberini, 2002; Suzuki *et al*, 2004; Finnie and Nader, 2012), perhaps because remote, well-trained memories are incorporated into rigid or extensive neuronal networks (McClelland *et al*, 1995; Frankland and

Bontempi, 2005). Thus, our negative findings may suggest either a time-limited involvement of the NACc or the resilience of NACc-dependent context-response-cocaine memory traces to destabilization, perhaps due to cocaine-induced metaplasticity (Lu *et al,* 2006; Kalivas and O'Brien, 2008; Finnie and Nader, 2012). However, it is also possible that the NACc is not critical for reconsolidating complex, instrumental context-response-cocaine memories. Consistent with this, re-exposure to a food-paired context fails to increase ERK2 activation in the NACc concomitant with food-seeking behavior (Shiflett *et al*, 2008). PKM- and PKC-zeta inhibition in the NACc, following re-exposure to a remifentanil-associated runway apparatus, fails to alter the speed of approach to the drug-paired goal box, which is considered a quasi-instrumental response (Crespo *et al*, 2012). However, it impairs the time spent in the goal box, suggesting intact Pavlovian conditioning. Similarly, *zif268* knockdown in the NACc disrupts the reconsolidation of a Pavlovian cocaine-memory, but not an CS-cocaine memory required for the acquisition of a new instrumental response (Théberge *et al*, 2010).

General Conclusion – Experiments 1a-e

These experiments and others (Théberge *et al*, 2010) suggest that the NACc and BLA mediate cocaine-memory reconsolidation under different experimental conditions. The NACc is critical in the restabilization of Pavlovian cocaine memories (Miller and Marshall, 2005; Théberge *et al*, 2010; Crespo *et al.,* 2012), while the BLA plays a more global role in the reconsolidation of Pavlovian and instrumental cocaine memories (Théberge *et al*, 2010; Sanchez *et al,* 2010; Fuchs *et al,* 2009), in part through ERK-dependent processes. Future studies will be needed to parametrically vary the extent of cocaine exposure and instrumental conditioning, as well as the length of the training-to-reactivation interval, in order to gain further insight into the

relative contributions of the NACc and the BLA to the restabilization of maladaptive cocaine memories. Nevertheless, the present findings support the idea that the treatment of cocaine addiction will benefit from the development of highly selective memory reconsolidation inhibitor treatments designed to assuage the impact of environmental contexts on cocaine craving.

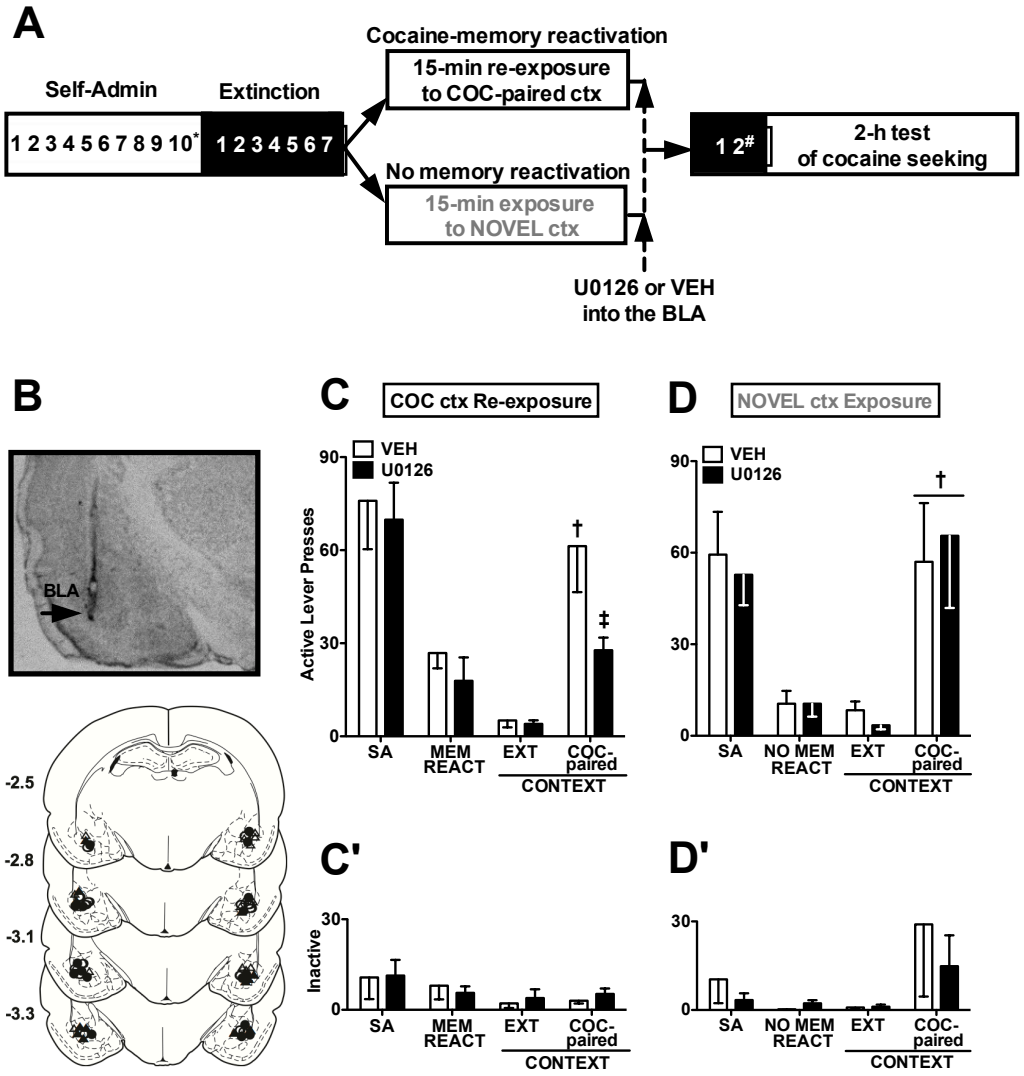


Fig 1.1 ERK inhibition in the BLA immediately after cocaine-memory reactivation impairs subsequent drug context-induced cocaine seeking, relative to VEH treatment. **(*A*)** Schematic depicting the timeline for Experiments 1a and 1b. Cocaine self-administration (SA) sessions took place in a distinct environmental context until rats reached the acquisition criterion (*10 infusions/session for a minimum of 10 sessions). Extinction training occurred in a distinctly different context. After extinction training, rats were re-exposed to the cocaine-paired context (Cocaine-memory reactivation) for 15 min to destabilize cocaine memories and stimulate memory reconsolidation (Experiment 1a), or they were exposed to a novel, unpaired context (No memory reactivation), to provide similar experience without explicit cocaine-memory reactivation (Experiment 1b). Immediately after the session, rats received bilateral microinfusions of the MEK/ERK inhibitor, U0126 (1.0 μg/ 0.5 μl/hemisphere) or 5% DMSO/6% TWEEN vehicle (VEH; 0.5 μl/hemisphere) into the BLA. Next, groups underwent additional extinction training until they met the extinction criterion (# ≤ 25 non-reinforced active lever responses/ session for two consecutive sessions). Cocaine seeking (non-reinforced active lever responding) was then assessed in the cocaine-paired context (COC-paired). **(*B*)** Photomicrograph and schematics depicting cannula placement. The symbols denote the most ventral point of the

injector cannula tracts for rats in Experiment 1a that received bilateral vehicle (VEH; *n = 7,* white circles) or U0126 (*n = 8,* black circles) infusions into the BLA following cocaine memory reactivation and for rats in Experiment 1b that received bilateral VEH (*n = 7,* white triangles) or U0126 (*n = 8,* black triangles) infusions following exposure to a novel, unpaired context. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997). **(*C*)** Mean (± SEM) active lever responses during SA (mean of last three training sessions), the cocaine-memory reactivation session (MEM REACT), and during the tests of cocaine seeking in the extinction (EXT; the last session before the test in the COC-paired context) and in the COC-paired context for rats in Experiment 1a. **(*C'*)** Mean (± SEM) inactive lever responses in Experiment 1a. **(*D*)** Mean (± SEM) active lever responses during SA, the novel-context no memory reactivation session (NO MEM REACT), and during the tests of cocaine seeking in the EXT and COC-paired contexts in Experiment 1b. **(*D'*)** Mean (± SEM) inactive lever responses in Experiment 1b. † denotes significant difference relative to responding in the extinction context (ANOVA context main and simple main effects, $P<0.05$). ‡ denotes significant difference relative to the respective VEH treatment (ANOVA treatment simple main effect, $P<0.05$).

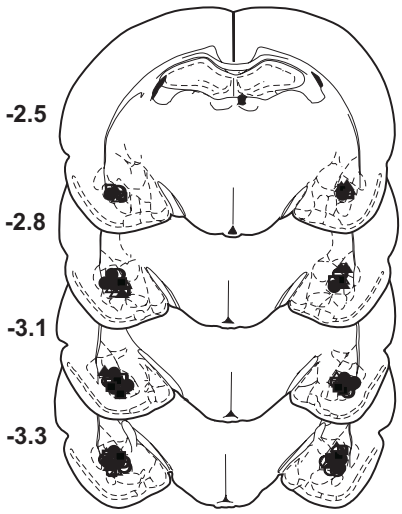


Fig. 1.2 Schematics depicting cannula placement. The symbols denote the most ventral point of the injector cannula tracts for rats in Experiment 1c. The rats received bilateral VEH (*n = 6,* open circles) or U0126 (*n = 8,* filled circles) infusions into the BLA following cocaine memory reactivation, for control rats that received bilateral VEH (*n = 8,* open triangles) or U0126 (*n = 6,* filled triangles) infusions following exposure to a novel context, and for control rats that received bilateral VEH (*n = 6,* open squares) or U0126 (*n = 5,* filled squares) infusions following exposure to the home cage. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997).

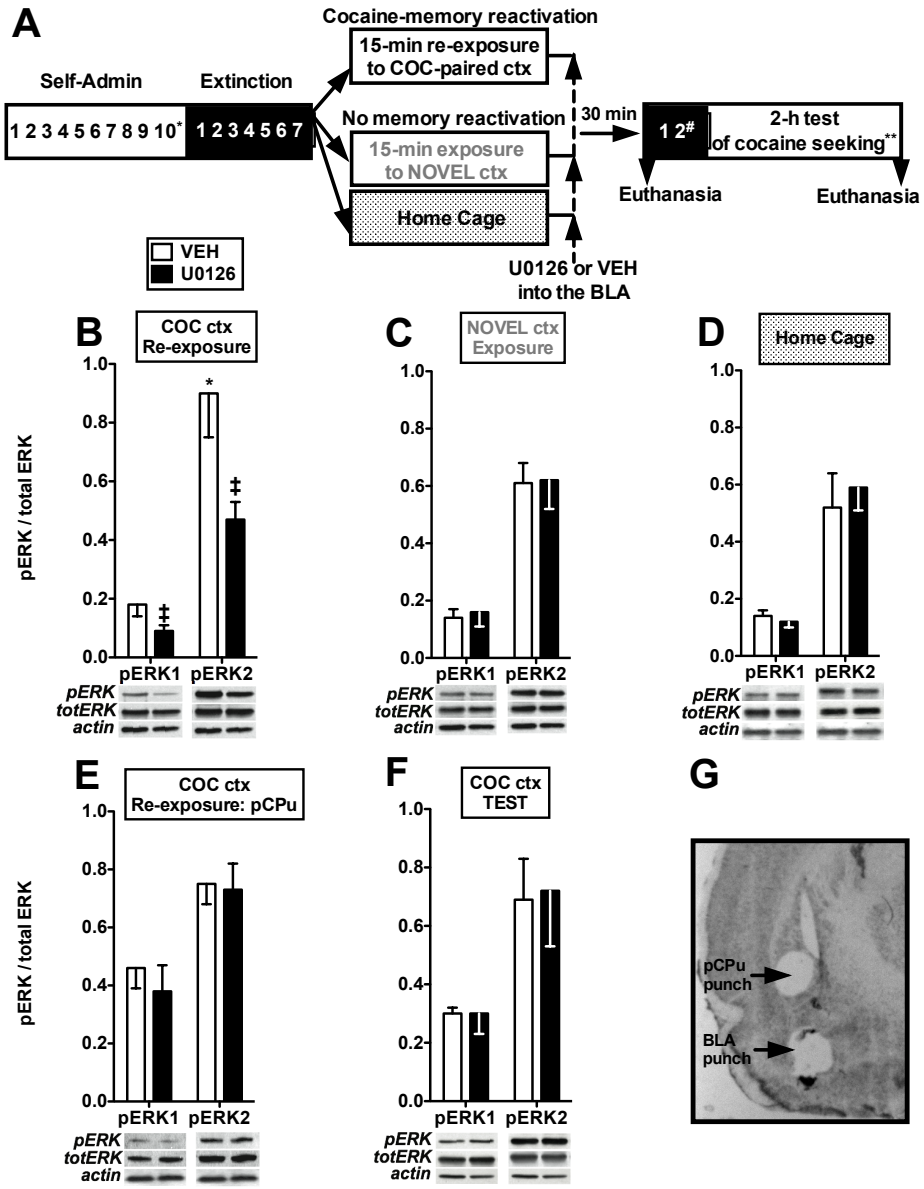


Fig. 1.3 U0126 in the BLA reduces ERK1 and ERK2 phosphorylation in a transient and reactivation-dependent manner. (*A*) Schematic depicting the timeline for Experiment 1c. Experimental parameters were identical to those in Experiment 1a, except that rats received bilateral microinfusions of U0126 (1.0 μg/ 0.5 μl/hemisphere) or VEH into the BLA following exposure to the cocaine-paired context (Cocaine-memory reactivation), the novel, unpaired context (No memory reactivation), or the home cage (No memory reactivation), and were sacrificed 30 min later. **Rats from Experiment 1a were sacrificed immediately after the test of drug context-induced cocaine seeking. (*B*) Mean (± SEM) ERK1/2 activation (expressed as the ratio of pERK to total ERK protein levels normalized to actin) in rats sacrificed 30 min after cocaine-memory reactivation and intracranial manipulations. Representative bands of pERK1, pERK2, total ERK1, total ERK2, and actin (i.e., loading control) are also provided. (*C*) Mean (± SEM) ERK1/2 activation and representative bands for rats sacrificed 30 min after novel context exposure and intracranial manipulations. (*D*) Mean (± SEM) ERK1/2 activation and

representative bands for home cage controls sacrificed 30 min after intracranial manipulations. (*E*) Mean (± SEM) ERK1/2 activation in the posterior caudate putamen (pCPu; anatomical control region) and representative bands for rats sacrificed 30 min after cocaine-memory reactivation and intracranial manipulations. (*F)* Mean (± SEM) ERK1/2 activation and representative bands for rats sacrificed immediately after the test of cocaine seeking in Experiment 1a. *(G*) Photomicrograph depicting the location of BLA and pCPu tissue punches that were used for Western blotting. * denotes significant difference relative to the respective home cage controls (planned, one-tail t-test, $P<0.05$). ‡ denotes significant difference relative to VEH treatment (Student's t-test, $P<0.05$).

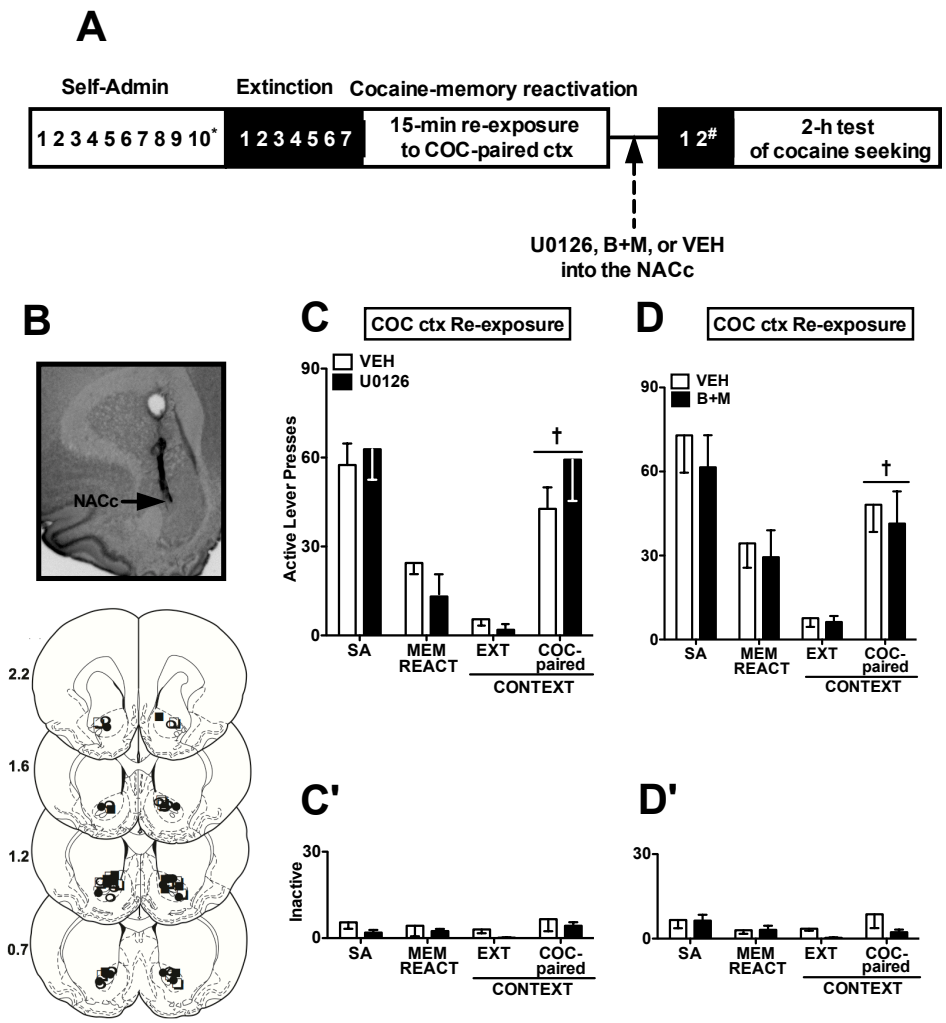


Fig. 1.4 ERK inhibition in, or neuronal inactivation of, the NACc after cocaine-memory reactivation fails to disrupt subsequent drug context-induced cocaine seeking. (*A*) Schematic depicting the timeline for Experiments 1d and 1e. Experimental parameters were identical to those in Experiment 1a, except that all rats in Experiment 1d received bilateral U0126 (1.0 μg/ 0.5 μl) or VEH infusions into the NACc following cocaine-memory reactivation, and rats in Experiment 1e received bilateral GABAA/B agonist cocktail baclofen+muscimol (B+M; 106.8/5.7 ng/0.5 μl/hemisphere) or phosphate buffered saline vehicle (VEH) infusions into the NACc following cocaine-memory reactivation. (*B*) Photomicrographic and schematic depiction of cannula placements. The symbols denote the most ventral point of the injector cannula tracts for rats that received bilateral VEH (*n = 7*, open circles) or U0126 (*n = 7,* filled circles) infusions into the NACc in Experiment 1d and for rats that received bilateral phosphate buffered saline VEH (*n = 7,* open squares) or B+M (*n = 6,* filled squares) infusions in Experiment 1e. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997). (*C*) Mean (± SEM) active lever responses during self-administration (SA; mean of last three training sessions), during the 15-min cocaine-memory reactivation session (MEM REACT), and during the tests of cocaine seeking in the extinction (EXT; the last session before the test in the COC-paired context) and cocaine-paired (COC-paired) contexts in Experiment 1d. (*C'*) Mean (± SEM) inactive lever responses in Experiment 1d. (*D*) Mean (±

SEM) active lever responses in Experiment 1e. (*D'*) Mean (± SEM) inactive lever responses in Experiment 1e. † denotes significant difference relative to responding in the extinction context (ANOVA context main effect, $P<0.05$).

