INVESTIGATING A NOVEL THALAMO-AMYGDALA CIRCUIT FOR THE REGULATION OF A DRUG-CUE-SPECIFIC MEMORY

by

Matthew Thomas Rich

B.S., University of Connecticut, 2008

M.S., University of Hartford, 2011

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This dissertation was presented

by

Matthew Thomas Rich

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and approved by

Committee chair: Susan Sesack, PhD., Professor, Dept. of Neuroscience

Yan Dong, PhD., Professor, Dept. of Neuroscience

Colleen McClung, PhD., Professor, Dept. of Psychiatry

Kenneth Fish, PhD., Associate Professor, Dept. of Psychiatry

Outside Examiner: Patricia Janak, PhD., Distinguished Professor, Dept. of Psychological and Brain Sciences; Dept. of Neuroscience, Johns Hopkins School of Medicine

Dissertation Advisor: Mary Torregrossa, PhD., Associate Professor, Dept. of Psychiatry

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Matthew T. Rich, PhD

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Addiction is a chronic, progressive disorder caused by repetitive drug use, which leads to long-lasting synaptic alterations in the brain. Drugs of abuse are powerful reinforcers that 'hijack' specific brain circuits that control reward-motivated behaviors, creating a maladaptive state that can trigger craving and relapse, even after prolonged periods of abstinence. Environmental contexts and cues that are present during drug use can later evoke drug-related memories that promote drug-seeking conditioned responses. Despite recent attempts to treat addiction with a combination of behavioral and pharmacological tools, success has been limited, mainly because the underlying mechanisms that govern drug-associated memories have not been fully elucidated. Studying the neural circuits and the synaptic and molecular underpinnings of drug-associated memories may uncover a means to reduce their salience, thereby reducing the likelihood of relapse.

The amygdala is important for integrating sensory information during cue-dependent learning and is activated by both fear- and drug-related stimuli. Preclinical models have shown that the amygdala activates behavioral responses to fear- or drug-conditioned cues. Neuroadaptations within amygdala-based circuits have been examined in models of aversive learning but have not been well studied during reward-based learning, especially in the context of drugs of abuse. The work presented in this dissertation focuses on understanding the circuit-specific synaptic and molecular changes that occur within the amygdala both during the formation of a drug-cue memory and upon subsequent re-exposure to drug-related cues. Through

a combination of electrophysiological, molecular, optogenetic, and behavioral techniques we find that repeated self-administration of cocaine, paired with an audiovisual cue, involves strengthening of auditory thalamic, but not cortical, synapses in the lateral amygdala. We also find that reversing drug-cue induced plasticity in this pathway, either through extensive cue re-exposure or by optical induction of postsynaptic LTD, inhibits cue-elicited relapse-like behavior. Finally, we show that specific pharmacological manipulations of an intracellular signaling pathway involved in bidirectional regulation of synaptic activity can interfere with reconsolidation and promote extinction of a drug-cue memory. Together, these studies suggest a projection-specific mechanistic approach for the inhibition of drug-cue memory, which may be informative for future pharmacobehavioral relapse prevention strategies.

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LIST OF COMMON ABBREVIATIONS

CaMKIIα	calcium-calmodulin dependent protein kinase II α subunit
CaN	calcineurin
CaM	calmodulin
LA	lateral amygdala
MGN	medial geniculate nucleus
T-LA	thalamo-amygdala
C-LA	cortico-amygdala
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
VTA	ventral tegmental area
SA	self-administration
IE	instrumental extinction
CS	conditioned stimulus
US	unconditioned stimulus
CR	conditioned response
LTP	long-term potentiation
LTD	long-term depression

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PREFACE

The past several years has been a period of immense intellectual and personal growth. The completion of this dissertation, while trying at times, has also been incredibly fulfilling. Of course, none of this would be possible without the help of others. I would therefore like to express my gratitude to several groups and individuals that have offered support, wisdom, and cheer through these past six years, and have undoubtedly made this a more rewarding experience. I owe the most to my mentor, Dr. Mary Torregrossa, who took a risk on me as her first graduate student. Mary has been an ideal mentor, offering a balance of guidance and independence, and her trust in me has helped me become more confident in my own abilities. Her careful knowledge of the ever-changing literature and her creativity are traits that I hope to emulate throughout the rest of my career, and I am thankful of the inspirational example she has set. I would also like to thank all members of the Torregrossa lab, past and present for their help, whether it be ordering supplies, helping with surgeries, running my behavior, or simply feeding my rats so I could enjoy an occasional weekend day off. Special shoutout to Dr. Megan Bertholomey, who has been in the lab since the beginning with me and has helped me finally get a somewhat decent grasp on statistical analysis. I am also thankful for the wonderful contributions of three undergraduates: Tara Cahanap, Alyssa Miller, and Emily Compagnoni. Their efforts in running behavioral experiments was critical in helping me simultaneously perform multiple experiments.

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1.0 INTRODUCTION: ADDICTION AS A DISORDER OF LEARNING AND MEMORY SYSTEMS

Within the brain, there exists a set of evolutionarily conserved neural systems responsible for controlling primitive biological functions that are vital for survival. One of these systems is known as the mesocorticolimbic system, a series of interconnected brain regions that govern reward, learning and memory, emotion, and executive function. Activity within the mesocorticolimbic system is necessary for the procurement of naturally-occurring positive reinforcers, such as food and sex, and is accompanied by a pleasurable sensation that motivates future reward-driven behaviors (Berridge and Kringelbach, 2015). Drugs of abuse, including psychomotor stimulants (like cocaine and methamphetamine) also activate the mesocorticolimbic system, which enhances locomotor activity aimed towards obtaining the drug (Kalivas et al., 2005; Yeh and Haertzen, 1991). Repeated drug exposure can result in behavioral sensitization as well as tolerance, which necessitates an escalation in drug use in order to achieve the original psychophysiological response (Morgan et al., 2006; Robinson and Berridge). Additionally, by engaging brain areas involved in learning and memory, individuals remember the circumstances surrounding the rewarding experience. In this manner, drug use is reinforced, thus promoting a chronic, progressive increase in substance use that induces long-lasting neuroadaptations within the mesocorticolimbic system. Over time, these changes to the neural

circuitry can result in a shift from reward-driven behavior to the more compulsive behaviors that are characteristic of addiction, or substance use disorders (SUDs) (Hyman et al., 2006; Piazza and Deroche-Gamonet, 2013).