CHAPTER 3

INVOLVEMENT OF AMYGDALAR NUCLEAR FACTOR–κB IN THE RECONSOLIDATION OF INSTRUMENTAL CONTEXTUAL COCAINE MEMORIES

INTRODUCTION

The role of gene transcription and protein synthesis in memory reconsolidation is hotly debated, and may reflect either the *de novo* synthesis of new proteins to effect structural changes that underlie plasticity during restabilization or the replacement of proteins that are modified or degraded during memory destabilization (Romano *et al,* 2006; Miller and Sweatt 2006). Regardless of their specific function, both gene transcription and protein synthesis are intrinsic to the reconsolidation of long-term memories, including associative memories that underlie environmentally triggered relapse (Tronson and Taylor 2007; Milton and Everitt 2010; Sorg 2012). Thus far, exploration into the molecular mechanisms of memory reconsolidation has not included rigorous examination of the transcription factors (TFs) involved. Instead, investigations focused primarily on post-translational modifications of existing cytoplasmic proteins whose activation leads up to, and is required for, subsequent gene transcription (for review, see Tronson and Taylor 2007; Sorg 2012). Identification of TFs that regulate gene expression critical for cocaine-memory reconsolidation will be paramount to the understanding of long-term contextual

stimulus control over relapse behavior and will likely inform the development of new pharmacotherapies designed to reduce relapse propensity.

Of the few TFs whose contributions to memory reconsolidation have been assessed, the nuclear factor-κB (NF-κB) family stands out as an important regulator of processes underlying the long-term stability of memory (Romano *et al,* 2006). TFs in the NF-κB family, including RelA/p65, Rel B, cRel/vRel, NF-κB1/p50, and NF-κB2, contain the Rel homology domain (RHD) (Romano *et al,* 2006). To be transcriptionally active, these proteins form obligatory hetero- or homodimers, the composition of which determines affinity for the κB DNA binding site as well as the specific genes targets (Marienfeld *et al,* 2003; Hoffman *et al,* 2006; Romano *et al,* 2006). Dimerization lends itself to an unprecedented amount of diversity in NF-κB-dependent transcriptional control (Romano *et al,* 2006). Consequently, NF-κB TFs can mediate the expression of over 500 genes in humans, many of which are related to inflammatory and immune responses (Baeuerle and Henkel 1994; Thanos and Maniatis 1995; Natoli *et al,* 2005; Hoffman *et al,* 2006) or oxidative stress (Schreck *et al,* 1992).

Several NF-κB-regulated genes are integral to activity-dependent synaptic plasticity and long-term memory, including NMDA (Richter *et al,* 2002) and AMPA (Yu *et al,* 2002) receptor subunits. Accordingly, NF-κB activation is required for the initiation of long-term potentiation (LTP; Yeh *et al,* 2002) and the consolidation of spatial (Dash *et al,* 2005; Kaltschmidt *et al,* 2006) and fear memories in various paradigms (Merlo *et al,* 2002; Yeh *et al,* 2002; Freudenthal *et al,* 2005; Anh *et al,* 2008, but see Levenson *et al,* 2004). More recently, NF-κB has been implicated in memory *re*consolidation. Specifically, systemic or intra-hippocampal inhibition of NF-κB with sulfasalazine (SSZ), a potent and selective inhibitor of the IκB kinase [IKK], the upstream kinase required for NF-κB activation and subsequent nuclear translocation (Zandi *et al,*

1997; Jacobs and Harrison 1998), impairs the reconsolidation of contextual fear memories and correspondingly reduces context-elicited freezing behavior in rats and the crab *Chasmagnathus* in the fear conditioning paradigm (Lubin and Sweatt, 2007; Lee and Hynds 2013 and Merlo *et al,* 2005, respectively) and latency to enter a footshock-associated context in the inhibitory avoidance paradigm (Boccia *et al, 2007*). Consistent with a critical role for NF-κB-mediated transcriptional activity, per se, intra-hippocampal administration of SN-50 or a κB decoy, both of which spare the activation but inhibit the transcriptional capacity of NF-κB, blocks contextual fear memory reconsolidation (Lubin and Sweatt 2007; de la Fuentes *et al,* 2011), similar to SSZ. Importantly, NF-κB is also critical for the reconsolidation of amygdala-dependent memories. Correspondingly, administration of either SSZ or SN-50 in the BLA prevents the reconsolidation of auditory fear memory (Si *et al,* 2012). Finally, intra-BLA SN-50 administration disrupts the reconsolidation of Pavlovian drug-related memories that underlie the expression of morphine CPP (Yang *et al,* 2011). However, the role of NF-κB in instrumental cocaine-related memory reconsolidation has not been similarly investigated.

Hence, the overarching objective for Experiment 2 was to elucidate the contribution of the NF-κB family of TFs in the BLA to the reconsolidation of contextual cocaine memories that drive drug context-elicited instrumental cocaine-seeking behavior in the extinction-reinstatement paradigm. Based on extensive literature in support of NF-κB-dependent memory reconsolidation, we postulated that NF-κB activation in the BLA would govern the successful reconsolidation of context-response-cocaine associative memories. To test this hypothesis, Experiment 2 evaluated the dose-dependent effects of SSZ-induced NF-κB inhibition in the BLA at the putative time of memory reconsolidation on subsequent drug context-induced cocaine-seeking behavior.

METHODS

Animals

Male Sprague-Dawley rats (N = 29; 275-300 g; Charles River Laboratories, Wilmington, MA) were individually housed in a temperature- and humidity-controlled vivarium on a reversed light-dark cycle. Rats were fed 20-25 g of rat chow per day, and water was available *ad libitum*. Protocols for the housing and treatment of rats followed the *Guide for the Care and Use of Laboratory Rats* (Institute of Laboratory Animal Resources on Life Sciences, 1996) and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Food training

To expedite cocaine self-administration, rats were trained to lever press under a continuous schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH, USA) in sound-attenuated operant conditioning chambers (26 x 27 x 27 cm high; Coulbourn Instruments, Allentown, PA, USA) during a 16-h overnight session. Responses on the right (active) lever were reinforced by one food pellet, whereas responses on the left (inactive) lever had no scheduled consequences. Food training occurred in a different set of chambers than those used for contextual conditioning during subsequent drug self-administration and extinction training sessions, and none of the contextual stimuli used were present during food training.

Surgery

Forty-eight h after food training, rats were fully anesthetized with ketamine hydrochloride (75 mg/kg, i.p.) and xylazine (5 mg/kg i.p.). Rats were implanted with chronic indwelling jugular catheters and with 26Ga stainless steel guide cannulae (Plastics One, Roanoke, VA), , as described previously (Fuchs *et al,* 2009). The cannulae were aimed bilaterally at the BLA (-2.7 mm AP, ± 5.1 mm ML, -6.7 mm DV, from bregma), using standard stereotaxic procedures. Stainless steel screws and cranioplastic cement secured the guide cannulae to the skull. When not in use, stylets (Plastics One) and tygon caps and obdurators (Plastics One) sealed the guide cannulae and catheter, respectively. To extend catheter patency and prevent infection, catheters were flushed daily with cefazolin (10.0 mg/ml of heparinized saline; Schein Pharmaceuticals, Albuquerque, NM, USA) and heparinized saline (70 U/ml; Baxter Health Care Corp, Deerfield, IL, USA), as described previously (Fuchs *et al,* 2009). Rats received 5 days of post-operative recovery before the start of self-administration training. Catheter patency was periodically assessed by administering propofol (1mg/0.1ml, i.v. Eli Abbott Lab, North Chicago, IL, USA) intravenously and confirming a rapid loss of muscle tone.

Self-administration and Extinction Training

All behavioral training and testing (i.e., self-administration and extinction training, as well as cocaine-memory reactivation and the test of drug context-induced cocaine seeking) were conducted in sound-attenuated operant conditioning chambers configured to one of two distinctly different contexts, as described previously (Fuchs *et al,* 2009; see **Table 1, Chapter 2:** Contexts A, B).

Rats were randomly assigned to either Context A or B for self-administration training, which took place during daily, 2-h sessions, during the rats' dark cycle. Active lever presses resulted in the delivery of cocaine infusions (cocaine hydrochloride; 0.15 mg/0.05 ml per infusion, i.v.; NIDA, Research Triangle Park, NC) under a FR-1/time-out-20s schedule, as described previously (Fuchs *et al,* 2009). Inactive lever presses were recorded but had no scheduled consequences. Training continued until rats reached the acquisition criterion (i.e., minimum 10 sessions with ≥10 cocaine infusions/session).

After the completion of cocaine self-administration training, rats received seven daily 2-h extinction-training sessions in the alternate context (A or B, different from the one used for self-administration training). During extinction training, both active and inactive presses were recorded but had no programmed consequences. Following the fourth extinction-training session, the rats were adapted to the intracranial microinfusion procedure (i.e., sham infusions). To this end, 33Ga injection cannulae (Plastics One) were inserted into the rats' guide cannulae to a depth of 2 mm below the tip of the guide cannulae and remained in place for 4 minutes. No liquid was infused.

Experiment 2

Experiment 2 evaluated whether SSZ-induced NF-κB inhibition in the BLA would dose-dependently impair instrumental context-cocaine memory reconsolidation. To this end, on the day following the completion of extinction training, rats were re-exposed to the cocaine-paired context for 15 min (i.e., cocaine-memory reactivation; see **Fig. 2A**) to trigger cocaine-memory destabilization and reconsolidation. This session length was carefully selected based on a previous parametric analysis (Fuchs *et al,* 2009). During cocaine-memory reactivation, levers

were extended to allow for similar tactile and motor experience as in self-administration training (Fuchs *et al,* 2009). However, lever presses were not reinforced with cocaine because cocaine acutely increases NF-κB transcriptional activity within 3 h post-treatment (i.e., within the memory reconsolidation time window; Nader *et al,* 2000a) in the pc-12 catecholaminergic cell line (Lepsch *et al,* 2009).

Immediately after cocaine-memory reactivation, rats received bilateral microinfusions of 20% DMSO/10 mM HEPES (pH 7.6) VEH or one of two doses of SSZ (2 or 5μg/0.5μl/hemisphere) into the BLA. The 2μg dose of SSZ was selected based on a previous report demonstrating that it impairs contextual fear memory reconsolidation when administered into the hippocampus (Lee and Hynds 2013). Treatment assignment was counterbalanced based on previous cocaine intake. During intracranial microinfusions, injection cannulae were lowered to a depth of 2 mm below the tip of the guide cannulae and remained in place for 1 min before and after the microinfusion, which was delivered over 2 min.

Post-reactivation extinction and test of drug context-induced cocaine seeking

Beginning on the day following cocaine-memory reactivation, rats received additional daily 2-h extinction-training sessions (2.1±0.1 days) until they met the extinction criterion (i.e., ≤25 active lever responses per session on a minimum of 2 consecutive days). Twenty-four h later, rats were placed into the previously cocaine-paired context for a 2-h test session, during which non-reinforced active and inactive lever presses were recorded. Non-reinforced active lever presses provided an index of motivation to obtain cocaine reinforcement.

Histology

Rats were overdosed with ketamine hydrochloride and xylazine (66.6 and 1.3 mg/kg, i.v. or 199.8 and 3.9 mg/kg, i.p., respectively, depending on catheter patency) and transcardially perfused with a 1x phosphate buffered saline (Fischer Scientific) plus 10% formaldehyde solution (Sigma). Brains were dissected out and stored in 10% formaldehyde solution until they were sectioned coronally using a vibratome. Cannula placements were verified on 75µm sections stained using cresyl violet (Kodak, Rochester, NY, USA). The most ventral portion of each cannula tract was mapped onto schematics from the rat brain atlas (Paxinos and Watson, 1997).

Data Analysis

Separate one-way ANOVAs were used to evaluate possible pre-existing differences in cocaine intake, in active and inactive lever responding during self-administration (mean of last 3 d), extinction (days 1 and 7), and during the memory reactivation session, as well as in the number of days required to reach the post-reactivation extinction criterion. Subsequent treatment (VEH, SSZ dose) was included as a between-subjects factor in the ANOVAs.

Separate mixed-factorial ANOVAs were conducted to examine the effects of intracranial microinfusions on active and inactive lever responses on the test days in the cocaine-paired and extinction contexts (the day preceding the test cocaine-paired context). In these analyses, treatment (VEH, SSZ dose) was included as the between-subjects factors, and testing context (EXT, COC-paired) was the within-subjects factor. Significant main and interaction effects were further examined using *post-hoc* Tukey tests. Alpha was set at 0.05.

RESULTS

Histology

Schematics and photomicrographs illustrating cannula placements are included in **2B.** The target brain region was defined as the lateral and basolateral nuclei of the amygdala (BLA). High power microscopy did not reveal tissue damage at injection sites. Only data from rats with correct cannula placements were included in the statistical analyses.

Behavioral History

Separate one-way ANOVAs confirmed that there were no pre-existing differences between groups that subsequently received VEH or either dose of SSZ into the BLA in cocaine intake, active or inactive lever responses during cocaine self-administration training, extinction training days 1 and 7, or the memory reactivation session, or in the number of days required to reach the extinction criterion (all $Fs_{(2,26)} \leq 1.97$, $Ps \geq 0.16$). Descriptive statistics are provided in **Table 3** below.

Exp./Group		Cocaine Intake	SA	EXT 1	EXT 7	Reactivation	Days to EXT
2	VEH (*N=11*)	24.5 ± 2.3	64.8 ± 10.1	84.2 ± 24.8	12.0 ± 3.7	25.6 ± 7.2	2.1 ± 0.1
	2 μg SSZ (*N=9*)	23.4 ± 2.5	58.3 ± 9.0	115.2 ± 20.3	12.8 ± 3.6	21.9 ± 4.1	2.0 ± 0.2
	5 μg SSZ (*N=9*)	27.3 ± 2.1	69.8 ± 9.1	55.3 ± 11.3	9.4 ± 2.2	31.0 ± 6.2	2.0 ± 0.2

Table 3: Behavioral History Cocaine intake (mean ± SEM infusions/session for the last 3 training sessions) and active lever responses during self-administration (SA; mean ± SEM for the last 3 training sessions), as well as active lever responses (mean ± SEM) during the first (Extinction 1) and last (Extinction 7) extinction training sessions, and during the 15-min memory reactivation (Reactivation) session. The number of days required to meet the extinction criterion (Days to EXT) is also included. Means are provided for rats in groups in Experiment 2 that received VEH or SSZ into the BLA after cocaine-memory reactivation.

Experiment 2: Effects of SSZ administration in the BLA following cocaine-memory reactivation on subsequent drug context-induced cocaine seeking

The IκK/NF-κB inhibitor, SSZ, administered into the BLA immediately after cocaine-memory reactivation failed to significantly alter subsequent cocaine-seeking behavior despite producing a non-significant trend for an increase in cocaine seeking at the 5μg dose (**Fig. 2C**; ANOVA context x treatment interaction, $F_{(2,26)}$=4.48, P=0.02; context main effect, $F_{(1,26)}$=63.08, P<0.001; NS treatment main effect, $F_{(2,26)}$=2.70, P=0.09). There was also no difference between the groups in active lever responding in the extinction context. Re-exposure to the cocaine-paired context increased active lever responding in the groups that had received VEH or either dose of SSZ following cocaine-memory reactivation, relative to responding in the extinction context on the preceding day (Tukey's test, P<0.05). Active lever responding did not differ between the groups that had received VEH or 2 μg of SSZ after cocaine-memory reactivation in the cocaine-paired context. Furthermore, despite a significant context x treatment interaction, the group that had received the 5μg SSZ dose following cocaine-memory reactivation exhibited only a non-significant trend for an increase in active lever responding in the cocaine-paired context during the test session, relative to group that had received VEH treatment (Tukey's test, P>0.05).

Inactive Lever Responding

Re-exposure to the cocaine-paired context at test produced a modest increase in inactive lever responding independent of treatment (**Fig. 2C';** ANOVA context main effect only, $F_{(1,26)}$=5.66, P=0.03; all treatment main and interaction effects, $F_{(1\text{-}2,26)}$=0.49-0.60, P=0.56-0.62).