Addiction is a highly prevalent disorder. Approximately 1 in 12 adults (8% of the population) struggles with substance abuse (National Household Survey on Drug Abuse, 2016). Drug use also represents a huge economic burden, whereby 600 billion dollars per year is lost due to substance abuse (National Household Survey on Drug Abuse, 2016). Addiction is characterized by repetitive cycles of drug use, withdrawal, abstinence, and relapse; throughout these periods, drug use continues despite the occurrence of sometimes severe, negative consequences (i.e. withdrawal, negative social relationships, economic hardship). A major problem facing individuals with a SUD is the dilemma of either engaging in drug-seeking behavior, which has immediate short-term rewarding effects, or maintaining abstinence, which has more long-term benefits. However, because drugs of abuse interact with and strengthen the connections within a system that is already evolutionarily hardwired for survival, SUDs are extremely difficult to overcome. Indeed, drug-induced alterations within the mesocorticolimbic system have been directly linked to craving and relapse-like behavior (Engblom et al., 2008; Mameli et al., 2011; Nugent et al., 2007). During early abstinence there is a progressive increase in craving during which time affected individuals are extremely vulnerable to relapse. Craving also remains elevated during prolonged abstinence (Gawin and Kleber, 1986; Kassani et al., 2015; Miller et al., 2012), so as the duration of abstinence increases, the risk of relapse remains. Recent surveys by the National Institute of Health estimate relapse rates to be between 40-60%, and even as high as 90% for alcohol (National Household Survey on Drug Abuse, 2016). This

high tendency for relapse is perhaps the biggest challenge for the successful treatment of addiction (Sinha et al., 2011).

Treatment of SUDs is therefore aimed at reducing the risk of relapse and sustaining abstinence. Over time, the weakening and strengthening of specific synapses by drugs of abuse generates abnormally strong, long-lasting memories of drug use. One strategy for preventing relapse has been to interfere with these "drug-associated memories." Oftentimes, neutral environmental stimuli, or cues, that are present during drug use can become predictors of drug availability, thus acting as conditioned reinforcers (Di Ciano and Everitt, 2003; Stewart, 1992). Specific drug-associated cues can vary widely across individuals but can include friends and family members with whom the individual engaged in drug-related behaviors, a specific context or location in which drugs were formerly used, and drug paraphernalia. Generally, these stimuli are grouped into either contextual or discrete cues (Back et al., 2014; Marchant et al., 2015; O'Brien et al., 1992). Evidence from clinical and preclinical research suggests that re-exposure to these drug-paired cues triggers drug-related memories that remind the individual of past drug use and activate the drug-adapted reward pathways, ultimately leading to craving and relapse.

Attempts to study the neural mechanisms that promote the activation of drug-associated memories have been undertaken with the hopes of developing an effective treatment medication; however, few pharmacological options have been found that can successfully prevent relapse across an extended period of time (Bossert et al., 2013; Tiffany and Conklin, 2002). Likewise, recent clinical efforts have utilized behavioral strategies aimed at suppressing drug-related memories, but behavioral approaches alone have also proven to be widely insufficient for maintaining abstinence. Interestingly, these strategies are highly effective at disrupting the emotionally-salient memories that form the basis of anxiety disorders, such as post-traumatic

stress disorder (PTSD). For example, cue exposure therapy (CET) can reduce the likelihood of fearful stimuli to evoke a detrimental stress response (Marin et al., 2015), and the effects of cue exposure can be enhanced by combining it with specific pharmacological compounds (Hofmann et al., 2006; Norrholm et al., 2016; Ressler et al., 2004). These same ideas have been utilized for the treatment of drug dependence; however, despite observed success of CET in the clinical setting, drug-seeking behavior is often renewed when the patient returns to the original drug-paired environment and is re-exposed to drug-associated stimuli (Crombag et al., 2008; Thewissen et al., 2006). Clinical efforts that use both behavioral therapies and treatment medication have yielded the highest success rates at reducing the strength of drug-associated memories (Cleva et al., 2010; Torregrossa and Taylor, 2016). Many of the neural processes that regulate fear-associated memories have proven to play a role in drug-related memories as well, which has been useful for proposing candidates for pharmacological intervention (Milton and Everitt, 2010; Monfils et al., 2009). Still, compounds that enhance CET in patients with PTSD, have had mixed results in individuals with a SUD (Price et al., 2013; Santa Ana et al., 2009), suggesting that there may be important differences in the neural mechanisms that regulate fear- vs. drug-associated memories. Continuing to study the specific circuits and cellular mechanisms involved in the formation, storage, and maintenance of drug-induced maladaptive memories may prove useful for the development of a successful intervention for drug addiction that has long-lasting inhibitory effects on relapse.

1.1 NEURAL MECHANISMS OF LEARNING AND MEMORY

In order to learn about their environment and survive, most organisms need acquired information to be stored as a memory that can later be accessed, updated, and modified as new information about the given environment is learned. The organism then utilizes this information to make decisions and direct their behavioral response. This type of learning, in which behavioral responses are guided by an environmental stimulus is known as associative learning. Two types of associative learning exist: classical (Pavlovian) conditioning and operant (instrumental) conditioning (Jones and Skinner, 1939; Pavlov, 1927; Robinson and Berridge). In classical conditioning, a neutral environmental stimulus is repeatedly paired with a biologically relevant unconditioned stimulus (US), so that an association between the two stimuli forms. The previously neutral stimulus then becomes a conditioned stimulus (CS) that, when presented alone, can trigger a conditioned response (CR) that is similar to the unconditioned response. The classic example is the pairing of a bell (CS) every time a dog is given food, which triggers reflexive salivation (US); eventually ringing of the bell alone can induce salivation (Pavlov, 1927). On the other hand, operant conditioning involves the modification of a behavioral response based on the outcome of a given action. The behavior can be increased if the response is reinforced (by reward) or decreased if the response is punished (Jones and Skinner, 1939). An example of this is when an individual learns that touching a hot stove burns his/her hand. As will be discussed in subsequent sections, both classical and operant conditioning are important principles that underlie drug-related memories and relapse-like behavior. However, it is important to first consider the cellular and molecular mechanisms that occur within neurons that enable these learning processes to occur. These mechanisms regulate the various stages of

learning and can serve as potential targets for manipulating the stability of maladaptive drug-related memories.