DISCUSSION

Experiment 2 assessed the involvement of the NF-κB family of TFs in context-response-cocaine memory reconsolidation. NF-κB exists in the cytoplasm of neurons, bound to an inhibitory κB protein (IκB) that prevents its nuclear localization by occluding the requisite nuclear localization sequence (NLS; Jacobs and Harrison 1998). Phospohrylation of IκB by IκK primes it for imminent degradation (Brockman *et al,* 1995; Finco and Baldwin 1995; Chen *et al,* 1996; DiDonato *et al,* 1996), allowing NF-κB to be translocated into the nucleus, where it can regulate gene transcription (Zandi *et al,* 1997). SSZ is a potent and highly specific inhibitor of IκK; therefore, it prevents the activation and successive translocation of NF-κB (Wahl *et al,* 1998). In the present study, contrary to our hypothesis, SSZ-induced inhibition of NF-κB in the BLA during cocaine-memory reconsolidation failed to inhibit subsequent drug context-induced cocaine-seeking behavior. Instead, at the highest dose, SSZ produced a non-significant, but observable, *enhancement* in drug context-induced cocaine seeking (**Fig. 2C**).

Specifically, SSZ microinfusions into the BLA after a 15-min exposure to the cocaine-associated context, which optimally engenders cocaine-memory destabilization and reconsolidation (Fuchs *et al,* 2009; Ramirez *et al,* 2009; Wells *et al,* 2011, 2013; Arguello *et al,* 2013b), did not *significantly* alter subsequent drug context-induced cocaine seeking, relative to VEH treatment (**Fig. 2C**). Remarkably, post-cocaine memory reactivation administration of the 2μg SSZ dose, which has a robust inhibitory effect on the reconsolidation of contextual fear memories in the hippocampus (Lee and Hynds 2013), failed to alter subsequent cocaine-seeking behavior relative to VEH. Conversely, the 5μg SSZ dose modestly *potentiated* subsequent cocaine-seeking behavior (**Fig. 2C**). This was indicated by a significant context x treatment

interaction effect, the source of which was likely a greater context-dependent increase in active lever responding exhibited by the group that received 5μg of SSZ following cocaine memory reactivation than the increase exhibited by the groups that received VEH or 2μg of SSZ. While nonsignificant, this trend is unlikely to reflect protracted hyperactivity or nonspecific increases in motivation, in that ongoing control experiments have demonstrated that intra-BLA 5μg SSZ fails to alter general motor activity in a novel context or cocaine-seeking behavior when administered in the absence of cocaine-memory reactivation (data not shown). Further, this modest effect depends on the BLA in that SSZ administration into the posterior caudate putamen (pCPu; expected negative anatomical control region; Fuchs *et al,* 2009) after cocaine memory reactivation does not alter cocaine-seeking behavior at test, relative to VEH (data not shown).

It is unclear whether insufficient dosing contributed to the modest effect of SSZ or the non-significant difference between the groups in the magnitude of cocaine-seeking behavior in the cocaine-paired context. On one hand, the 5μg SSZ dose is considerably higher than the highest reported dose (i.e., 2μg in Lee and Hynds 2013) used in the literature to impair memory reconsolidation. However, the 5μg dose of SSZ in the BLA may have been insufficient to appreciably disrupt the extent of NF-κB activation putatively required during the reconsolidation of context-response-cocaine memories *per se*. This possibility might be related to well-documented cocaine-related increases in basal levels of NF-κB and to a subsequent decrease in signal to noise ratio. In support of this, chronic cocaine administration increases delta fosB-dependent expression of NF-κB in the nucleus accumbens core (Ang *et al,* 2001, but see Muriach *et al,* 2010), and these increases likely persist during drug-free periods, similar to delta fosB (Hope 1998).

Another possibility is that a ceiling effect may have precluded the observation of a genuine group difference, given that the VEH group exhibited full reinstatement of the behavior (**Fig. 2C**). Similarly, in a previous study, the memory reconsolidation–enhancing effects of the PKA activator 6 – BNZ-cAMP were undetectable unless BNZ-cAMP was administered following repeated memory reactivation sessions for 4 consecutive days (Tronson *et al,* 2006). Regrettably, in the present study, we did not similarly adjust our experimental parameters to account for a ceiling effect, because we had postulated that SSZ would *impair,* rather than *enhance,* cocaine-memory reconsolidation.

Future studies will be needed to determine the effects of SSZ under conditions in which behavioral enhancement is more easily detected. Positive findings will have important implicatons for our understanding of the role of NF-κB in memory. In the only other study that explored the contribution of NF-κB to drug-memory reconsolidation to date, NF-κB inhibition in the BLA following reactivation of a Pavlovian morphine-related memory prevented the expression of morphine CPP 24 h and up to 7 d later (Yang *et al,* 2011). The apparent discrepancy between this and the present study may reflect differences in cocaine- versus morphine-induced metaplasticity in NF-κB signaling that differentially impacted the ability of NF-κB inhibitors to suppress memory reconsolidation-related activity. Consistent with this possibility, unlike cocaine, both morphine administration (Hargrave *et al,* 2003; Borner *et al,* 2012, but see Hou *et al,* 1996) and morphine withdrawal have a *suppressive* effect on NF-κB (Kelschenbach *et al,* 2013).

In addition to drug-based differences, the recruitment of NF-κB transcriptional regulation during the memory reconsolidation may differ as a function of memory type, age, and strength (Alberini *et al,* 2011; Besnard *et al,* 2012). Similar to its effects on the reconsolidation of

morphine CPP (Yang *et al,* 2011, described above), NF-κB inhibition in the DH or BLA after contextual and auditory fear memory reactivation, respectively, robustly inhibits subsequent conditioned freezing behavior (Lubin and Sweatt 2007; Si *et al,* 2012; Lee and Hynds 2013), which is suggested, in part, to depend on changes in histone acetylation after pharmacological manipulations to NF-κB signaling (Si *et al,* 2012). Unlike in fear conditioning and morphine CPP studies, cocaine-memory reactivation in the present study took place a minimum of 18 d following initial context-response-cocaine memory consolidation and after extensive context-response-drug pairing over the course of self-administration training (see **Fig. 2A** for experimental timeline). Therefore, the target memory at the time of manipulation was likely more crystallized and cortically distributed (McClelland *et al*, 1995; Frankland and Bontempi, 2005) and this, as well as extensive conditioning and drug exposure, perhaps have led to NF-κB-dependent suppression of memory reconsolidation.

It is important to note that the regulation of gene transcription by NF-κB is extremely complex. NF-κB controls the expression of over 500 genes (Natoli *et al,* 2005; Hoffman *et al,* 2006), but the specific genes regulated and the manner in which their expression is altered (i.e., activation or repression) following NF-κB activation is multiply determined by a number of intracellular factors, including dimeric composition of NF-κB TFs and the specific pathway leading to IKK/NF-κB activation (Romano *et al,* 2006). Thus, while the majority of studies investigating the role of NF-κB in memory reconsolidation have suggested NF-κB is a promoter of memory reconsolidation (Lubin and Sweatt 2007; Boccia *et al,* 2007; de la Fuentes *et al,* 2011; Yang *et al,* 2011, Si *et al,* 2012; Lee and Hynds 2013), and notably, the reconsolidation of Pavlovian morphine-related memories that subserve morphine CPP (Yang *et al,* 2011), it is conceivable that under certain conditions, NF-κB, can act as an endogenous *negative* regulator of

the memory reconsolidation processes underlying the long-term motivational effects of cocaine-related environmental stimuli, as the modest findings in Experiment 2 suggest. Future studies will be needed in order to identify (A) experimental parameters under which NF-κB involvement in context-response-cocaine memory reconsolidation is significant and (B) cellular factors that determine whether NF-κB has a facilitative or inhibitory effect on memory reconsolidation. In light of the discrepancies between the present results and the larger memory reconsolidation literature, it is clear that cross-model validation of experimental results will be imperative in order to extend preclinical findings toward development of useful pharmacotherapies that capitalize on memory reconsolidation inhibition as a viable treatment for drug addiction and other neuropsychiatric disorders characterized by pathogenic memories.

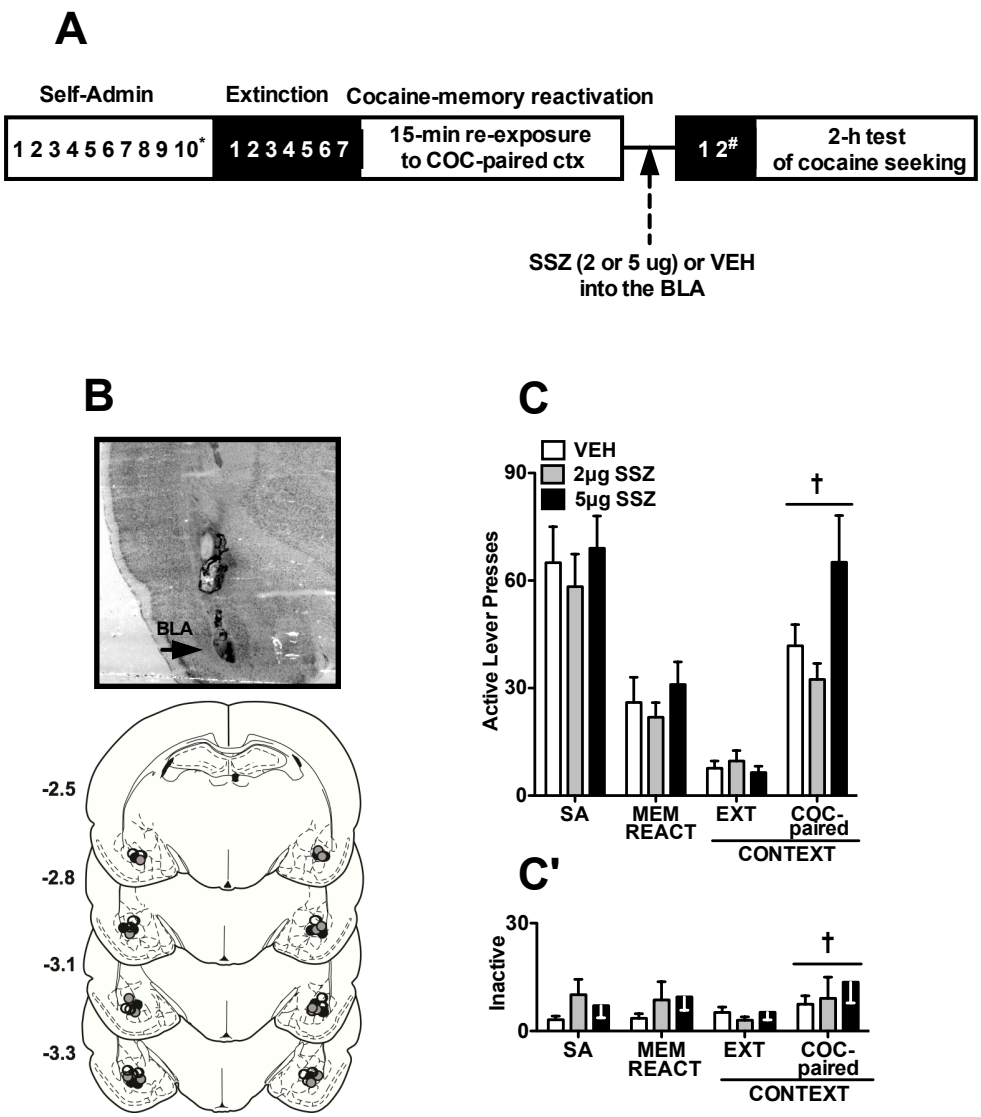


Fig 2 IκK/NF-κB inhibition in the BLA following cocaine-memory reactivation modestly, but non-significantly, alters subsequent drug context-induced cocaine seeking in a dose-dependent manner **(*A*)** Schematic depicting the timeline for Experiment 2. Cocaine self-administration (SA) sessions took place in a distinct environmental context until rats reached the acquisition criterion (*10 infusions/session for a minimum of 10 sessions). Extinction training occurred in a distinctly different context. After extinction training, rats were re-exposed to the cocaine-paired context (Cocaine-memory reactivation) for 15 min to trigger cocaine-memory destabilization and reconsolidation. Next, rats received bilateral microinfusions of the the IκK/NF-κB inhibitor, SSZ (2 or 5 µg/ 0.5 µl/hemisphere) or 20% DMSO/10 mM HEPES (pH 7.6) vehicle (VEH; 0.5 µl/hemisphere) into the BLA. On the day after memory reactivation, rats received additional extinction training until they met the extinction criterion (# ≤ 25 non-reinforced active lever responses/ session for two consecutive sessions). Cocaine seeking (non-reinforced active lever responding) was assessed in the cocaine-paired context (COC-paired) on the following day. **(*B*)** Photomicrograph and schematics depicting cannula placement. The symbols denote the most ventral point of the injector cannula tracts for rats in Experiment 2a that received bilateral

microinfusions of vehicle (VEH; *n = 11,* white circles), 2μg, (*n = 9,* grey circles), or 5 μg SSZ (*n = 9,* black circles) into the BLA following cocaine memory reactivation. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997). **(*C*)** Mean (± SEM) active lever responses during SA (mean of last three training sessions), the cocaine-memory reactivation session (MEM REACT), and during the tests of cocaine seeking in the extinction (EXT; the last session before the test in the COC-paired context) and in the COC-paired context for rats in Experiment 2a. **(*C'*)** Mean (± SEM) inactive lever responses in Experiment 2. † denotes significant difference relative to responding in the extinction context (ANOVA context main effect, $P<0.05$).

CHAPTER 4

CONTRIBUTION OF DORSAL HIPPOCAMPAL SRC-FAMILY TYROSINE KINASES TO THE RECONSOLIDATION OF INSTRUMENTAL CONTEXTUAL COCAINE MEMORIES

INTRODUCTION

The dorsal hippocampus (DH) is an integral substrate for the cellular consolidation of new declarative memories (Eichnembaum 1999), and it is thought to be a repository for spatial/contextual information, in particular (Eichenbaum *et al,* 1999; Morris *et al,* 2003). Accordingly, the DH is recruited during the expression of *context-elicited* drug-seeking behavior *per se*, including the expression of cocaine CPP (Meyers *et al,* 2006, Haghparast *et al,* 2013; Otis *et al,* 2013) and reinstatement of instrumental cocaine-seeking behavior (Fuchs *et al,* 2005, 2007; Xie *et al,* 2013). Conversely, the involvement of the DH in the reconsolidation of cocaine memories, the maintenance and availability of which is required for the ability of a cocaine-associated context to evoke cocaine-seeking behaviors (Tronson and Taylor 2007; Milton and Everitt 2013; Sorg 2012), is poorly understood (Ramirez *et al,* 2009; Otis *et al,* 2013). Illustrating this, both B-adrenergic receptor antagonism and global protein synthesis inhibition in the DH at the time of putative contextual cocaine-memory reconsolidation fail to impact the subsequent expression of cocaine CPP (Otis *et al,* 2013) and drug context-induced instrumental cocaine seeking (Ramirez *et al,* 2009), respectively. Remarkably, however, tetrodotoxin (TTX)-

induced neuronal inactivation of the DH and functional disconnection of the DH from the basolateral amygdala (BLA), a cite of protein synthesis dependent memory restabilization, are both sufficient to impair contextual cocaine-memory reconsolidation and attenuate instrumental cocaine-seeking behavior (Ramirez *et al,* 2009 and Wells *et al,* 2011, respectively). These findings highlight the possibility that the DH critically contributes to cocaine-memory reconsolidation by permitting protein synthesis-dependent cocaine-memory restabilization in extra-hippocampal locations, like the BLA (Fuchs *et al,* 2009; Wells *et al,* 2011). Therefore, delineating the importance of specific memory reconsolidation-related signal transduction cascades within the DH will allow for a refined understanding of the role of the DH in cocaine-memory reconsolidation.

To date, the role of Src family of tyrosine kinases (SFKs) in memory reconsolidation has been unexplored. Five distinct members of the Src tyrosine kinase family – Src, Fyn, Lyn, Yes, and Lck – are densely expressed in the hippocampus (Salter and Kalia 2004). Interestingly, both upstream signals from, and downstream substrates of, SFK activation are necessary for memory reconsolidation. For instance, activation of hippocampal SFKs can be achieved following stimulation of mGluR1 (Heidinger *et al,* 2002) or dopamine D1 receptors (MacDonald *et al,* 2007; Stramiello and Wagner 2008; Yang *et al,* 2012), or other G-protein coupled receptors that elicit the activation of cyclic AMP/Protein kinase A (PKA; MacDonald *et al,* 2007). Each of these signaling events has been implicated in memory reconsolidation (see Auber *et al,* 2013 for a current review), and PKA signaling, in particular, is necessary for successful reconsolidation of cocaine memories underlying both cue- and context-elicited cocaine-seeking behavior in the BLA (Sanchez *et al,* 2010 and Arguello *et al,* 2013b, respectively), albeit the involvement of PKA in drug-memory reconsolidation has not been similarly evaluated in DH.

Following activation, SFKs tightly regulate the activity of several neurotransmitter receptors by phosphorylating residues on their C-termini (Ohnishi *et al,* 2011). These receptors include NR2a- (Nakazawa *et al* 2001; Taniguchi *et al,* 2009) and NR2b-containing NMDARs (Nakazawa *et al,* 2001; Prybylowski *et al,* 2005; Zhang *et al,* 2008), as well as GluR2-containing AMPARs (Hayashi *et al* 1999, Hayashi and Huganir 2004). NMDARs, in particular, have been critically implicated in cocaine-memory reconsolidation in a variety of paradigms (Brown *et al,* 2008; Milton *et al,* 2008; Milton *et al,* 2013), including in our context-based extinction-reinstatement model (Healey, Wells, and Fuchs, unpublished). Importantly, SFKs are required for activity-dependent changes in synaptic efficacy, in particular long-term potentiation (LTP; Lu *et al,* 1998; Huang *et al,* 2001; Stramiello and Wagner 2008) and long-term depression (LTD; Hayashi and Huganir 2004; Fox *et al,* 2007; Yang *et al,* 2012), via their effects on NR2a and NR2b subunits, respectively (Yang *et al,* 2012). And, these SFK-mediated processes have been directly linked to learning and memory (for reviews, see Purcell and Carew 2003 and Ohinishi *et al,* 201). Particularly relevant to the present experiment, pharmacological inhibition of SFKs in the dorsomedial striatum or the DH attenuates the expression of drug-primed ethanol- and context-elicited cocaine-seeking behavior, respectively (Wang *et al,* 2010 and Xie *et al,* 2013, respectively).