1.1.1 Synaptic and molecular mechanisms of learning and memory

Hebbian learning suggests that memories form through the simultaneous activation of a set of neurons, which increases the synaptic strength between those neurons (Hebb, 1949). Hebb's theory forms the basis of spike-timing dependent plasticity (STDP), whereby connections between two neurons are strengthened or weakened in a temporally-specific manner. This input-specificity ensures that only synapses experiencing correlated activity will be modified. Typically, a synapse tends to be strengthened if, over time, a postsynaptic neuron depolarizes and fires an action potential immediately (10-20 ms) after receiving synaptic input from a given presynaptic neuron (Levy and Steward, 1983; Magee and Johnston, 1997; Markram et al., 1997). Conversely, synapses can be weakened if the order of firing reverses so that the postsynaptic neuron tends to fire prior to receiving the synaptic input (Bi and Poo, 2001). The refinement of synapses via STDP is an important hallmark of development and occurs universally throughout the nervous system (Katz and Shatz, 1996). A diverse set of cellular machinery is required at both the synaptic and molecular level for synaptic modifications to occur. Organisms have also developed specific neuroanatomical systems that specifically utilize these mechanisms for the formation, storage, and updating of memories. Importantly, drugs of abuse have actions at the neuroanatomical systems involved in memory (e.g. cocaine and amphetamine acting at the dopamine transporter; See Section 1.2) and exert their effects through many of the same cellular and molecular substrates, which accounts for drug-dependent learning.

The strengthening and weakening of synapses occurs through mechanisms known respectively as long-term potentiation (LTP) and long-term depression (LTD) (Bi and Poo, 1999; Malenka and Bear, 2004). Activity-dependent synaptic plasticity takes place universally throughout the nervous system at both glutamatergic and GABAergic synapses, and at both the pre- and postsynaptic membrane. A discussion of GABAergic synaptic plasticity is beyond the scope of this dissertation; however, it should be noted that activity at local inhibitory synapses is likely an important factor in the regulation of memory (Vogels et al., 2011). Excitatory synapses are referred to as asymmetric synapses because of the enriched postsynaptic density (PSD) (Carlin et al., 1980; Walikonis et al., 2001) that is host to an array of structural proteins (F-actin, integrins, AKAP5), intracellular signaling proteins (protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), calcineurin (CaN), and protein phosphatase 1 (PP1)), and receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR)) (Bosch et al., 2014; Gipson et al., 2014; Malenka et al., 1989; Moczulska et al., 2013). Glutamatergic postsynaptic sites are typically contained within transient, specialized structures known as dendritic spines. The shape, size, and number of dendritic spines are all indicators of synaptic strength (Meyer et al., 2014; Oh et al., 2013). Large, mushroom-shaped spines are thought to be more stable, and an increase in spine density is characteristic of a potentiated-state.

The most commonly observed forms of long-term plasticity at glutamatergic synapses are NMDAR-dependent LTP and LTD (Kauer and Malenka, 2007; See Figure 1). NMDA-dependent LTP and LTD both require presynaptic glutamate release, concurrent with time-dependent AMPAR-mediated depolarization of a postsynaptic neuron. Postsynaptic depolarization displaces Mg^{2+} ions from the channel pore of NMDAR, allowing Ca^{2+} and other ions to enter the

postsynaptic neuron and subsequently activate protein kinases and phosphatases, which have downstream phosphorylation or dephosphorylation activity, respectively (Klann et al., 1991; Levy and Steward, 1983; Malenka et al., 1989). Depending on the concentration of Ca^{2+} and the balance between protein kinase and phosphatase activity, either LTP or LTD can occur. Synapses that receive the most consistent input undergo structural alterations that result in their stability (LTP), while those that are activated less frequently may be weakened or destabilized (LTD) (Hasegawa et al., 2015; Lüscher et al., 2000). Typically, LTP is triggered through high-frequency presynaptic burst activity that results in transiently-elevated Ca^{2+} levels, although there are instances of LTP also being induced from low-frequency stimulation (Malenka et al., 1999). High levels of Ca^{2+} preferentially activates kinases (PKA, PKC, and CaMKII) that result in upregulated gene expression, increased protein synthesis, and/or the phosphorylation of several synaptic proteins, including AMPAR (Abel et al., 1997; Boehm et al., 2006; Sanhueza and Lisman, 2013). The phosphorylation of specific amino acids on AMPAR initiate its transport, insertion, and stability at the postsynaptic membrane (Lee et al., 2000; Malinow and Malenka, 2002; Whitehead et al., 2013). Experimentally, measurements of LTP include increases in spine size and density as well as increased AMPA:NMDA ratios (Chen and Roche, 2007; Collin et al., 1997; Hayashi, 2000).

NMDA-dependent LTP involves activity of CaMKIIα (**Figure 1**), a protein that is necessary for various learning tasks (Coultrap et al., 2014; Lisman et al., 2012; Sanhueza et al., 2011). For example, CaMKIIα knockout mice have deficits in LTP that correlate with impairments in hippocampal-dependent spatial learning (Silva et al., 1992a, 1992b). CaMKIIα is a dodecameric protein (consisting of 12 subunits) that is activated following Ca^{2+} influx through NMDAR. Ca^{2+} forms a complex with calmodulin (CaM) that binds CaMKIIα, initiating auto-

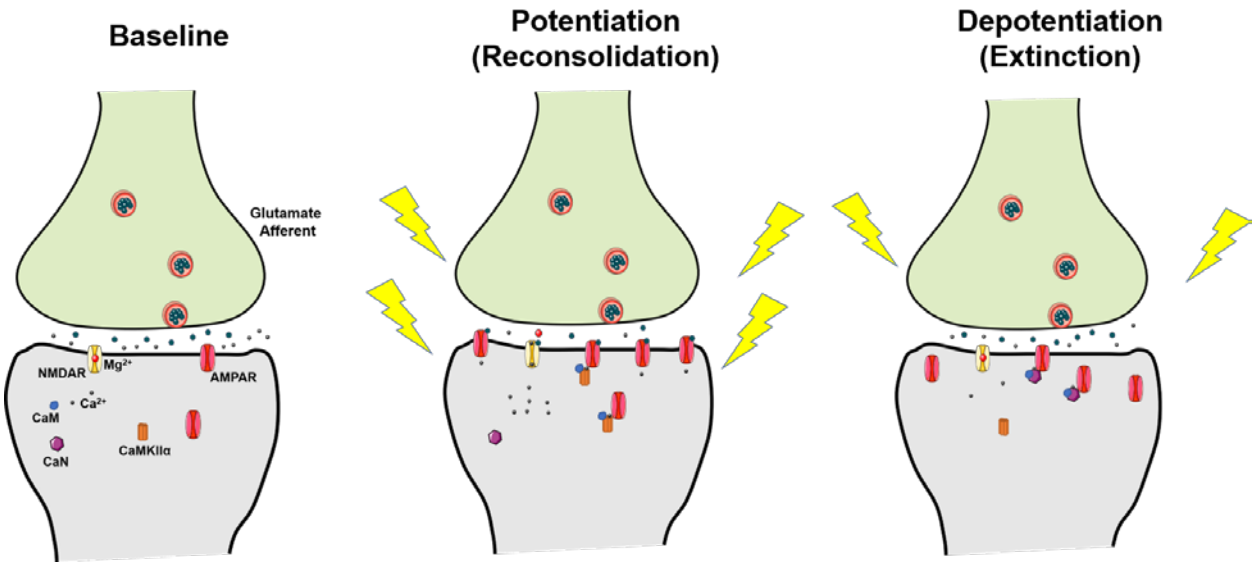

Figure 1. Mechanisms of excitatory synaptic plasticity.

Simplified schematic of cellular mechanisms that regulate NMDAR-dependent forms of synaptic plasticity. Glutamatergic afferents form synapses primarily at dendritic spines. Under baseline conditions, the hyperpolarized postsynaptic neuron is minimally activated due to the block of NMDAR by Mg^{2+} ions, which prevents the influx of Ca^{2+} and other ions. However, under conditions of increased activity (brief, high frequency stimulation) postsynaptic depolarization removes Mg^{2+} ions from the NMDAR pore. When glutamate binds the NMDAR, Ca^{2+} ions enter the postsynaptic neuron. The relatively high level of Ca^{2+} activates kinases, including CaMKIIα, which is also dependent on CaM. CaMKIIα autophosphorylates and then phosphorylates several downstream proteins, including AMPAR GluA1 subunits. The phosphorylation of AMPAR results in its translocation and insertion in the synaptic membrane, leading to an increase in AMPA:NMDA and potentiation. Similar mechanisms underlie the reconsolidation of associative memories. Under conditions of sustained, subthreshold activity (long durations of low frequency stimulation) the postsynaptic is not as strongly depolarized, leading to a smaller influx of Ca^{2+} ions, for example through L-type voltage-gated Ca^{2+} channels (not pictured). The relatively low level of Ca^{2+} preferentially activates phosphatases, including CaN, which is also dependent on CaM activity. CaN activation causes the dephosphorylation of other phosphatases and results in the dephosphorylation of AMPAR GluA1 subunits, followed by the internalization of AMPAR and decrease in AMPA:NMDA. This mechanism of depotentiation is likely responsible for the extinction of associative memories. *CaMKII: calcium/calmodulin-dependent protein kinase II; CaN: calcineurin; CaM: calmodulin.*

phosphorylation at Thr286 (Baucum et al., 2015; Rodrigues, 2004), and leads to the translocation of CaMKIIα to the synapse, where it directly phosphorylates the AMPAR subunit GluA1 at Ser831 which primes AMPAR for synaptic insertion, thereby increasing channel conductance (El Gaamouch et al., 2012; Kristensen et al., 2011; Lemieux et al., 2012; Lu et al., 2010). The complex structure of CaMKIIα and its capacity for auto-phosphorylation makes it suitable for the regulation of synaptic activity. Once a single subunit is phosphorylated at Thr286, neighboring subunits can subsequently be phosphorylated, giving rise to the autonomous activity of the protein (Coultrap and Bayer, 2012). CaMKIIα can function autonomously even after its substrate for activation, Ca^{2+}, falls below baseline levels. As a result, the protein can retain long-term modification and cause a cascade of molecular events over a longer course of time following a brief activation period, consistent with the theory for how LTP shapes learning and memory formation. CaMKIIα acts to mediate dendritic spines through its actions at synaptic proteins including the actin-binding protein, α-actinin, the guanine nucleotide exchange factor, kalirin-7, and cyclin-dependent kinase 5 (Hosokawa et al., 2006; Ma et al., 2008; Walikonis et al., 2001). CaMKIIα also forms a complex with NMDAR, by binding to the GluN2B subunit, that is thought to provide synaptic stability and contribute to long-term information storage (Coultrap and Bayer, 2012). CaMKIIα activity influences other signaling pathways that are involved in memory processes, including the PI3K/mTOR pathway (Joyal et al., 1997).

NMDAR-dependent LTD arises from low-frequency presynaptic activity combined with a smaller-magnitude postsynaptic depolarization that results in a sustained, but lower Ca^{2+} concentration (**Figure 1**). This form of LTD is also dependent on activation of L-type voltage-gated Ca^{2+} channels (LTCC), which are activated subsequent to NMDAR and because of their long activation duration, allow a more sustained increase in intracellular Ca^{2+} that preferentially

activates phosphatases (Bi and Poo, 1998; Ghosh et al., 2017). One phosphatase that is particularly important for this form of plasticity is CaN, a Ca^{2+}/CaM-dependent phosphatase that, when activated initiates a sequence that leads to dephosphorylation of AMPAR, initiating their internalization. (Baumgartel and Mansuy, 2012; Mulkey et al., 1994). CaN is often described as a negative regulator of CaMKIIα activity (Wen et al., 2004) because the frequency and duration of Ca^{2+} input controls the relative activation of the two proteins and is an important predictor of the direction of synaptic activity (LTP vs LTD) (Lüscher and Malenka, 2012). CaN is a heterodimer composed of a catalytic A subunit and a regulatory B subunit (Mumby and Walter, 1993). Sequential conformational changes following binding of Ca^{2+} and calmodulin to CaN_B activates the phosphatase by allowing displacement of a CaN_A autoinhibitory domain (Shen et al., 2008). CaN has both direct and indirect actions at postsynaptic proteins that promote depotentiation. CaN directly dephosphorylates GluA1 at Ser845 (the PKA phosphorylation site). Phosphorylation of Ser845 drives AMPAR membrane insertion and is associated with enhanced AMPAR current (Beattie et al., 2000; Man et al., 2007; Roche et al., 1996). CaN also directly dephosphorylates the A-type K^+ channel, Kv4.2, which is itself a negative regulator of plasticity (Jung et al., 2008). Dephosphorylation of Kv4.2 by CaN leads to its stabilization in the membrane, thereby promoting LTD. Finally, CaN dephosphorylates a peptide known as inhibitor-1, which subsequently activates PP1 and protein phosphatase 2A, which function to inactivate LTP-promoting kinases (Mulkey et al., 1994). The internalization of AMPAR is linked to changes in spine morphology, decreased spine density and decreased AMPA:NMDA ratios, which are features of LTD (Beattie et al., 2000; Lu et al., 2000; Sanderson et al., 2016).