Expanding upon these findings, Experiment 3a was designed to test the hypothesis that SFKs in the DH regulate the reconsolidation of contextual cocaine memories required for the sustained ability of a cocaine-associated environmental context to drive instrumental cocaine-seeking behavior. To determine this, Experiment 3a explored the effects of SFK inhibition in the DH following explicit reactivation of a context-response-cocaine memory trace on the subsequent expression of cocaine-seeking behavior. Additionally, Experiment 3b determined

whether the effects of SFK inhibition were memory reactivation-dependent. Finally, Experiment 3c utilized quantitative Western blotting to characterize changes in the phosphorylation-dependent activation of three known SFK substrates – NR2a NMDA subunits (Taniguchi *et al,* 2009),NR2b NMDAR (Nakazawa *et al,* 2001; Prybylowski *et al,* 2005; Zhang *et al,* 2008), and GluR2 AMPAR subunits (Hayashi *et al* 1999, Hayashi and Huganir 2004) – at the putative time of memory reconsolidation and following SFK inhibition (Clarke *et al,* 2010; Milton *et al,* 2013).

METHODS

Animals

Male Sprague-Dawley rats (*N =92*; 275-300 g; Charles River Laboratories, Wilmington, MA) were individually housed in a temperature- and humidity-controlled vivarium on a reversed light-dark cycle. Rats were fed 20-25 g of rat chow per day, and water was available *ad libitum*. Protocols for the housing and treatment of rats followed the *Guide for the Care and Use of Laboratory Rats* (Institute of Laboratory Animal Resources on Life Sciences, 1996) and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Food training

To expedite cocaine self-administration, rats were trained to lever press under a continuous schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH, USA) in sound-attenuated operant conditioning chambers (26 x 27 x 27 cm high; Coulbourn Instruments,

Allentown, PA, USA) during a 16-h overnight session. Active lever responses were reinforced by one food pellet, whereas inactive lever responses had no scheduled consequences. None of the contextual stimuli used for self-administration and extinction training were present during food training.

Surgery

Forty-eight h after food training, rats were fully anesthetized with ketamine hydrochloride (75 mg/kg, i.p.) and xylazine (5 mg/kg i.p.) Rats were implanted with chronic indwelling jugular catheters, as described previously (Fuchs *et al,* 2009) and next with 26-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA), aimed bilaterally at the DH (angled laterally by 15°; AP -3.4 mm, ML +/-3.1 mm, DV -2.15 mm), based on Paxinos and Watson (1997), using standard stereotaxic procedures. Stainless steel screws and cranioplastic cement were used to secure the guide cannulae to the skull. Stylets (Plastics One) and Tygon caps and obdurators (Plastics One) were used to seal the catheter and guide cannulae, respectively.

To extend catheter patency and prevent infection, catheters were flushed daily with cefazolin (10.0 mg/ml of heparinized saline; Schein Pharmaceuticals, Albuquerque, NM, USA) and heparinized saline (70 U/ml; Baxter Health Care Corp, Deerfield, IL, USA), as described previously (Fuchs *et al,* 2009). Rats received 5 days of post-operative recovery before the start of self-administration training. Catheter patency was periodically assessed by administering propofol (1mg/0.1ml, i.v. Eli Abbott Lab, North Chicago, IL, USA) intravenously and confirming a rapid loss of muscle tone.

Self-administration and Extinction Training

Behavioral training and testing (i.e., self-administration, extinction, cocaine-memory reactivation, and reinstatement) were conducted in sound-attenuated operant conditioning chambers configured to one of two distinctly different contexts, as described previously (Fuchs *et al,* 2009; see **Table 1, Chapter 2:** Contexts A and B).

Rats were randomly assigned to either Context A or B for self-administration training. Training took place during daily, 2h sessions in the rats' dark cycle. Active lever presses resulted in cocaine reinforcement (cocaine hydrochloride; 0.15 mg/0.05 ml per infusion, i.v.; NIDA, Research Triangle Park, NC) under a FR-1/time-out-20s schedule, as described previously (Fuchs *et al,* 2009). Responses on the other (inactive) lever were recorded but had no scheduled consequences. Training continued until rats reached the acquisition criterion (i.e., minimum 10 sessions with ≥10 cocaine infusions/session).

After meeting the acquisition criterion, rats received seven daily 2-h extinction-training sessions in the alternate context (A or B; different from the one used for self-administration training).. During extinction training, both active and inactive presses were recorded but had no programmed consequences. Following the fourth extinction-training session, rats were adapted to the intracranial microinfusion procedure (i.e., sham infusions). To this end, 33Ga injection cannulae (Plastics One) were inserted into the rats' guide cannulae to a depth of 1 mm below the tip of the guide cannulae and were left in place for 4 minutes. No liquid was infused. In Experiment 3c, rats, whose brains were collected for western blot analysis of protein levels following memory reactivation and intracranial manipulations, were extensively acclimated to the microinfusion procedure, in order to circumvent the effects of infusion stress on protein expression at the time of tissue collection. These rats received sham infusion following the sixth

and seventh extinction training sessions. However, the injection cannulae fully lowered only once, as for all other rats, in order to minimize cell loss/damage.

Experiment 3a

Experiment 3a was designed to determine whether administration of PP2, an ATP-competitive inhibitor of SFKs (Hanke *et al,* 1996; Brandvoid *et al,* 2012), into the DH at the putative time of cocaine-memory reconsolidation would attenuate subsequent drug context-induced cocaine-seeking behavior. On the day after the 7th extinction training session, rats were re-exposed to the cocaine-paired context for 15 min (i.e., cocaine-memory reactivation; see **Fig. 3.1A**) to trigger the destabilization and reconsolidation of context-response-cocaine associative memories (Fuchs *et al,* 2009). During this session, the levers were extended to allow for similar behavioral experience as during self-administration training (Fuchs *et al,* 2009), but lever responses were not reinforced because cocaine produces an acute crease in phosphorylation of NR2a NMDAR subunits in the ventral tegmental area in a SFK-dependent manner (Schumann *et al,* 2009), although its actions on other SFK-dependent interactions remain unclear. Immediately after cocaine-memory reactivation, rats received bilateral microinfusions of 0.1% DMSO VEH or PP2 (62.5 ng/0.5 μl/hemisphere) into the DH. This dose of PP2 was selected because it significantly attenuates the expression of drug context-induced cocaine-seeking behavior when microinfused into the DH at test (Xie *et al,* 2013). During intracranial microinfusions, injection cannulae were lowered to a depth of 1 mm below the tip of the guide cannulae. The injection cannulae remained in place for 1 min before and 1 min after the microinfusion, which was delivered over 2 min. Treatment assignment was counterbalanced based on previous cocaine intake.

Post-reactivation Extinction and Test of Drug Context-induced Cocaine Seeking

Beginning on the day following cocaine-memory reactivation, rats received additional daily 2h extinction-training sessions (2.14±0.14 days) until they reached the extinction criterion (i.e., ≤25 active lever responses per session on a minimum of 2 consecutive days). Twenty-four h later, rats were placed into the cocaine-paired context for a 1-h test of drug context-induced cocaine seeking, during which, non-reinforced active lever presses were recorded and provided an index of motivation to obtain cocaine reinforcement.

Experiment 3b

Experiment 3b tested whether the effects of PP2 administration in Experiment 3a reflected a *bona fide* memory reconsolidation impairment and, therefore, would fail to manifest in the absence of explicit cocaine-memory reactivation (Nader *et al,* 2000b). The experimental design (see **Fig. 3.2A**) was identical to that in experiment 3a, except that the groups were exposed to the novel, unpaired context (i.e., No-reactivation control context) for 15 min prior to receiving bilateral microinfusions of VEH or PP2 into the DH. The unpaired context distinctly differed from Contexts A and B (**Table 1**). Exposure to the unpaired context provided a similar behavioral experience to that during the cocaine-memory reactivation session in Experiment 3a without explicit cocaine-memory reactivation.

Experiment 3c

SFKs tightly regulate the cell surface expression and functional state of several neurotransmitter receptors involved in synaptic plasticity, learning, and memory (for review see

Ohnishi *et al,* 2011). SFKs phosphorylate tyrosine residues on the c-terminus of the NR2a NMDAR (Taniguchi *et al,* 2009), NR2b NMDAR (Nakazawa *et al,* 2001, 2002, 2006; Lavezzari *et al,* 2003; Prybylowski *et al,* 2005; Zhang *et al,* 2008), as well as the GluR2 AMPAR, subunits (Hayashi *et al* 1999; Hayashi and Huganir 2004). Experiment 3c was designed to ascertain whether cocaine-memory reconsolidation is associated with activation of NR2a, NR2b, or GluR2 subunits in the DH and whether PP2-induced SFK inhibition prevents this effect. Additionally, Experiment 3c evaluated whether potential changes in substrate activation in the DH would similarly manifest in the dorsally adjacent trunk region of the somatosensory cortex (SStr), as this region represents the most likely target of drug diffusion out of the DH. Experimental parameters were identical to those in Experiment 3a except that rats were exposed to the cocaine-paired context or remained in their home cages, prior to receiving bilateral microinfusions of VEH or PP2 into the DH (see **Fig. 3.3A)**. The home cage condition was expected to provide an index of basal levels of SFK substrate activation. Groups were euthanized either 15 min or 1 h following intracranial microinfusions in order to capture a range of possible activation kinetics of downstream SFK substrates (Hayashi and Huganir 2004; Schumann *et al,* 2008, 2009; Xie *et al,* 2013) related to memory reconsolidation *per se* (Pedreira and Maldonado 2003). Immediately following rapid decapitation, brains were quickly removed and flash frozen in isopentane before being stored at -80°C.

Western blotting.

Tissue punches for Western blot analyses were taken from the DH or SStr (expected negative anatomical control based on Ramirez *et al,* 2009) using 19Ga neuropunches (Fine Science Tools) from 40μm tissue sections. The tissue sections were also collected to verify

cannula placement. Punched tissue was stored at -80°C in lysis buffer containing 10 mM HEPES, 1% SDS, and 1x protease and phosphatase inhibitor cocktails (Sigma Aldrich, St. Louis, MO). Hippocampal samples were thawed, manually homogenized, and boiled for 10 min on a block heater at 100 °C. Protein concentrations were determined using the Biorad DC protein assay. For each sample, 30 μg of protein were electrophoresed on a 7.5% Tris-HCL polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane for 1 h at 100 V. Membranes were blocked in 5% nonfat milk for 1 h and incubated in polyclonal antibodies developed in rabbit against phosphorylated NR2b (pTyr1472; 1:1000, Sigma Aldrich, St. Louis, MO), NR2a (pTyr1325; 1:1000, Rockland Inc., Gilbertsville, PA), or GluR2 (pTyr876; 1:1000, Millipore Co., Billerica MA) overnight (16-20 h) at 4 °C. Membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (1:10,000, GE Healthcare, Piscataway, NJ), for 1 h followed by development with an enhanced chemiluminescence (ECL) system (Pierce Biotech, Rockford, IL). Membranes were incubated with stripping buffer (62.5 mM Tris-HCL at pH 6.7, 2% SDS, 100 mM beta-mercaptoethanol) to permit re-probing with antibodies developed in rabbit against total NR2b (1:1000, Millipore Co.) or NR2a (1:1000, Millipore Co.), or developed in mouse against total GluR2 (1:1000, Millipore Co.), and later, the loading control, actin (1:25,000, Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated and total protein, as well as actin, levels were quantified by densitometry, using NIH Image J software. To evaluate changes in protein activation, phosphorylated protein was normalized to total protein and actin. Subsequent discussion of protein activation refers to normalized levels. Western blot analysis of activation of these substrates is currently being assessed in tissue punches from the SStr, to determine whether there was appreciable diffusion of PP2 out of the DH at the time of microinfusions in Experiment 3a.

Histology

In Experiments 3a-c, rats were overdosed with ketamine hydrochloride and xylazine (66.6 and 1.3 mg/kg, i.v. or 199.8 and 3.9 mg/kg, i.p., respectively, depending on catheter patency) and transcardially perfused with a 1x phosphate buffered saline (Fischer Scientific) plus 10% formaldehyde solution (Sigma). Brains were dissected out and stored in 10% formaldehyde solution until they were sectioned coronally using a vibratome. Cannula placements were verified on 75μm sections stained using cresyl violet (Kodak, Rochester, NY, USA). The most ventral portion of each cannula tract was mapped onto schematics from the rat brain atlas (Paxinos and Watson, 1997).

Data Analysis

Separate t-tests were used to evaluate possible pre-existing differences in cocaine intake, in active and inactive lever responding during self-administration (mean of last 3 d), extinction (days 1 and 7), and during the memory reactivation session, as well as in the number of days required to reach the post-reactivation extinction criterion for rats in Experiments 3a-c that would subsequently receive either VEH or PP2 treatment following cocaine-memory reactivation or the no memory reactivation condition.

Separate mixed-factorial ANOVAs were conducted to examine the effects of intracranial microinfusions on active and inactive lever responses on the test days in the cocaine-paired and extinction contexts (the day preceding the test cocaine-paired context). In these analyses, treatment (VEH, PP2) was the between-subjects factor and testing context (EXT, COC-paired)

was the within-subjects factor. Significant main and interaction effects were further examined using Tukey *post-hoc* tests.

In Experiment 3c, separate t-tests were used to examine the effects of context (cocaine-paired, home cage) or treatment (VEH, PP2) on the activation (i.e., ratio of phosphorylated to total protein) of NR2b, NR2a, and GluR2 in the DH or in the SStr (data not shown; ongoing). An *a priori*, one-tail t-test was used to examine the effects of PP2 on NR2a activation following cocaine-memory reactivation. Alpha was set at 0.05.

RESULTS

Histology

Schematics and photomicrographs illustrating cannula placements or anticipated placements are included in **Figs 3.1-3.2B** and in **3.4.** The target brain region was defined as the dorsal hippocampus proper (DH). High power microscopy did not reveal tissue damage (i.e., extensive cell loss or gliosis) at injection sites. Only data from rats with correct cannula placements were included in statistical analyses.

Behavioral History

There were no pre-existing differences between subsequently VEH- and PP2-treated rats in cocaine intake or in active or inactive lever responses during cocaine self-administration training, extinction training days 1 and 7, or during the memory reactivation session. Additionally, there were no differences between treatment groups in the number of days required

to reach the extinction criterion (all $Ts \leq 1.76$, $Ps \geq 0.10$). Descriptive statistics are provided in **Table 4** below.

Table 4. Behavioral History

Exp./Group		Cocaine Intake	SA	EXT 1	EXT 7	Reactivation	Days to EXT
3a	VEH (*N*=6)	23.9 ± 2.2	64.2 ± 10.8	66.3 ± 18.9	4.0 ± 1.6	25.3 ± 13.8	2.0 ± 0.0
	PP2 (*N*=8)	21.7 ± 2.8	65.8 ± 15.0	60.4 ± 14.3	6.6 ± 2.0	17.1 ± 7.2	2.3 ± 0.3
3b	VEH/NR (*N*=7)	23.5 ± 1.7	54.0 ± 13.2	27.3 ± 10.3	7.7 ± 3.4	6.0 ± 3.3	2.0 ± 0.0
	PP2/NR (*N*=7)	25.7 ± 3.7	66.9 ± 21.6	50.1 ± 21.3	13.6 ± 8.1	3.7 ± 1.1	2.0 ± 0.0
3c	(15 m) VEH (*N*=7)	28.9 ± 4.1	56.8 ± 9.0	51.6 ± 12.0	7.0 ± 1.1	21.5 ± 8.4	N/A
	(15 m) PP2 (*N*=7)	25.3 ± 2.4	49.0 ± 5.0	58.1 ± 8.7	17.3 ± 7.7	17.3 ± 5.5	N/A
	(15 m) VEH/HC (*N*=7)	23.9 ± 3.5	54.1 ± 10.2	77.6 ± 29.2	18.7 ± 4.8	N/A	N/A
	(15 m) PP2/HC (*N*=5)	21.3 ± 2.2	44.1 ± 4.7	66.0 ± 23.0	10.6 ± 3.0	N/A	N/A
	(1h) VEH (*N*=6)	22.7 ± 1.9	54.7 ± 7.7	74.1 ± 28.1	10.1 ± 3.4	28.6 ± 12.3	N/A
	(1h) PP2 (*N*=8)	26.4 ± 2.6	55.1 ± 6.4	137.1 ± 40.3	10.4 ± 3.3	29.6 ± 7.0	N/A
	(1h)VEH/HC (*N*=7)	23.8 ± 2.7	51.0 ± 7.4	104.0 ± 28.2	5.7 ± 1.1	N/A	N/A
	(1h) PP2/HC (*N*=7)	23.0 ± 2.2	52.1 ± 10.2	77.8 ± 19.8	11.4 ± 3.9	N/A	N/A

Table 4. Cocaine intake (mean ± SEM infusions/session for the last 3 training sessions) and active lever responses during self-administration (SA; mean ± SEM for the last 3 training sessions), as well as active lever responses (mean ± SEM) during the first (Extinction 1) and last (Extinction 7) extinction training sessions, and during the 15-min memory reactivation (Reactivation) session. The number of days required to meet the extinction criterion (Days to EXT) is also included. Means are provided for rats that received PP2 or 0.1% DMSO vehicle (VEH) into the DH after cocaine-memory reactivation, novel context exposure (Nov, no reactivation controls), or confinement to their home cages (HC, no reactivation controls) and were euthanized 15 minutes (15 m) or 1 hour (1 h) later in Experiment 3c.