In addition to CaMKII and CaN, several other intracellular signaling molecules can contribute to LTP and LTD. The activation of G-protein coupled metabotropic receptors (e.g.,

metabotropic glutamate receptors; mGluRs, dopamine receptors) can activate or inhibit PKA via effects on adenylyl cyclase and cyclic adenosine monophosphate (cAMP). PKA phosphorylates GluA1 at Ser845, which promotes insertion into the postsynaptic membrane (Zhang et al., 2016). Other metabotropic receptors (e.g., serotonin receptors, mGluR1) can activate phospholipase C (PLC), an important enzyme in the production of inositol 1,4,5-triphosphate (IP_3), which induces release of Ca^{2+} from internal stores (Fukami et al., 2010). Diacyl glycerol (DAG), a byproduct of this reaction, induces PKC activation, which also has LTP-like effects (Malinow et al., 1989). Additionally, PLC initiates synthesis of endocannabinoids that retrogradely activate presynaptic CB1 receptors and leads to a presynaptic form of LTD characterized by decreased glutamate release (Andrade-Talavera et al., 2016). Finally, LTP can also be induced presynaptically, when repetitive synaptic activity increases presynaptic Ca^{2+} activation of PKA, leading to phosphorylation of proteins (Rab3a, RIM1α) that increase presynaptic glutamate release (Wong and Stanley, 2010). Increases and decreases in glutamate release probability are associated with presynaptic potentiation and depression, respectively. Experimental measures of presynaptic plasticity include paired pulse ratio (PPR) and frequency of spontaneous excitatory postsynaptic currents (EPSC). PPR is inversely correlated with presynaptic strength, such that decreases in PPR are associated with increased neurotransmitter release probability, and vice versa (Kleschevnikov et al., 1997). Conversely, measurements of postsynaptic plasticity include AMPA:NMDA ratio and EPSC amplitude. As will be described throughout this dissertation, these measurements are important for helping deduce the underlying mechanisms responsible for changes in synaptic plasticity.

Finally, other neurotransmitters, especially neuromodulators such as dopamine (DA) have a prominent role in the regulation of synaptic plasticity at glutamatergic synapses (Ciranna,

2006; Hamilton et al., 2010; Otani et al., 2015; Sun et al., 2005). There are two primary classes of DA receptors: D1- and D2-like. D1-like receptors (D1R) are Gs/Gq-coupled and stimulate the production of adenylyl cyclase, which increases cAMP and enhances neural activity. Conversely, D2-like receptors (D2R) are Gi/o-coupled proteins that when activated, have opposing effects to D1R, and are therefore associated with decreased neural activity (Beaulieu and Gainetdinov, 2011; Li and Rainnie, 2014). Both D1R and D2R mediate several downstream effectors, which facilitates synaptic changes that can ultimately result in LTP or LTD (Otani et al., 1998; Roggenhofer et al., 2010). D1R increase the activity of protein kinases (Chergui and Lacey, 1999; Gutierrez-Arenas et al., 2014; Matsuyama et al., 2002), enhancing downstream phosphorylation; for example, phosphorylation of DARPP-32 (DA and cAMP-regulated phosphoprotein, 32kDA) by PKA regulates excitatory transmission through effects at LTCC, as well as AMPAR and NMDAR (Bibb et al., 1999; Flores-Hernandez, 2002; Nishi et al., 1997). D2R activation inhibits phosphorylation of PKA targets (DARPP-32). Additionally, presynaptically-located D2R activate G-protein-coupled inwardly rectifying potassium (GIRK) channels, which inhibit subsequent DA release (Martel et al., 2011; Michaeli and Yaka, 2010). As will be discussed below, drugs of abuse increase dopaminergic transmission particularly in areas involved in the expression and maintenance of reward sensitivity and memory, such as the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and lateral amygdala (LA). Together this leads to alterations in normal memory processes and promotes addictive-like behaviors.

1.1.2 Consolidation, reconsolidation, and extinction of memories

The formation, maintenance, and modification of drug-cue associations depend on a series of processes known as consolidation, reconsolidation, and extinction (Lee et al., 2005; Marchant et al., 2013; Tronson and Taylor, 2007). These processes have been extensively studied for various types of classical and instrumental learning (Dudai, 2012; Todd et al., 2014), and are prominent during emotionally-salient learning, for example, auditory or contextual fear conditioning (Debiec et al., 2002; Duvarci et al., 2008; Schafe and LeDoux, 2000) as well as drug-cue learning. Consolidation is a time- and protein synthesis-dependent process that occurs following initial learning and is necessary for stabilizing and storing new information after it has been acquired. Over time, consolidated memories can be reactivated, which retrieves and destabilizes the memory, causing it to enter a transiently labile state. After reactivation, the malleable memory must then undergo reconsolidation to be re-stabilized (Dudai, 2012; Rodriguez et al., 1993). As with consolidation, blocking protein synthesis prior to re-stabilization impairs the memory (Nader et al., 2000). Reconsolidation exists as a mechanism to update or strengthen memories, for example, the detection and encoding of environmental changes. Induction of reconsolidation is therefore strongest when new information is presented at the time of memory retrieval (e.g., reactivation in a novel context) (Haubrich et al., 2015; Lee et al., 2009). However, the extent to which reconsolidation affects pre-established memories is currently unclear. Some studies support reconsolidation as necessary to strengthen and update memories, while others fail to show memory-enhancing effects (Bonin and De Koninck, 2015; Fukushima et al., 2014). At the very least, successful reconsolidation seems to preserve memories that have been destabilized after recall. To this end, pharmacological agents that affect reconsolidation have been used as a

means for altering the strength of memories (Besnard et al., 2012; Finnie and Nader, 2012). For example, Tronson and colleagues demonstrated that stimulating PKA in the amygdala immediately after retrieval of a conditioned fear memory increases the degree of freezing during subsequent exposures to the CS (Tronson et al., 2006). An opposing effect was observed after intra-amygdala infusion of PKA inhibitors following cue retrieval: CS-elicited freezing was reduced, suggesting that reconsolidation was disrupted. Importantly, PKA inhibitors given in the absence of CS reactivation are insufficient to block the fear memory, verifying that retrieval is required as a precursor to reconsolidation, which can then be interfered with by amnestic agents. Utilizing this approach clinically could indeed prove beneficial for treating maladaptive memory disorders (Agren et al., 2012; Lee et al., 2005).