Experiment 3a: Effects of PP2 microinfused into the DH following cocaine-memory reactivation on subsequent drug context-induced cocaine seeking

Microinfusions of PP2 administered into the DH after cocaine-memory reactivation attenuated subsequent cocaine seeking in a context-dependent fashion (**Fig. 3.1C**; ANOVA context x treatment interaction, $F_{(1,12)}$=5.87, P=0.03; context main effect, $F_{(1,12)}$=25.13, P<0.001; treatment main effect, $F_{(1,12)}$ =4.88, P<0.05). Upon exposure to the cocaine-paired context during the test session, there was an increase in active lever responding in the group that had previously received VEH into the DH following cocaine-memory reactivation, relative to responding in the extinction context (Tukey's test, P<0.05). In contrast, the group that had previously received PP2 into the DH after cocaine-memory reactivation exhibited less active lever responding the cocaine-paired context at test, but not in the extinction context, relative to VEH-treated group (Tukey's test, P<0.05). Furthermore, active lever responding in this group did not differ between the extinction and cocaine-paired contexts.

Experiment 3b: No reactivation control experiment

Microinfusions of PP2 administered into the DH following exposure to a novel, unpaired context – i.e., in the absence of explicit cocaine-memory reactivation - did not alter subsequent cocaine-seeking behavior (**Fig. 3.2C**). Upon exposure to the cocaine-paired context at test, there was an increase in active lever responding, relative to responding in the extinction context (ANOVA context main effect only, $F_{(1,12)}$=33.50, P<0.001). Furthermore, there was no difference in active lever responding between VEH- and PP2-treated groups in either context (all treatment main and interaction effects, $F_{(1,12)}$=0.25-0.42, P=0.53-0.63).

Experiment 3c: Effects of PP2 microinfused into the DH on activation of NR2a, NR2b, and GluR2

Quantitative Western blot analyses indicated that PP2 treatment in the DH preferentially interfered with the phosphorylation-dependent activation of NR2a NMDAR subunits. Importantly, this effect depended on time and explicit memory reactivation and was specific to the DH. Cocaine-memory reactivation failed to alter activation of NR2a, NR2b, or GluR2 in VEH-treated rats, relative to home cage exposure (data not shown; all *Ts < 1.5, Ps > 0.16*). Intra-DH administration of PP2 following cocaine-memory reactivation significantly suppressed the activation of NR2a ($t(12)$=1.91, P=0.04; planned one-tailed t-test), but not NR2b ($t(11)$=0.17, P=0.866) or GluR2 ($t(11)$=0.56, P=0.58), in the DH, relative to VEH (**Fig. 3.3C**) when assessed 15 min post treatment. Consistent with minimal diffusion out of the DH, intra-DH PP2 did not similarly alter NR2a activation in the dorsally adjacent SStr (**Fig. 3.3C, inset,** $t(12)$=0.10, P=0.92). Further, unlike at the 15 min euthanasia time point, there was no difference between the PP2- and VEH-treated groups in the activation of any of these substrates at 1 h post treatment (**Fig. 3.3D**; all *Ts < 0.96, Ps > 0.36*). Further, PP2 treatment following home cage exposure did not alter the activation of any of these substrates at either euthanasia time point, relative to VEH (**Fig. 3.3C', D'**; all *Ts < 1.58, Ps > 0.14)*. Current analyses will determine whether post-memory reactivation intra-DH infusions of PP2 will similarly or differently alter NR2a activation in the dorsally adjacent SStr, in order to evaluate the anatomical specificity of PP2 effects.

Inactive Lever Responding

Inactive lever responding was minimal in all experiments and did not significantly differ as a function of treatment group or testing context (all *Fs*<3.017, *Ps*>0.108; **Figs. 3.1-3.2 C').**

DISCUSSION

Results from the present experiments are the first to demonstrate that hippocampal SFKs are important regulators of the memory reconsolidation process. In particular, our findings reveal that SFK activation in the DH is necessary for the reconsolidation of cocaine-related memories that drive drug context-induced instrumental cocaine-seeking behavior in the extinction-reinstatement animal model of drug relapse.

In Experiment 3a, administration of PP2, a nonselective inhibitor of SFKs (Hanke *et al,* 1996; Brandvoid *et al,* 2012), into the DH following a 15-min re-exposure to the cocaine-associated context, which reliably destabilizes and permits the reconsolidation of context-response-cocaine associative memories (Fuchs *et al,* 2009; Ramirez *et al,* 2009; Wells *et al,* 2011, 2013; Arguello *et al,* 2013b), significantly reduced subsequent drug context-elicited cocaine seeking, assessed ~72 h later, relative to VEH treatment (**Fig. 3.1C**). This finding is consistent with an authentic memory reconsolidation impairment, as PP2 did not produce general, context-independent amnesia or a nonspecific deficit in motivation. In support of this, PP2 administration following exposure to a novel, unpaired context - thus in the *absence* of explicit cocaine-memory reactivation – was not sufficient to disrupt the ability of the cocaine-paired context to elicit cocaine-seeking behavior on the test day, relative to VEH (**Fig. 3.2C**) Moreover, post-memory reactivation intra-DH PP2 did not alter active lever responding in the

extinction context (**Fig. 3.1C**), or inactive lever responding in either context, relative to VEH (**Fig. 3.1C'**). Together, these findings suggest that PP2 exerted a targeted disruption of memory reconsolidation processes *per se.*

The effects of PP2 in Experiment 3a can most likely be attributed to an anatomically selective inhibition of SFKs in the DH. In support of this, we have previously demonstrated that TTX-induced neuronal inactivation of the SStr, a brain region that lies just dorsal relative to the DH and is consequently in the most likely path of unintended diffusion of PP2 along the injector cannula shaft (Baker *et al,* 1996; Neisewander *et al,* 1998), after cocaine-memory reactivation fails to alter cocaine-seeking behavior at test, suggesting that the integrity of this brain region is not required for the *reconsolidation* of cocaine-related contextual memories (Ramirez *et al,* 2009). It is improbable that a more selective manipulation, like inhibition of SFKs within the DH, would have different effects than global inaction on the dorsally adjacent SStr. In support of this, administration of PP2 into the DH during cocaine-memory reconsolidation failed to alter activation of NR2a subunits in the SStr, relative to VEH (**Fig. 3C, inset**). This is consistent with negligible drug diffusion (Wells *et al,* 2013). Thus, the current findings most likely point to SFK signaling within the DH as an integral component of the cellular processes that subserve the long-term maintenance of cocaine-related memories.

The present study extends previous research demonstrating DH involvement in cocaine-memory reconsolidation (Ramirez *et al,* 2009; Wells *et al*, 2012) by identifying an intracellular mechanism – SFK activation - through which the DH might regulate functional and structural changes at synapses that support protein-synthesis dependent cocaine-memory restabilization in other brain regions, like the BLA, (Fuchs *et al,* 2009; Wells *et al,* 2011). SFKs are well positioned near the cell membrane to tightly regulate the activity of NMDARs, AMPARs, and

other receptors, and thereby either depress or facilitate local synaptic strength (for review, see Ohinishi *et al,* 2011). Evidence from the present experiments supports SFK-dependent regulation of NR2a receptors as the critical mechanism underlying DH-dependent cocaine-memory reconsolidation (**Fig. 3.3**). Specifically, PP2 treatment attenuated NR2a, but not NR2b or GluR2, activation in the DH, when assessed following cocaine-memory reactivation and therefore, during the period of putative cocaine-memory reconsolidation (**Fig. 3.3C**). Importantly, the same manipulation, administered following home cage exposure, failed to alter basal activation of NR2a (**Fig. 3.3C').** Furthermore, these changes were transient and observable at the 15 min, but not the 1 h, euthanasia time point (**Fig. 3.3D)** consistent with NR2a phosphorylation kinetics (Schumann *et al,* 2009). Remarkably, cocaine memory reactivation did not result in an increase in NR2a subunit activation in the DH in VEH-treated rats, relative to home cage exposure (data not shown). These data may reflect our inability to detect changes in the phosphorylation state of a small pool of NR2a subunits or rapid de- and re-phosphorylation following cocaine context exposure. In support of the latter, the activation and deactivation kinetics of NR2a are substantially faster than those of NR2b (Vincini *et al,* 1998). Alternatively, NR2a subunit activation in the DH may play a permissive role such that, instead of a rise in NR2a activation, a threshold level of NR2a activation is required in the DH in order for cocaine memory reconsolidation to occur. For instance, a minimum NMDA current through NR2a-containing receptors in the DH may be required for setting in motion robust ERK1/2 activation in the BLA that is required for cocaine memory reconsolidation (Wells *et al,* 2013; see **Chapter 2**).

Previous literature has identified the SFK, Src, as responsible for phosphorylating Tyr-1325 of the NR2a NMDAR subunit (Yang and Leonard 2001; Taniguchi *et al,* 2009; Yang *et al,* 2012). While additional Src phosphorylation sites on the C-terminus of NR2a have been

identified in HEK293 cells (Yang and Leonard 2001), we chose to focus on Tyr-1325, as it is the most abundantly phosphorylated residue in neuronal cultures and has been functionally linked to NMDAR trafficking and learning that underlies depressive-like behavior (Taniguchi *et al,* 2009). SFK-dependent phosphorylation of Tyr-1325 contributes to the potentiation of NMDAR currents in the striatum *in vivo* (Taniguchi *et al,* 2009) and LTP at CA1 synapses *in vitro* (Yang *et al,* 2012). Moreover, activation of NR2a subunits *per se* is required for the reconsolidation of fear conditioned associative memories (Milton *et al,* 2013). The present findings significantly expand upon this previous research by identifying SFK-mediated NR2a activation as a possible mechanism for cocaine-memory restabilization. As PP2 nonselectively inhibits all 5 SFK isoforms (Hanke *et al,* 1996; Brandvoid *et al,* 2012), it will be imperative to follow up on the present findings by using transgenic models with either a selective deletion of Src or site directed mutagenesis of Tyr-1325 to establish a causal relationship between this specific SFK interaction and successful context-response-cocaine memory reconsolidation.

The non-significant effects of cocaine-context exposure and intra-DH PP2 treatment on NR2b in Experiment 3c were unexpected. NR2b phosphorylation by the SFK, Fyn (Nakazawa *et al,* 2001; Prybylowski *et al,* 2005; Zhang *et al,* 2008), reduces clatharin-mediated endocytosis of NMDARs (Yaka *et al,* 2002; Lavezzari *et al,* 2003; Prybylowski *et al,* 2005) and promotes the appropriate localization of these receptors to the cell membrane (Yaka *et al,* 2002). Such augmentation of NR2b subunit function is hypothesized to underlie SKF-mediated enhancements in NMDAR currents and long-term potentiation (LTP) in hippocampal cultured neurons and slice preparations (Wang and Salter 1994; Yu *et al,* 1994; Yu *et al,* 1997; Stramiello and Wagner 2008). Additionally, we have previously demonstrated that SFKs in the DH control the expression of drug context-induced cocaine-seeking behavior in a NR2b activation-dependent

manner (Xie *et al,* 2013). Therefore, overall, the previous literature suggests that SFK-mediated phosphorylation of NR2b positively regulates synaptic efficacy and memory stability.

In contrast, the present findings support the alternative hypothesis that SFKs may differentially recruit NR2a, NR2b, and GluR2 subunits for synaptic depotentiation and potentiation. For instance, Lyn-mediated phosphorylation of GluR2 AMPAR subunits and subsequent AMPAR internalization can facilitate long-term depression (LTD; Hayashi and Huganir 2004; Fox *et al,* 2007; Shinohara *et al,* 2008; Liu *et al,* 2009). Interestingly, SFK-dependent facilitation of NR2b subunit surface expression (Yaka *et al,* 2002; Lavezzari *et al,* 2003; Prybylowski *et al,* 2005) can also produce LTD (Liu *et al,* 2004; Kim *et al,* 2005; Yang *et al,* 2012), likely by promoting NR2b-mediated AMPAR internalization (Shi *et al,* 2001). Notably, a recent report points to a double dissociation of NR2b and NR2a-containing NMDARs in auditory fear memory destabilization and reconsolidation, respectively (Milton *et al,* 2013). Evidence suggests that LTD-like processes, including synaptic depotentiation and protein degredation, mediate memory destabilization (Lee *et al,* 2008; Mallert *et al,* 2010; Clarke *et al,* 2010), whereas LTP-like processes, including synaptic potentiation and protein synthesis promote memory reconsolidation (Nader *et al,* 2000a; Alberini 2006; Finnie and Nader 2012). Hence, SKFs in the DH may promote the transition of a memory trace from a destabilized to a reconsolidated state by initially regulating NR2b-mediated memory destabilization and later supporting NR2a-dependent memory reconsolidation. The present findings are consistent with this hypothesis, as administration of PP2 *following* cocaine-memory reactivation interferes with memory restabilization *per se*. Future studies will be instrumental in pinpointing the exact role of SFKs in cocaine-memory *destabilization*.

General Conclusion – Experiments 3a-d

SFKs in the DH are required for the expression of drug context-induced cocaine-seeking behavior (Xie *et al,* 2013), and the present experiments demonstrate that these kinases are also responsible for the restabilization of drug-related memories that contribute to cocaine-seeking behavior. Interestingly, hippocampal SFKs regulate these distinct phenomena via the activation of different NMDAR subunits, with SKF-mediated NR2b subunit activation controlling the behavioral expression of context-elicited motivation (Xie *et al,* 2013) whereas SKF-mediated NR2a subunit activation permitting cocaine memory restabilization (present study). These phenomena may involve different SFKs, since it has recently been suggested that Src and Fyn selectively control activation of NR2a versus NR2b NMDAR subunits, respectively, via signaling pathways that are mutually exclusive, starting at the level of cell-surface receptor stimulation (Yang *et al,* 2012). Src-NR2a interactions follow upstream stimulation of $G_{\alpha Q}$-coupled GPCRs, including M1 muscarinic acetylcholine (Lu *et al,* 1999) or group 1 mGluRs (Lan *et al,* 2001; Heidinger *et al,* 2002; Kotecha *et al,* 2003), whereas Fyn-NR2b interactions are downstream from the stimulation of $G_{\alpha S}$-coupled GPCRs, including the D1 dopamine receptors (MacDonald *et al,* 2007; Stramiello and Wagner 2008; Yang *et al,* 2012) or, alternatively, pituitary adenylate cyclase activating peptide 1 receptors (PAC1Rs; Yaka *et al,* 2003). While the present findings are consistent with the former pathway of activation, future studies will be required to delineate the precise pathway through which SFKs are activated to control cocaine-memory reconsolidation. Overall, however, the present findings begin to explicate the complex mechanisms through which the DH controls cocaine-memory reconsolidation and will likely have important implications for the development of treatments intended to perturb the long-term maintenance of maladaptive drug-related memories.

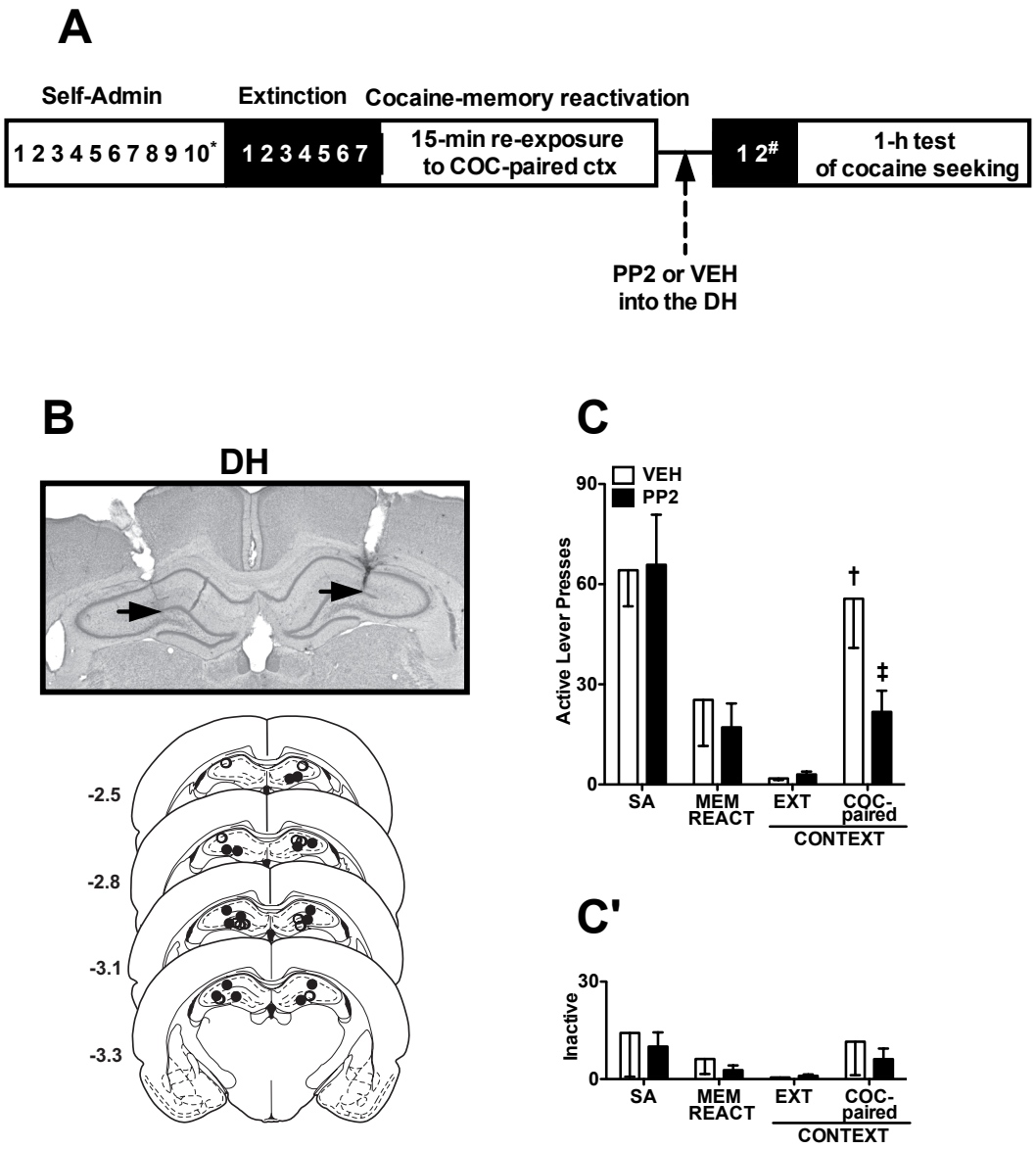


Fig 3.1 Inhibition of SFKs in the DH following cocaine-memory reactivation attenuates subsequent drug context-induced cocaine seeking. **(*A*)** Schematic depicting the timeline for Experiment 3a. Cocaine self-administration (SA) sessions took place in a distinct environmental context until rats reached the acquisition criterion (*10 infusions/session for a minimum of 10 sessions). Extinction training occurred in a distinctly different context. After extinction training, rats were re-exposed to the cocaine-paired context (Cocaine-memory reactivation) for 15 min to trigger the destabilization and subsequent reconsolidation of cocaine memories. Immediately after the session, rats received bilateral microinfusions of the nonselective SFK inhibitor, PP2 (62.5 ng/ 0.5 µl/hemisphere) or 0.1% DMSO vehicle (VEH; 0.5 µl/hemisphere) into the DH. Next, groups underwent additional extinction training until they met the extinction criterion (# ≤ 25 non-reinforced active lever responses/ session for two consecutive sessions). Cocaine seeking (non-reinforced active lever responding) was then assessed in the cocaine-paired context (COC-

paired). **(*B*)** Photomicrograph and schematics depicting cannula placement. The symbols denote the most ventral point of the injector cannula tracts for rats in Experiment 3a that received bilateral vehicle (VEH; *n = 6,* white circles) or PP2 (*n = 8,* black circles) infusions into the DH following cocaine memory reactivation. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997). **(*C*)** Mean (± SEM) active lever responses during SA (mean of last three training sessions), the cocaine-memory reactivation session (MEM REACT), and during the tests of cocaine seeking in the extinction (EXT; the last session before the test in the COC-paired context) and in the COC-paired context for rats in Experiment 3a. **(*C'*)** Mean (± SEM) inactive lever responses in Experiment 3a. † denotes significant difference relative to responding in the extinction context (ANOVA context main and simple main effects, $P<0.05$). ‡ denotes significant difference relative to the respective VEH treatment (ANOVA treatment simple main effect, $P<0.05$).

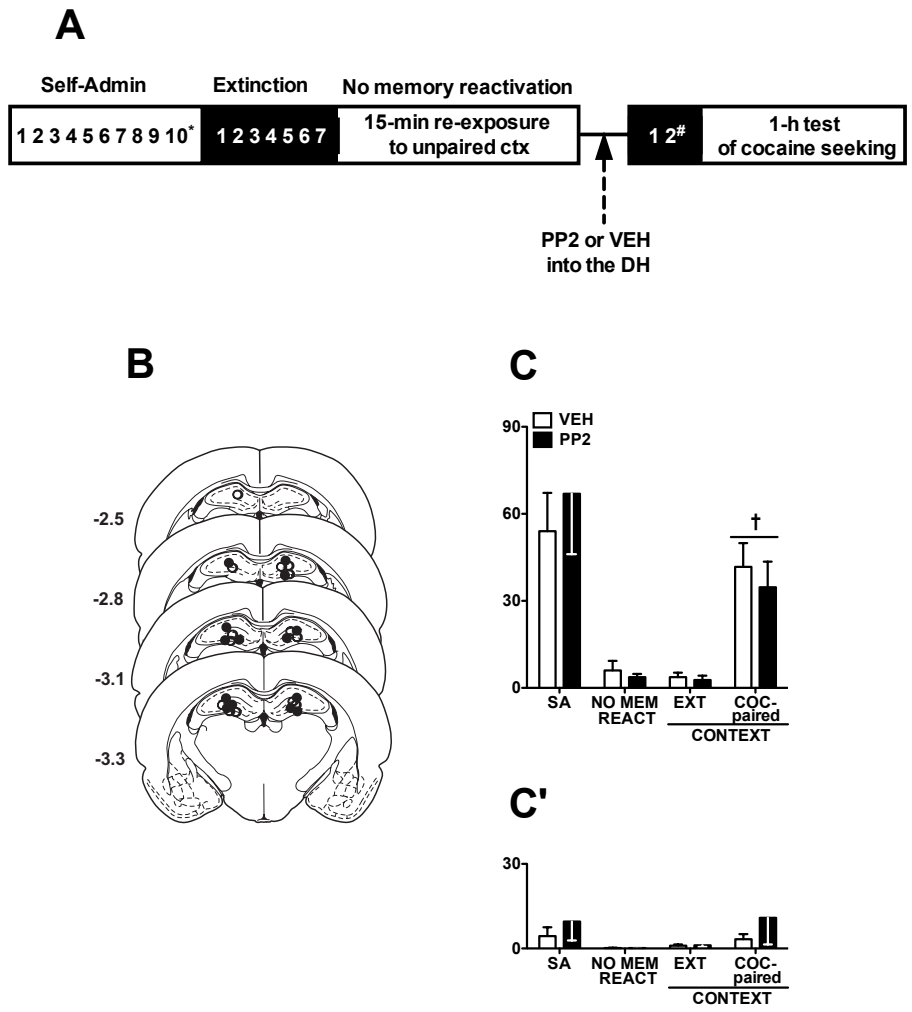


Fig. 3.2 Inhibition of SFKs in the DH in the absence of explicit cocaine-memory reactivation fails to alter subsequent drug context-induced cocaine seeking. **(*A*)** Schematic depicting the timeline for Experiment 3b. Experimental parameters were identical to those in Experiment 3a, except that rats in Experiment 3b were placed into a novel, unpaired context (No memory reactivation) for 15 min to provide similar behavior experience to rats in Experiment 3a without reactivating cocaine memories prior to receiving bilateral microinfusions of PP2 (62.5 ng/ 0.5 µl/hemisphere) or VEH (0.5 µl/hemisphere) into the DH. **(*B*)** Schematics depicting cannula placement. The symbols denote the most ventral point of the injector cannula tracts for rats in Experiment 3b that received bilateral vehicle (VEH; *n = 7,* white circles) or PP2 (*n = 7,* black circles) infusions into the DH following novel context exposure. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997). **(*C*)** Mean (± SEM) active lever responses during SA (mean of last three training sessions), novel, unpaired context exposure (NO MEM REACT), and during the tests of cocaine seeking in the extinction (EXT; the last session before the test in the COC-paired context) and in the COC-paired context for rats in Experiment 3b. **(*C'*)** Mean (± SEM) inactive lever responses in Experiment 3b. † denotes significant difference relative to responding in the extinction context (ANOVA context main and simple main effects, $P<0.05$).

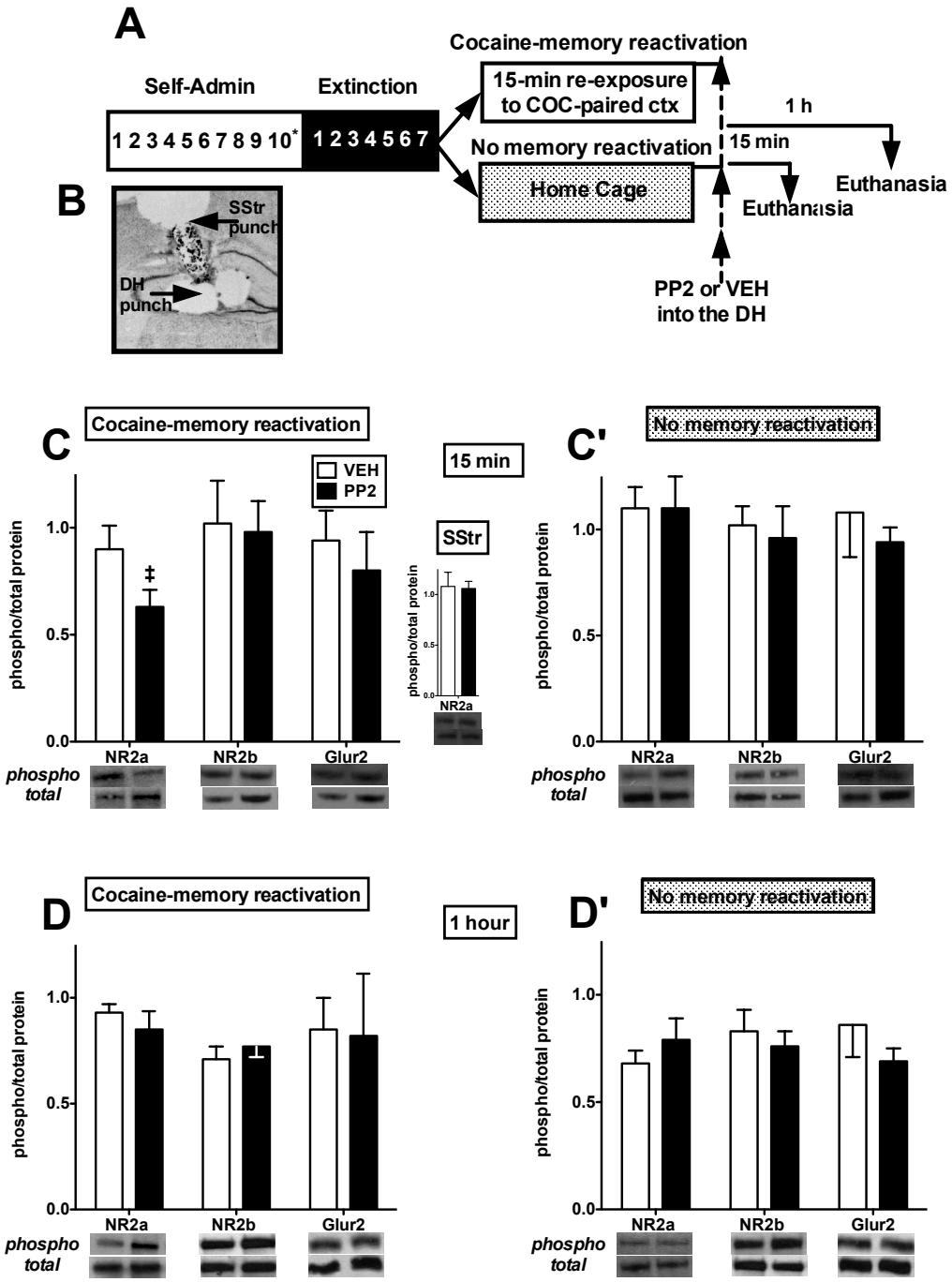
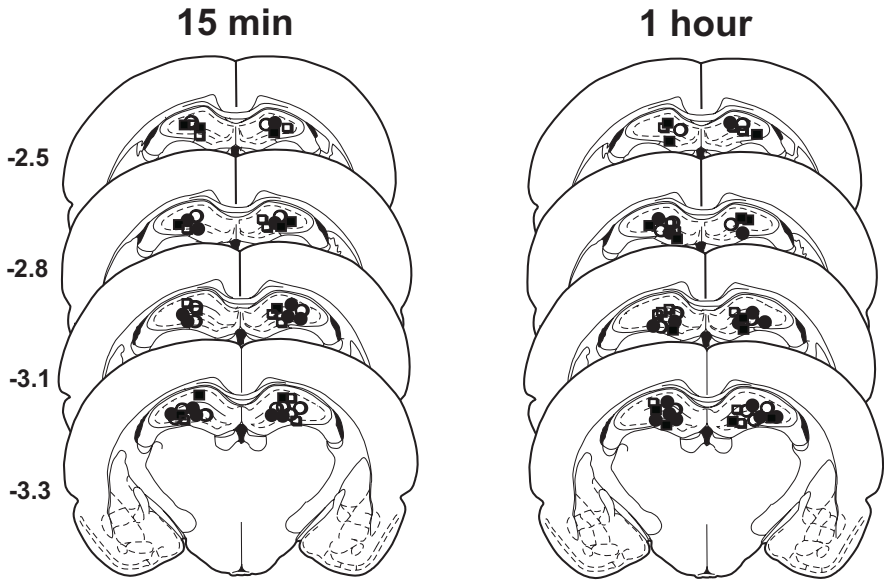


Fig. 3.3 SFK inhibition in the DH preferentially suppresses phosphorylation-dependent activation of NR2a subunits in the DH, but not the SStr, a time- and memory reactivation-dependent fashion. (*A*) Schematic depicting the timeline for Experiment 3c. Experimental parameters were the same as those used in Experiment 3a, except that rats received bilateral microinfusions of PP2 (62.5 ng/ 0.5 µl/hemisphere) or VEH into the DH after re-exposure to the cocaine-paired context (Cocaine-memory reactivation) or following retention in the home cage (No memory reactivation). These rats were euthanized either 15 min or 1 h post-infusion. *(B)*

Photomicrograph depicting the location of tissue punches taken from the DH and overlying SStr that were used for Western blotting. (*C, C'*) Mean (± SEM) NR2a, NR2b, and GluR2 activation (expressed as the ratio of phosphorylated to total protein levels normalized to actin) in rats euthanized 15 min after cocaine-memory reactivation (*C*) or home cage exposure (*C'*) and intracranial manipulations. Representative bands of phospho and total NR2a, NR2b, and GluR2 are provided. (*D, D'*) Mean (± SEM) NR2a, NR2b, and GluR2 activation and representative protein bands in rats sacrificed 1 h after cocaine-memory reactivation (*D*) or home cage exposure (*D'*) and intracranial manipulations. ‡ denotes significant difference relative to VEH treatment (Planned, one-tailed t-test, $P<0.05$).



∫**Fig. 3.4** Schematics depicting cannula placement. The symbols denote the most ventral point of the injector cannula tracts for rats in Experiment 3c. The left panel contains placements from rats that were euthanized 15 minutes after receiving cocaine-memory reactivation plus bilateral VEH (*n = 7,* open circles) or PP2 (*n = 7,* filled circles) infusions into the DH or following exposure to the home-cage plus bilateral VEH (*n = 7,* open squares) or PP2 (*n = 5,* filled squares) infusions. The right panel contains placements from rats that were euthanized 1 hour after cocaine-memory reactivation and bilateral VEH (*n = 6* open circles) or PP2 (*n = 8,* filled circles) infusions into the DH or following exposure to the home-cage plus bilateral VEH (*n = 7,* open squares) or PP2 (*n = 7,* filled squares) infusions. Numbers indicate the distance from bregma in mm, according to the rat brain atlas of Paxinos and Watson (1997).

CHAPTER 5

GENERAL DISCUSSION

Summary of Experimental Findings

Several lines of research have begun to elucidate the intracellular mechanisms of drug-memory reconsolidation, but overall, this phenomenon is poorly understood. Thus, the overarching objective of this dissertation was to make a significant contribution to our understanding of the molecular mechanisms of context-response-cocaine memory reconsolidation within two critical neural substrates of this phenomeonon, the BLA and DH (Fuchs *et al,* 2009; Ramirez *et al,* 2009; Wells *et al,* 2011). The first in these series of experiments ascertained the specific involvement of the MEK/ERK signal transduction cascade within the BLA and NACc. Previous research has critically implicated ERK signaling in the NACc in the reconsolidation of Pavlovian cocaine-related memories that underlie expression of cocaine CPP (Miller and Marshall 2005) and core elements of the MEK/ERK pathway, like expression of the immediate early gene *zif268* in the BLA, in the reconsolidation of both Pavlovian cocaine memories and instrumental cocaine memories that maintain the expression of conditioned reinforcement, respectively (Theberge *et al,* 2010). Based on these studies, we postulated that phosphorylation-dependent activation of ERK in the BLA and NACc would be necessary for the restabilization of cocaine memories in our context-based extinction-

reinstatement paradigm. To test this hypothesis, rats in Experiment 1a were briefly re-exposed to the cocaine-paired context in order to promote the reactivation and reconsolidation of cocaine-related memories and next received bilateral microinfusions of U0126, a potent MEK/ERK inhibitor, or VEH into the BLA or the NACc. The effects of these manipulations on drug context-induced cocaine-seeking behavior were assessed after ~2 days of additional extinction training – approximately 72 h after intracranial manipulations. Interestingly, ERK inhibition in the BLA, but not the NACc, reduced subsequent drug context-induced cocaine-seeking behavior, relative to VEH. This effect in the BLA was accompanied by a transient inhibition of ERK1/2 phosphorylation and depended on memory reactivation, given that the same manipulation administered in conjunction with novel context exposure – in the absence of explicit cocaine-memory reactivation – did not similarly alter context-elicited cocaine seeking. Remarkably, similar to U0126, B+M-induced neural inactivation of the NACc following cocaine-memory reactivation, failed to alter subsequent cocaine seeking. These findings demonstrate that ERK activation in the BLA, but not the NACc, is required for the reconsolidation of context-response-cocaine associative memories. Together with prior research, these results suggest that contextual drug-memory reconsolidation in Pavlovian and instrumental paradigms involves distinct neuroanatomical substrates.