Reconsolidation occurs following just brief interaction with the CS, as short exposure durations are sufficient to reactivate CS-associated memories. Repeated or long-term exposure to CS in the absence of the associated US instead promotes memory extinction (Monfils et al., 2009; Rich et al., 2016; Tronson et al., 2012a). Extinction is a separate and opposing process that involves the formation of a competing association, where the CS no longer predicts the US (Lee et al., 2006; Morgan et al., 1993; Quirk et al., 2010). Like reconsolidation, extinction was first characterized in studies of aversive learning, in which it effectively attenuated fear-like behaviors (Fucich et al., 2016; Santini, 2004). Despite learning of a new association, extinction does not result in erasure of the original memory. In fact, re-exposure to the US can promote reinstatement of the extinguished response (Rescorla and Heth, 1975). Additionally, extinction is highly context-dependent, so if the extinction context and the training context are different, responding to the CS can be renewed upon return to the original training context (Effting and Kindt, 2007; Todd et al., 2014). Finally, extinguished responses will often spontaneously

recover, suggesting there is competition between the original memory and the extinction memory. These characteristics (reinstatement, renewal, and spontaneous recovery) are especially problematic for memory-based relapse prevention therapies, which highlights the importance of therapeutic strategies aimed at inhibiting reconsolidation and enhancing extinction. The use of preclinical animal models of addiction has shed light on several of the neural mechanisms responsible for these memory processes, which have a great deal of overlap with the cellular and synaptic mechanisms involved in promoting LTP and LTD.

1.2 NEURAL SUBSTRATES UNDERLYING DRUG-CUE MEMORY

The strength of drug-associated memories is indicative of their potential to cause relapse; therefore, interfering with reconsolidation or strengthening extinction have been proposed as therapeutic strategies to prevent relapse (Taylor et al., 2009; Torregrossa and Taylor, 2013). These processes can be evoked in an animal model of operant conditioning where rodents learn to self-administer (SA) drugs of abuse. In this model, an operant response (nose poke or lever press) results in delivery of the drug (US) in unison with a discrete cue (CS). Over the course of many trials, the drug-cue association is consolidated and stored in memory (Hernandez et al., 2002; Schafe and LeDoux, 2000; Wilensky et al., 1999). Next, the operant response is extinguished (instrumental extinction) by allowing animals to make nonreinforced responses. Over time, the animals adapt their behavior to cease performing the operant response, as it no longer results in a rewarding drug infusion. During this phase, operant responses are not paired with CS presentation, so that, although the operant response is extinguished, the drug-cue

association remains intact. Finally, the now extinguished behavioral response is reinstated, either by re-exposure to the CS, the reinforcing substance (drug), or a stressful stimulus. Importantly, instrumental responses made during reinstatement occur in the absence of drug reinforcement, thereby serving as a measure of risk of drug-seeking behavior. Typically, the number of operant responses is much higher than the number of responses under extinction conditions.

The reinstatement paradigm closely mirrors patterns of human drug use in which subjects take a drug under their own volition (as opposed to experimenter administered drugs), and drug-seeking occurs after a period of abstinence (Koob, 2000). Cue-induced reinstatement enables measurement of the subject's behavioral and neural response to drug-associated CS. Similar behavioral strategies are often used clinically in drug-dependent individuals to measure physiological responses and subjective craving in response to relapse-promoting cues, making the reinstatement paradigm a valid translational model (Epstein and Preston, 2003; Katz and Higgins, 2003; Shaham et al., 2003). Reinstatement has been utilized almost universally across drug classes, including stimulants, opioids, nicotine, alcohol, and cannabinoids (Bertholomey et al., 2016; Feltenstein et al., 2012; Kirschmann et al., 2017; Shalev et al., 2002). Recently, our lab and colleagues have developed a slight modification to the model that provides an opportunity to separate instrumental extinction from the more clinically-relevant Pavlovian cue extinction, as well as to examine the neural correlates and behavioral effect of drug-cue memory reconsolidation (Rich et al., 2016; Torregrossa et al., 2010). Following instrumental extinction, subsequent, brief exposures to CS (3 CS presentations) trigger memory retrieval, followed by reconsolidation. If reconsolidation is successful, the original drug-cue association should be strengthened or at least maintained, and subjects will display high levels of reinstatement (**Figure 2**). Alternatively, if the CS is presented in sufficient number or over a long enough

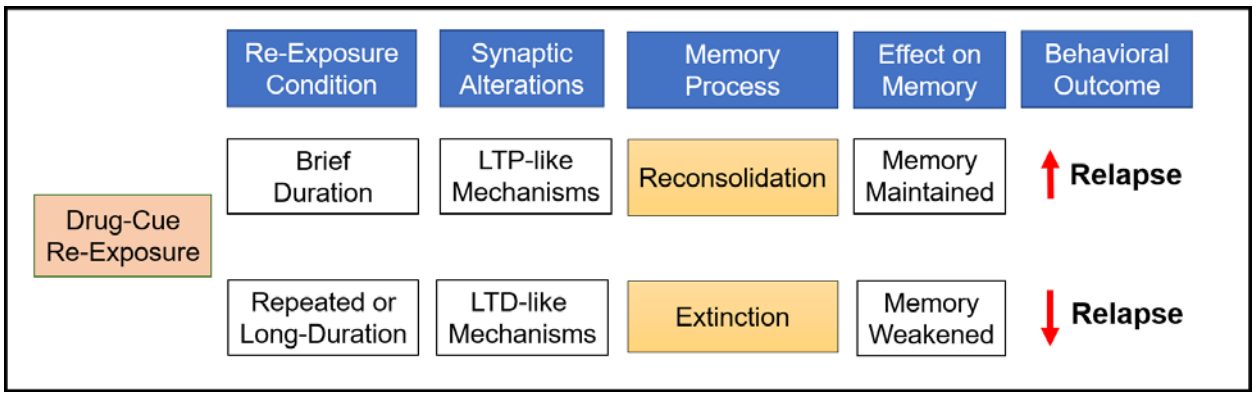

Figure 2. Schematic representing events that follow re-exposure to drug-associated cues.