Both gene transcription and protein synthesis are integral to the structural changes accompanying memory restabilization (Miller and Sweatt 2006; Romano *et al,* 2006). Downstream from MEK/ERK, activation of the TF CREB has been theorized to play a critical role in this process (Miller and Marshall 2005; Kim *et al,* 2011; Tronson *et al,* 2012). In addition to CREB, NF-κB, a TF involved in immune and inflammatory responses (Natoli *et al,* 2005; Hoffman *et al,* 2006), has also emerged as an vital regulator of fear- (Romano *et al,* 2006; Boccia

et al, 2007; Si *et al,* 2012), and notably, morphine-related memory reconsolidation required for the sustained expression of morphine CPP (Yang *et al,* 2011). However, whether NF-κB similarly regulates the reconsolidation of contextual *cocaine-related* memories that permit contextual stimulus control over *instrumental* cocaine-seeking behavior, was uncertain. Based on a wealth of evidence critically implicating NF-κB in a number of learning and memory processes across model organisms and experimental paradigms (for review, see Romano *et al,* 2006), we hypothesized that IκK-dependent activation of NF-κB in the BLA would be obligatory for instrumental contextual cocaine-memory reconsolidation. To evaluate this hypothesis, rats in Experiment 2 received bilateral microinfusions of one of two doses of SSZ, an inhibitor of IκK/NF-κB activation (Zandi *et al,* 1997; Jacobs and Harrison 1998) or VEH into the BLA after cocaine-memory reactivation and were tested for drug context-induced reinstatement of cocaine seeking ~72 h later. Interestingly, SSZ treatment failed to significantly alter subsequent drug context-induced cocaine-seeking behavior, relative to VEH, irrespective of dose. However, interestingly, the higher, 5μg dose of SSZ modestly *potentiated* cocaine-seeking behavior, compared to VEH. This finding, albeit nonsignificant, may point to a fundamentally dissimilar role for NF-κB transcriptional regulation in the reconsolidation of cocaine memories underlying context-elicited motivation for cocaine and in the reconsolidation of conditioned fear (Lubin and Sweatt 2007; Boccia *et al,* 2007; de la Fuentes *et al,* 2011; Si *et al,* 2012) or Pavlovian morphine-related memories (Yang *et al,* 2011).

A third aim of this dissertation was to begin to probe the molecular underpinnings of DH involvement in instrumental context-cocaine memory reconsolidation by assessing the contribution of SFKs in the DH to this phenomenon. Previous findings from our laboratory have suggested that the functional integrity of, but surprisingly, not protein synthesis within, the DH is

necessary for cocaine-memory reconsolidation (Ramirez *et al,* 2009). Moreover, we have demonstrated that intrahemispheric information processing by the BLA and DH critically governs cocaine-memory reconsolidation (Wells *et al,* 2011). Based on these findings, we have tenatively proposed that the contextual representation of cocaine memory traces is transiently maintained by the DH during the putative cellular restabilization of context-response-cocaine memories in the BLA (Fuchs *et al,* 2009), or alternatively, that the connection between these brain regions during cocaine-memory reconsolidation may be required for the establishment of retrieval links (Wells *et al,* 2011). However, direct assessment of such a hypothesis would be premature before first identifying potential molecular mechanisms of DH-mediated cocaine-memory reconsolidation. Given that SFKs in the DH regulate the *expression* of drug context-induced cocaine-seeking behavior in a NR2b-dependent fashion (Xie *et al,* 2013) and NMDA receptor stimulation in the DH appears to be involved in cocaine memory reconsolidation (Healey, Wells, and Fuchs unpublished), Experiment 3 evaluated the hypothesis that SFKs activation would be similarly required in the DH for the reconsolidation of context-response-cocaine memories. To this end, rats received bilateral microinfusions of the SFK inhibitor, PP2, or VEH into the DH after memory reactivation and were tested for reinstatement of cocaine seeking ~72 h later. Consistent with our hypothesis, post-cocaine memory reactivation intra-DH PP2 disrupted cocaine-memory reconsolidation, and in turn, attenuated subsequent drug context-induced cocaine-seeking behavior, relative to VEH. These effects depended on explicit cocaine-memory reactivation and SFK inhibition in the DH *per se*, in that PP2 was without effect when administered after exposure to an unpaired context (i.e., without explicit cocaine-memory reactivation), and previous global inactivation of the dorsally adjacent SStr after cocaine-memory reactivation was similarly ineffective in attenuating subsequent cocaine-seeking

behavior (Ramirez *et al,* 2009). Furthermore, SFK inhibition in the DH selectively interfered with the phosphorylation-dependent activation of NR2a, but not NR2b or GluR2 subunits (i.e., downstream effectors of SFKs; Ohnishi *et al,* 2011), even though memory reconsolidation was not associated with an increase in NR2a activation, relative to baseline. These findings suggested that NR2a subunit-containing NMDAR might play a permissive role in cocaine memory reconsolidation.

Molecular Mechanisms of Cocaine-Memory Reconsolidation in the BLA

The present experiments, considered in concert with a larger body of literature, begin to delineate the specific molecular signaling cascades that lead to gene transcription and protein synthesis during drug-memory reconsolidation. Specifically, MEK/ERK, NF- κB, and SFKs potentially function within a larger, more complex, milieu of intracellular interactions that take place during cocaine-memory reconsolidation and are likely affected by cocaine-induced adaptations.

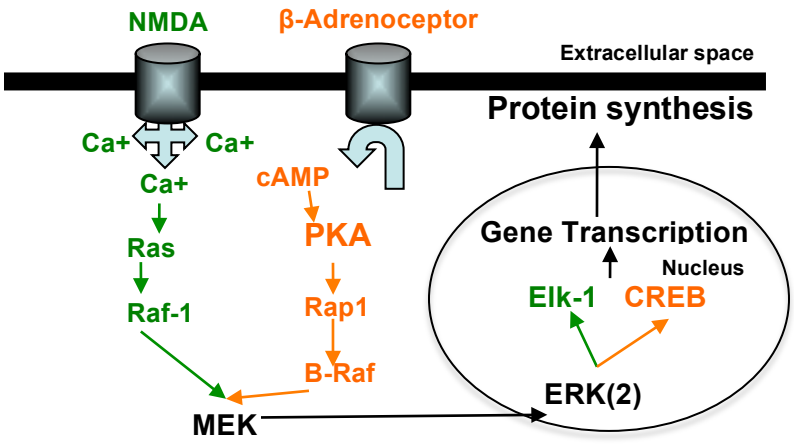
Findings in Experiment 1 suggest that ERK1/2 in the BLA is required for context-response-cocaine memory reconsolidation (Wells *et al,* 2013), and contribute to a larger literature implicating MEK/ERK signaling in synaptic plasticity, long-term memory consolidation (English and Sweatt 1996, 1997; Schafe *et al,* 1999, 2000; Sweatt 2001) and reconsolidation (Kelly *et al,* 2003; Miller and Marshall 2005; Duvarci *et al,* 2005). Interestingly, the importance of ERK in maintaining maladaptive drug related memories after their retrieval (Miller and Marshall 2005; Wells *et al,* 2013) compared to non-drug memories (Kelly *et al,* 2003; Duvarci *et al,* 2005) is likely augmented following repeated exposure to cocaine, in that cocaine produces dramatic adaptations in ERK signaling, especially that of the ERK2 isoform

(Valjent *et al,* 2005), which subserves the conditioned effects of cocaine (Mazzucchelli *et al,* 2002; Ferguson *et al,* 2006). Thus, ERK2 may represent a mechanism for the development of pathologically strong or intrusive cocaine-related memories. In support of this, cocaine CPP and cocaine-induced locomotor sensitization are *enhanced* in constitutive ERK1 knockouts, likely related to increased ERK2 phosphorylation by MEK (Mazzucchelli *et al,* 2002; Ferguson *et al,* 2006). Moreover, there is a robust increase in ERK2, but not ERK1 phosphorylation in the NACc of rats during the reconsolidation of Pavlovian cocaine memories (Miller and Marshall 2005) and in the BLA during the reconsolidation of instrumental context-cocaine memories in Experiment 2c (see **Fig. 1.3**). This likely reflects a cocaine-induced biasing of stimulus-elicited MEK phosphorylation toward ERK2 over ERK1, contributing to ERK2-dependent facilitation of pathological drug-related memories (Mazzucchelli *et al,* 2002; Ferguson *et al,* 2006; Girault *et al,* 2007).

ERK1/2 has been described as a coincidence detector (Girault *et al* 2007) because it integrates several converging inputs at the level of cytoplasmic activation (Adams and Sweatt 2002; Davis and Laroche 2006). While the canonical MEK/ERK pathway requires upstream activation of growth factors and mitogens (Cooper and Hunter 1982; Davis and Laroche 2006), ERK activation can also be triggered by numerous cell-surface signals that have been implicated in other forms of memory reconsolidation, including β-adrenergic (Roberson *et al,* 1999), dopamine D1 (Lu *et al,* 2006), and NMDA receptor stimulation (English and Sweatt 1996; Impey *et al,* 1998). ERK activation following β-adrenergic or dopamine D1 receptor stimulation depends on PKA (Roberson *et al,* 1999), whereas ERK phosphorylation downstream from NMDAR stimulation requires rapid calcium transients (Bading and Greenberg 1991; Rosen *et al,* 1994). Importantly, these distinct pathways of ERK activation differentially alter gene

transcription (Davis and Laroche 2006). In particular, calcium influx through NMDARs results in successive GTPase activation, transient ERK phosphorylation, and preferential stimulation of Elk-1 in the cell nucleus (Murphy *et al,* 2002). Conversely, PKA activation selectively activates the GTPase, Rap-1 (Vossier *et al,* 1997; York *et al,* 1998). Rap-1, in turn, induces a more sustained state of ERK phosphorylation and biases ERK-dependent activation of CREB, via the 40S ribosomal protein S6 kinase (RSK; Murphy *et al,* 2002). The involvement of ERK in the reconsolidation of instrumental cocaine memories in the BLA in Experiment 1a likely reflects PKA-Rap-1-, but not NMDA-Ca^{2+}-dependent activation (depicted as green and orange pathways, respectively in **Illustration 1**). In support of this, we have previously demonstrated that PKA, but not Ca2+/calmodulin-dependent protein kinase II (CaMKII) in the BLA regulates contextual cocaine-memory reconsolidation (Arguello *et al,* 2013b). PKA activation likely follows β-adrenergic receptor stimuliation, in that the BLA receives dense adrenergic projections from the locus coeruleus (Asan *et al,* 1998; Chen and Sara 2007; see **Illustration 3**) and β -adrenergic receptor stimuliation itself is necessary for the reconsolidation of instrumental cocaine memories (Milton *et al,* 2008a). Moreover, NMDAR-mediated ERK activation in the hippocampus is required for the consolidation, but *not the reconsolidation,* of a contextual fear memory (Lee and Hynds 2013). It is possible that distinct pools of ERK are activated during the initial consolidation and the subsequent reconsolidation of long-term memories, consistent with several non-overlapping mechanisms underpinning memory stabilization and restabilization (von Hertzen and Giese 2005; Tronel *et al,* 2005; Lee *et al,* 2004; Barnes *et al,* 2012).

Illustration 1. Putative MEK/ERK signaling in cocaine-memory reconsolidation



Similar to ERK1/2, the role of NF-κB in cocaine-memory reconsolidation may reflect cocaine-induced adaptations in learning and memory processes. Specifically, findings from Experiment 2 revealed that SSZ-induced IκK/NF-κB inhibition in the BLA modestly enhances cocaine seeking at the highest dose (see **Fig. 2C**). This was particularly noteworthy, as NF-κB TFs critically promote synaptic plasticity, memory formation and consolidation (Barger *et al,* 2005; Romano *et al,* 2006a, b; Oikawa *et al,* 2012), as well as memory reconsolidation (Merlo *et al,* 2005; Lubin and Sweatt, 2007; de la Fuentes *et al,* 2011; Si *et al,* 2012; Lee and Hynds 2013), including that of morphine-related CPP memories (Yang *et al,* 2012). However, the role of NF-κB in memory stability may change after repeated cocaine exposure. Specifically, chronic cocaine administration elevates levels of basal NF-κB in a delta fosB-dependent manner (Ang *et al,* 2001, but see Muriach *et al,* 2010). Under normal physiological conditions, activation of the p65 (RelA)/p50 (NF-κB1) heterodimer, which is abundantly expressed in the mammalian nervous system and exclusively acts as a transcriptional activator (Romano *et al,* 2006), triggers the expression of the p50 homodimer, which can negatively regulate, via transcriptional repression, subsequent expression of p65 (Tong *et al,* 2004). As such, the p50 homodimer is an essential compenent within a negative feedback loop of NF- κB activation (Zhong *et al,* 2002). It

is possible that this compensatory feedback mechanism may become less effective with repeated, cocaine-induced increases in NF-κB, which would putatively result in the further build-up of intracellular NF-κB. Nevertheless, it is still somewhat unclear why this would have a deleterious effect on memory, as the present study suggests. However, given the many transcriptional targets of NF-κB, cocaine-induced changes in the activation of this TF may have profound effects on which transcriptional targets of NF-κB become regulated, which could conceivably transform its role in memory reconsolidation from a promoter to a repressor.

One possibility is that at higher intracellular concentrations, NF-κB preferentially activates certain gene transcripts that negatively regulate memory reconsolidation. In support of this, NF-κB is a transcriptional activator for several proinflammatory cytokines (Hiscott *et al,* 1993) known to inhibit long-term potentiation (LTP) and memory consolidation, including IL-1β (O'Connor and Coogan 1999; Ikegaya *et al,* 2003; Ross *et al,* 2003; Moore *et al,* 2009, but see Goshen *et al,* 2007 and Barnes *et al,* 2012). Except for one report (Barnes *et al,* 2012), the role of interleukins, like IL-1β, in memory reconsolidation has not been investigated. Future studies will be needed to clarify how interplay between interleukins and NF-κB can influence drug-memory reconsolidation. Additionally, NF-κB activation has been demonstrated to counteract oxidative stress, via activation of certain genes, like inducible nitric oxide synthase (iNOS; Hatano *et al,* 2001; Bayir *et al,* 2005). Notably, iNOS has been demonstrated to have amnesic effects on recently acquired fear memories (Udayabanu *et al,* 2008, but see Palumbo *et al,* 2007). Memory reconsolidation presumably requires some of the same cellular events that take place during oxidative stress – high levels of cellular excitation, NMDAR agonism, calcium influx, etc. (see Tronson and Taylor 2007 for review), and these processes may become even more exacerbated following withdrawal from chronic cocaine administration, which confers an upregulation of

some glutamate receptors (i.e, mGluR1s and NR2b NMDAR subunits; Schmidt and Pierce 2010). Therefore, it is possible that during cocaine-memory reconsolidation in Experiment 2, there was an increase in NF-κB-dependent iNOS expression, which effectively hampered subsequent memory processing. However, the direct involvement of IL-1B and iNOS in drug memory reconsolidation has not been ascertained.

Notably, there is evidence to suggest that both PKA and MEK/ERK can modulate IKK/NF-κB (Romano *et al,* 2006). Specifically, PKA activation, which has been demonstrated to be required for contextual cocaine-memory reconsolidation (Arguello *et al,* 2013b) facilitates NF- κB – CREB binding protein (CBP) interactions at the level of the nucleus, which allows for subsequent activation of gene transcription (Gerritsen *et al,* 1997). Interestingly, ERK has the opposite effect on NF-κB-dependent gene transcription. ERK2, the ERK isoform most impacted by chronic exposure to drugs of abuse (Mazzucchelli *et al,* 2002; Ferguson *et al,* 2006; Girault *et al,* 2007) and preferentially activated during the reconsolidation of both Pavlovian and instrumental cocaine memories (Miller and Marshall 2005; Wells *et al,* 2013), can inhibit NF-κB activity. Specifically, ERK2 can disrupt gene transcription by inhibiting phosphorylation-dependent activation of the TATA-binding protein (TBP; Carter and Hunninghake 2000). This prevents important interactions between NF-κB and TBP, which are speculated to control transcriptional activation (Kerr *et al,* 1993; Xu *et al,* 1993; Carter *et al,* 1999). In addition to tightly regulating NF-κB-dependent transcription, ERK1/2 activation can modulate the activation/translocation of this TF at the level of the cytoplasm. Although the exact mechanism underlying this relationship is unclear, increases in ERK1/2 phosphorylation is correlated with reduced IκB phosphorylation and the cytoplasmic retention of NF-κB (Lu *et al,* 2010). In summary, the non-significant and seemingly paradoxical effects of NF- κB in the BLA on

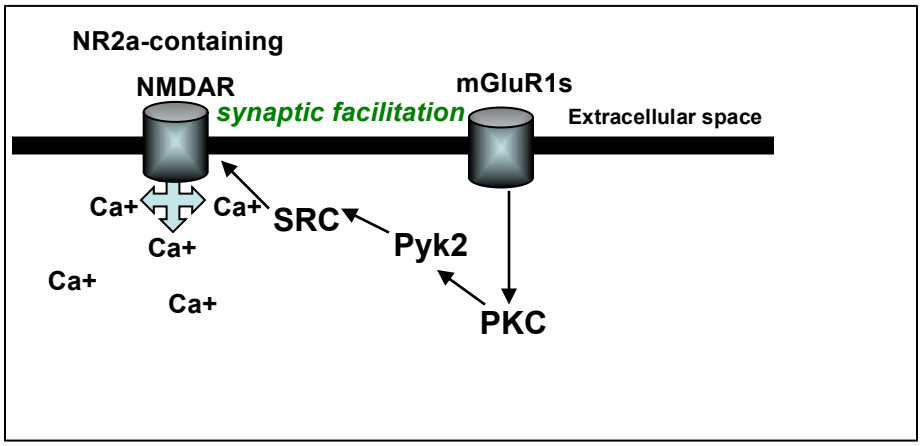
memory reconsolidation in Experiment 2 may reflect NF- κB at the front and center of cocaine-related neuroadaptations and/or complex horizontal cross-talk between other pathways that control cocaine-memory reconsolidation.

The Role of Hippocampal SFKs in BLA-dependent Cocaine-Memory Reconsolidation

We have previously demonstrated that intrahemispheric information processing by the BLA and DH is required for contextual cocaine-memory reconsolidation (Wells *et al,* 2011), likely via intermediate relays, given minimal direct connections between these two structures (Pikkarainen *et al,* 1999). However, unlike the BLA (Fuchs *et al,* 2009), the DH is not a locus of protein synthesis-dependent cocaine-memory reconsolidation. The present findings highlight the critical involvement of SFKs in the DH (Experiment 3; **Figs. 3.1-3.5)** in cocaine-memory reconsolidation and enhance our understanding of the enigmatic role the DH assumes in this process. Specifically, in Experiment 3, we demonstrate that intra-DH PP2, administered at the time of cocaine-memory reconsolidation, has a robust inhibitory effect on cocaine-seeking behavior, measured ~72 h later (**Fig. 3.1C**). Further, we demonstrate that these effects are associated with Src-dependent phosphorylation of the NR2a NMDAR subunit (**Fig. 3.3C**). As SFKs are compartmentalized near the plasma membrane by scaffolding proteins (Yaka *et al,* 2002), these kinases are ideally situated to control synaptic, but not genomic, activity (Ohinishi *et al,* 2011), providing insight into their potential involvement in protein synthesis-*independent* cocaine-memory restabilization in the DH (Ramirez *et al,* 2009). Considered together with previous literature (Narayanan *et al,* 2007a, b; Ramirez *et al,* 2009; Wells *et al,* 2011), the possibility remains that SFKs promote cocaine-memory reconsolidation by strengthening synapses containing polysynaptic afferents from the BLA (Fuchs *et al,* 2009; Wells *et al,* 2011)

or other loci of protein synthesis-dependent memory restabilization (i.e., neocortical regions; Einarsson and Nader 2011). This putatively requires glutamatergic neurotransmission, in that projection neurons from the entorhinal cortex (EC), which relay information from the neocortex *and* the BLA to the DH (Pikkarainen *et al,* 1999), are predominately glutamatergic pyramidal neurons (Peters *et al,* 1999). Interestingly, glutamatergic stimulation of group I metabotropic glutamate receptors (mGluR1s) activate Src via Pyk2 (Huang *et al,* 2001; MacDonald *et al,* 2007 Lan *et al,* 2001; Heidinger *et al,* 2002; Kotecha *et al,* 2003; **Illustration 2**), resulting in Src-mediated rapid phosphorylation of NR2a-containing NMDARs, and enhancement in NMDAR channel conductance (Kohr and Seeberg 1996; Zheng *et al,* 1998; Yang *et al,* 2012), consistent with synaptic reinforcement. In support of this, Src phosphorylation of Tyr-1325 on the NR2a NMDAR subunit is necessary for the induction of LTP (Yang *et al,* 2012). Whether SFKs act locally at regions innervated by polysynaptic afferents from the DH is unknown but would presumably also strengthen connections between the DH and other memory-processing loci.

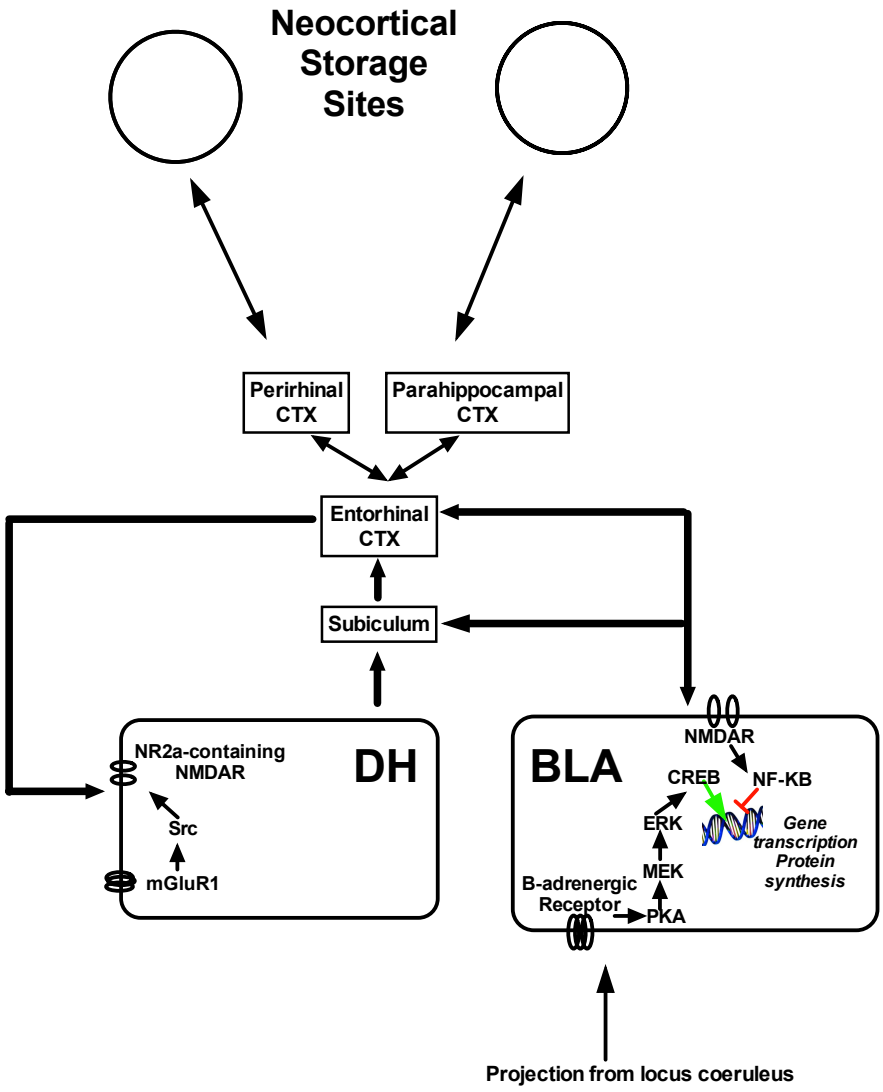
Illustration 2. Mechanisms of Src-mediated cocaine-memory reconsolidation



The functional consequence underlying this framework of DH involvement may be to establish or re-establishing retrieval links with other brain regions that are involved in long-term

memory storage while conveying specific contextual information to restabilization sites, like the BLA (see **Illustration 3**). In support of the development of DH-dependent retrieval links, anatomical studies have suggested that the DH is highly reciprocally connected to neocortical modules for memory storage (Frankland and Bontempi 2005), via the perirhinal cortex, parahippocampal cortex, and EC, as well as the subiculum (i.e., exclusively for outputs; Bird and Burgess 2008; **Illustration 3**).

Illustration 3. Possible mechanisms of BLA/DH interactions during cocaine-memory reconsolidation



As such, facilitation of protein synthesis-dependent memory restabilization by the DH may be achieved by indexing cortical locations during the maintenance of contextual representations, while the context-response, response-reward, and context-reward associations are restrengthened elsewhere in the brain, perhaps in the BLA (Fuchs *et al,* 2009). In support of this, due to volumetric differences, the neocortex is better suited for the long-term storage of remote memories, consistent with systems consolidation theory (Nadel 2007). Consequently, it is expected that with the passage of time, the primary site of memory storage changes from hippocampal ensembles, to highly distributed cortical, networks (McClelland *et al,* 1995; Frankland and Bontempi 2005). The memory acquisition-to-memory reactivation interval in our studies is consistent with predominantly neocortically-maintained memory storage. Yet, the involvement of the DH in memory reconsolidation has been shown to be independent of time. For instance, even after 45 days, remote fear-related associations that are putatively stored in neocortical ensembles become once again dependent on the DH at memory reactivation (Debiec *et al,* 2002). Additionally, ample evidence exists to support the hippocampal index theory, which postulates that the hippocampus functions as a directory for sensory-rich memories, binding specific sensory information contained within the cortex and activating the appropriate cortical locations for a "replay" of sensory experience during retrieval (Teyler and Discenna 1986; Teyler and Rudy 2007). This depends on dense reciprocal projections with the EC (Burwell *et al,* 2004; Chrobak *et al*, 2000). Interestingly, in our hands, manipulations to the EC fail to impact cocaine-memory reconsolidation and subsequent drug context-induced cocaine-seeking behavior (Arguello, Hodges, and Fuchs, unpublished), but it is likely that given the complexity of information flow into, and out of, the DH (Frankland and Bontempi 2005), achievement of an

observable disruption in cocaine-memory reconsolidation requires inhibiting information processing at multiple relays within DH/neocortex/BLA circuitry, including, perhaps, the subiculum (Szalay *et al,* 2011) or areas 35 and 36 of the perirhinal cortex (Pitkanen *et al,* 1997). Nevertheless, regardless of the direction of information processing and specific neural locations activated, the present findings are consistent with the long-term, retrieval-dependent involvement of the DH in cocaine-memory reconsolidation.

Differences in Pavlovian and Instrumental Drug-Memory Reconsolidation

A secondary aim of this dissertation was to follow up on findings using the Pavlovian CPP in an instrumental model of cocaine seeking. Interestingly, while the activation of ERK and *zif268* in the NACc are required for Pavlovian cocaine-memory restabilization (Miller and Marshall 2005 and Theberge et al. 2010, respectively), neither ERK signaling within nor functional activation of the NACC was necessary for instrumental cocaine-memory reconsolidation and drug context-induced operant responding in Experiment 1d. Moreover, NF-κB activation in the BLA mediates the reconsolidation of morphine associations subserving morphine CPP, whereas the results from Experiment 2 loosely suggest that NF-κB acts as an endogenous inhibitor of the processes serving long-term memory maintenance of instrumental cocaine-related memories. Boundary conditions may account for these incompatible findings, and are discussed below. However, these discrepancies may also reflect the differential recruitment of certain neural and intracellular substrates in Pavlovian versus instrumental drug-memory reconsolidation, related to partially non-overlapping circuitry mediating each form of learning (Hatfield *et al,* 1996; Cardinal *et al,* 2002; Balleine *et al,* 2003). For instance, as the memory trace to be disrupted in our model involved context-reward, context-response, and

response-reward (i.e., action-outcome) relationships, it is likely that disruption of context-reward associations, critically mediated by the NACC (Cardinal *et al,* 2002), alone, is insufficient for behavioral suppression and rather, behavioral manifestation of a memory impairment is additive and requires suppression of more than one of these contingencies, which may require the BLA (Cardinal *et al,* 2002).

Alternatively, differences in the role of NF-κB in Experiment 2 versus morphine memory reconsolidation in the CPP model (Yang *et al,* 2011) may be independent of experimental paradigm, and rather, might reflect divergent neuroadaptations following cocaine versus morphine abuse. However, while the reconsolidation of different drug memories presumably involves at least a subset of non-overlapping molecular mechanisms, the larger literature has identified a set of common requirements for long-term memory reconsolidation across classes of drugs of abuse, including protein synthesis and NMDAR stimulation in the reconsolidation of alcohol (von de Goltz *et al,* 2009; Wouda *et al,* 2010), opiate- (Robinson and Franklin 2007; Wu *et al,* 2012a, b), and cocaine-related memories (Brown *et al,* 2008; Milton *et al,* 2008a, b; Fuchs *et al,* 2009; Ramirez *et al,* 2009). Thus, despite differences in the neural circuitry mediating acute and chronic drug effects for each of these (Koob and Volkow 2010), it is possible that the same core elements of the circuit (i.e. , the BLA) are recruited during memory processing per se. Nevertheless, inconsistent findings across models of relapse highlight the importance of careful selection of preclinical model and of cross-model validation when testing the efficacy of possible memory reconsolidation inhibitor treatments.

Disruption of Drug Memory Reconsolidation As a Treatment for Addiction

Since Nader and colleagues provided convincing evidence for a post-retrieval period of memory lability (Nader *et al,* 2000a) the mechanisms of memory reconsolidation have been meticulously studied in a variety of model organisms and across several different forms of memory (Nader and Einarsson 2010). The popularity of this line of research owes to the therapeutic promise of memory reconsolidation inhibitors. With the power to change the integrity of harmful memories, like traumatic or drug-related associations, memory reconsolidation inhibitors have the potential to be extremely effective in the treatment of disorders characterized by pathogenic memories, like PTSD and addiction (Taylor *et al,* 2009; Milton and Everitt 2010, 2012). Further, disruption of memory reconsolidation offers several advantages over behavioral/pharmacological enhancement of extinction consolidation (Taylor *et al,* 2009) - an alternative approach used in the treatment of disordered behavior triggered by pathological memories (Taylor *et al,* 2009). Typically, enhancement of extinction consolidation involves extensive non-reinforced exposure to a fear- or drug-conditioned CSs followed by pharmacological treatment with a cognitive enhancer, like the glycine/NMDAR partial receptor agonist, D-cycloserine (Walker *et al,* 2002; Nic Dhonhadchada *et al,* 2010), which, in turn, facilitates the consolidation of new extinction learning (Taylor *et al,* 2009; Auber *et al,* 2013). Extinction learning is theorized to engender a distinct memory trace – one encoding the absence of a predictable CS-US relationship - from the original memory trace (Bouton 2004). As such, the original memory can resurface under a number of conditions (Bouton 2004), including following the passage of time (i.e., spontaneous recovery; Franken *et al,* 1999; Di Ciano and Everitt 2002), in a new context (i.e., renewal; Mineka and Ohman 2002; Collins and Brandon 2002; Thewissen *et al,* 2006), and following presentation of the US alone (i.e., reinstatement;

Rescorla and Heth 1975). These phenomena limit the efficacy of extinction enhancing strategies in the treatment of PTSD or addiction in both preclinical and clinical studies. At least in theory, memory reconsolidation inhibition should circumvent these limitations, in that it is expected to result in a permanent impairment of the target memory trace (Nader and Einarsson 2010). Consistent with this, several groups have reported enduring suppression of maladaptive behaviors following memory reconsolidation inhibition (Miller and Marshall 2005; Lee *et al,* 2006; Brown *et al,* 2008; Wells *et al,* 2011; Schiller *et al,* 2012). Notably, β-adrenergic receptor antagonism following Pavlovian cocaine-memory reactivation abolishes subsequent cocaine CPP even following a cocaine priming injection in rats (Brown *et al,* 2008)! Also encouraging, a non-pharmacological intervention – introduction of extinction learning within the reconsolidation time window – disrupts fear memory in humans for up to 1 year post-treatment (Schiller *et al,* 2012).

Regardless of their apparent efficacy, however, the specific actions of reconsolidation inhibitors are still uncertain; in particular, what aspect of a complex memory is disrupted following reconsolidation impairment (e.g., declarative memory, emotional memory, or both). Preferential disruption of fear-related physiological responses, but not declarative memory for fearful events, in humans (Brunet *et al,* 2008; Kindt *et al,* 2009) and suppression of context-elicited perseverative cocaine seeking, despite evidence for memory recovery after extended abstinence, in rats (Wells *et al,* 2011) suggest that memory reconsolidation inhibitors are capable of disrupting the intrusive, emotionally salient features of memory while sparing declarative memory, per se. However, it is important to note that attentional processes likely impact which aspects of the target, or even alternate, memories are most vulnerable at retrieval.

The success of memory reconsolidation inhibitor manipulations in preclinical models of addiction is encouraging from a treatment perspective (Taylor *et al,* 2009). However, there remain several challenges to the development of these manipulations as viable treatment options for addiction. This is emphasized by the limited number of clinical studies using reconsolidation inhibitors for craving prevention (1; Saladin *et al,* 2013) and the failure of post-cocaine-cue memory retrieval propranolol treatment to disrupt cue-elicted craving after only 1 week in this particular study (Saladin *et al,* 2013). This is in direct opposition of preclinical findings, where, for instance, post-memory reactivation *zif268* knockdown in the BLA disrupts stimulus-elicited drug-seeking behaviors for up to ***50 d*** in rats (Lee *et al,* 2006). This underscores the importance of careful selection of preclinical model and of cross-model validation when testing the efficacy of possible memory reconsolidation inhibitor treatments. Preclinical models should adequately capture boundary conditions (i.e., memory age and strength), which reduce the effectiveness of memory reconsolidation inhibitors (Milekc and Alberini 2002; Eisenberg *et al,* 2003; Biedenkapp and Rudy 2004; Cammarota *et al,* 2004; Rossato *et al,* 2006; Sevenster *et al,* 2012, 2013) or render them only transiently effective (Judge and Quartermain 1982; Lattal and Abel 2004; Amaral *et al,* 2007; Wells *et al,* 2011). As addicts typically engage in cycles of use and abstinence for months to *years* prior to seeking treatment (Gawin and Kleber 1986), the cocaine-memory trace likely becomes incorporated into expansive, diffuse, and cortically-dependent networks (Buhl *et al,* 2002; Frankland and Bontempi 2005), and consequently, becomes less vulnerable to disruption (Alberini *et al,* 2006; Amaral *et al,* 2008). Regrettably, even in the extinction-reinstatement paradigm, where drug administration is more extensive than in CPP, relapse testing is typically administered following a subchronic training regimen, and likely fails to capture the age and strength of the cocaine-memory trace in the human condition (Fuchs *et al,*

2008b). Additionally, this subchronic regimen does not adequeately match the extent of habit learning and inflexible cocaine-seeking behavior expected following extensive drug use in addiction (Everitt *et al,* 2008). Habit memories are presumably more resistant to disruption than those underlying goal directed behavior (Everitt *et al,* 2008), although some studies have suggested the opposite- namely, that habit memories can be disrupted by reconsolidation inhibitors, which returns behavior to a flexible, outcome-dependent state (Wells *et al,* 2011; Pauli *et al,* 2012). Nevertheless, future studies will be required to evaluate memory vulnerability following a chronic drug administration regimen.

Conclusions

The experiments described in this dissertation identify novel molecular substrates that govern the reconsolidation of context-response-cocaine memories requisite for contextual stimulus control over maladaptive, cocaine-seeking behavior in the rodent extinction-reinstatement paradigm. These data significantly extend previous investigations into intracellular control of drug-memory reconsolidation, by indicating that 1) ERK signaling within the BLA and 2) activation of SFKs in the DH are both essential for successful contextual cocaine-memory reconsolidation. They additionally underscore a complex contribution by NF-κB in the BLA to this phenomenon, which warrants further investigation. Importantly, identification of SFKs as novel hippocampal regulators of cocaine-memory reconsolidation is particularly important, in that the role of the DH in memory reconsolidation, in general, has been poorly understood (Debiec *et al,* 2002; Amaral *et al,* 2007; Ramirez *et al,* 2009; Wells *et al,* 2011). Specifically, this finding begins to illuminate potential mechanisms through which the DH supports protein synthesis-dependent cocaine-memory restabilization in the BLA and in other extrahippocampal

locations (Einarsson and Nader 2011). Future research into the neural circuitry and molecular mechanisms of drug-memory reconsolidation, in general, will undoubtedly impact the development of novel therapeutics designed to alleviate the ability of drug-associated stimuli to control maladaptive conditioned behaviors.

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