Brief re-exposure initiates LTP-like synaptic mechanisms. These changes lead to memory reconsolidation and the maintenance or strengthening of drug-associated memory, which increases relapse potential upon subsequent presentation of cues. Repeated, or a longer exposure to cues causes LTD-like synaptic changes that induce memory extinction. The drug-cue memory is destabilized and weakened, resulting in lower relapse potential when the cues are re-encountered (From Rich and Torregrossa, 2017).

period without drug reinforcement (60-120 CS presentations), extinction can occur (Pedreira and Maldonado, 2003). In this scenario, CS is no longer predictive of drug delivery, and expression of the original drug-cue memory is blocked (Bouton and Moody, 2004). Thus, after sufficient extinction, subsequent encounters with the stimuli are less likely to trigger the relapse-promoting CR, and levels of reinstatement will be decreased (**See Figure 2**). Recent studies have utilized the reinstatement model in combination with optogenetic techniques to determine if stimulation or inhibition of specific neural circuits can promote or attenuate relapse-like behavior (Arguello et al., 2017; Ma et al., 2014; Stefanik and Kalivas, 2013), which has shed light on the underlying anatomical circuitry and neural mechanisms that are activated following re-exposure to drug-associated cues. Combining these novel approaches with manipulations of reconsolidation and extinction may reduce the efficacy of drug-cue memories to trigger relapse.

1.2.1 Central circuitry governing reward-related behavior

Behavioral responses to addictive substances require coordinated communication between several areas of the mesocorticolimbic system as well as various sensorimotor brain regions. By serving as a mediator of reward-motivated behaviors, the mesocorticolimbic system plays an important role in drug-seeking and relapse-like behavior. Midbrain dopamine (DA) neurons located in the ventral tegmental area (VTA) send projections to cortical and subcortical limbic structures, including the medial mPFC, NAc, hippocampus (HPC) and amygdala (Lüscher and Malenka, 2011; Pierce and Kumaresan, 2006; Wise, 2004). Collectively, the projections from the midbrain form part of the medial forebrain bundle (MFB). Stimulation of the MFB results in

increased locomotor activity, which drives reward seeking (Wise, 1987). Animals will also learn to complete tasks that result in self-stimulation of the MFB, suggesting a role for this pathway in motivated behavior (Gallistel, 1978; Sagara et al., 2010). The brain regions that receive VTA projections are also coupled by dense excitatory and inhibitory projections, forming a complex, interconnected network that functions to control adaptive behaviors, which can be modulated by DA (Geisler and Zahm, 2005; Sesack and Grace, 2010). Drugs of abuse, as with naturally-occurring rewards, increase the firing rate of VTA DA neurons, resulting in locally-elevated DA levels (Tomasi et al., 2015; Volkow et al., 2009). Additionally, psychostimulants block or reverse the presynaptic DAT, allowing postsynaptic activation of DA receptors to be enhanced (Harris and Baldessarini, 1973). These rapid pharmacodynamic effects of drugs enhance goal-directed behaviors that serve to promote reinforcement learning and increase motivation for drugs of abuse over natural reinforcers (Montague et al., 2004; Robbins and Everitt, 2002). Drugs and drug-related stimuli also induce expression of the immediate early gene Fos in glutamtergic neurons throughout the mesocorticolimbic system (Ciccocioppo et al., 2001; Neisewander et al., 2000; Pierce and Kumaresan, 2006). Fos is a marker of neural activity, which further implicates these brain regions in the control of drug-related behaviors.

Human imaging studies have revealed that drug-induced increases in DA release are accompanied by a subjective feeling of euphoria (Drevets et al., 2001), which creates an expectation for future reward, increasing the likelihood of future drug use, causing DA neurons to fire when reward is predicted (Schultz et al., 2000). This mechanism of anticipatory firing enables environmental stimuli to acquire incentive motivational properties that trigger future drug use. Re-exposure to drug-paired stimuli augment excitatory transmission to the VTA, increasing DA neuron firing rate even if the drug itself is withheld (Phillips et al., 2003; Stuber et

al., 2008). Based on theories of reward prediction error, if anticipated rewards are withheld following cue presentation, firing of DA neurons is inhibited (Schultz et al., 2000), and if repeated frequently enough, may shape behavior toward an inhibition of drug-seeking, thus acting as a signal mediating the extinction of drug-related memories. In addition to changes in firing rate, drugs of abuse induce synaptic changes in VTA DA neurons. For example, a single experimenter-administered cocaine injection is sufficient to rapidly increase excitatory transmission, a change that can last for up to 5 days (Ungless et al., 2001). Similar synaptic changes have been observed following cocaine SA even after 3 months of withdrawal (Chen et al., 2008). Drug-induced enhancements of VTA synaptic strength appear to be driven by alterations in the subtype of synaptic glutamate receptors. Specifically, cocaine increases the expression of GluN3A/GluN2B NMDAR, which have decreased conductance and are less permeable to Ca^{2+}, and also increases the expression of synaptic GluA2-lacking, Ca^{2+}-permeable AMPAR (Creed et al., 2016). Overall, drug-induced synaptic changes in VTA excitatory transmission may lead to the increases in intrinsic excitability that enhances DA release in downstream limbic structures (mPFC, amygdala, NAc) and helps perpetuate drug-seeking behavior.

Two of the most well-studied brain regions in terms of understanding addictive behaviors are the mPFC and the NAc. The mPFC functions in the control of working memory and is specialized for the representation of abstract information that guides behaviors related to executive function and cognition (Goldman-Rakic, 1987). The mPFC forms excitatory synapses with several GABAergic brain regions and is therefore an important source of “top-down” inhibitory control over unwanted or unnecessary behaviors (Kesner and Churchwell, 2011; Quirk and Mueller, 2008). Abnormal DA signaling within the PFC has been demonstrated in human

addicted individuals (Volkow et al., 2009). Furthermore, chronic cocaine SA modulates membrane excitability of PFC pyramidal neurons (Dong, 2005). Drug-induced adaptations in the PFC may account for the failure to inhibit drug-seeking even in the face of negative consequences, although pre-existing dysfunction in the PFC may also make individuals more susceptible to risky decisions that increase vulnerability to addiction (Blum et al., 2015; Cheng and Lee, 2016). The rodent PFC can be subdivided anatomically into a dorsal prelimbic cortex (PL) and a ventral infralimbic cortex (IL), which are analogs of human dorsomedial and ventromedial PFC, respectively. PL and IL are often viewed as functionally distinct brain regions that have opposing effects on behavior. Typically, the PL has been shown to promote drug-seeking behavior, while IL activity causes inhibition of drug-seeking (Peters et al., 2009).

The mPFC projects to the NAc, which is a major site of sensorimotor integration that functions in the assignment of motivational significance to rewarding substances (and reward-related cues) to drive goal-directed motor activity (Kalivas and Volkow, 2005). The NAc receives afferent information from the VTA, hippocampus, mPFC, and amygdala, and projects to the dorsal striatum and ventral pallidum. The NAc also has reciprocal connections with the VTA, which functions as an important feedback mechanism to control dopaminergic activity (Gibson et al., 2018). The major output neurons of the NAc are GABAergic medium spiny neurons (MSN), which represent approximately 90-95% of all neurons in the NAc (Kauer and Malenka, 2007). The NAc can be subdivided into two distinct subregions (core and shell), which may have different roles in the control of drug-related behavior. For example, lesions of the NAc shell attenuate unconditioned behavioral responses to psychostimulants, but do not prevent the acquisition of conditioned drug-seeking behavior. Conversely, lesions to the NAc core impair the ability of drug-associated reinforcers to maintain drug-seeking behavior (Ito et al., 2004). The

core and shell also exhibit distinct responses to drugs of abuse and drug-related stimuli that are underscored by different patterns of synaptic activity. Re-exposure to drugs or drug-paired cues result in a rapid potentiation of AMPAR-mediated glutamatergic signaling in the core, but a reduction in AMPAR expression in the shell (Gipson et al., 2014; Shen et al., 2011).

One possible explanation for the functional differences of the core and shell may be that the two regions receive a separate set of afferent projections. A common belief is that the PL projects to the NAc core to promote drug-seeking, while the IL innervates the NAc shell and functioned to suppress drug-seeking (Peters et al., 2009). Instrumental extinction results in activation of the IL-NAc shell, and inhibition of the IL is sufficient to induce a robust increase in relapse-like behavior (Peters et al., 2008). However, anatomical studies have contradicted this behavioral dichotomy, as there are also strong connections from IL to NAc core and from PL to NAc shell (Brog et al., 1993; Ma et al., 2014; Sesack et al., 1989). New behavioral evidence suggests that *both* the PL and IL can promote relapse. Activation of glutamatergic projections from IL to NAc shell can induce context-induced reinstatement of heroin seeking (Bossert et al., 2012). Likewise, clusters of neurons within the IL respond to cues predictive of reward availability to promote reward seeking (Suto et al., 2016). These conflicting reports may be due to a cell-type specific circuit in which behavioral output (enhancement or inhibition of drug-seeking) depends on the subtype of MSN that is predominately activated (D1- vs. D2-containing MSNs). Recent technological developments (e.g., optogenetics, DREADDs) have begun to shed light upon these discrepancies, such as studies demonstrating that D1-MSN activity promotes, and D2-MSN activity suppresses drug-seeking behaviors (Bock et al., 2013; Heinsbroek et al., 2017; Ortinski et al., 2015). For example, specific inhibition of D2-MSNs increases motivation for cocaine, while activation of these neurons suppresses cocaine SA. The antagonistic effects on

drug-seeking are mediated by the different projection targets of D1- and D2-MSNs, whereby D1-MSNs project to the substantia nigra (direct pathway) and D2-MSNs primarily project to the ventral pallidum (indirect pathway) (Kravitz et al., 2012). It should be noted that most of the studies that have investigated the role of the mPFC-NAc circuit on the extinction of drug-related behavior have done so using a model of instrumental extinction. Therefore, relatively little is known about the circuitry that mediates Pavlovian cue extinction, and likewise reconsolidation, of drug-associated memories. However, based on other studies of cue-related learning, the amygdala, which might be acting as a mediator between the mPFC and NAc, has emerged as a likely candidate for the control of memories evoked by drug-associated cues.

1.2.2 Control of cue-dependent memory by the amygdala

The amygdala is a phylogenetically-primitive collection of cortical and subcortical nuclei located within the temporal lobe that has been heavily implicated in the control of emotionally-salient memories, acting to establish emotional and motivational associations between CS and US (Cador et al., 1989; Johansen et al., 2011; Maren and Quirk, 2004). Early lesion studies showed that the amygdala was important for establishing behavioral responses to reinforcing stimuli as animals with amygdala lesions failed to exhibit typical freezing behavior in response to footshock-predictive cues (Weiskrantz, 1956). Similarly, amygdala lesions prevent various reward-related behaviors. Lesioned animals fail to exhibit conditioned place preference for methamphetamine (Hiroi and White, 1991). Additionally, amygdala lesions have no effect on responding during cocaine SA, but they do alter responding during cue extinction and abolish cue-induced reinstatement (Meil and See, 1997; See, 2002), suggesting a specific role in

mediating cue-dependent behaviors. More recent imaging studies in humans have demonstrated activation of the amygdala by drug-related stimuli, as well as when subjects are told to think about drug use, which correlates with physiological and subjective measures of craving (Childress et al., 1999; Garavan et al., 2000; Jasinska et al., 2014). Electrophysiological recordings show that amygdala neurons fire in response to reward-predictive cues and are also activated during goal-directed seeking of both natural rewards and drugs of abuse (Schoenbaum et al., 1999; See, 2002). Finally, increased Fos expression is observed in the amygdala following acute exposure to cocaine and cocaine-associated stimuli as well as cocaine-cue extinction, suggesting that intracellular changes within the amygdala occur during drug-induced memory processes (Ciccocioppo et al., 2001; Neisewander et al., 2000; Nic Dhonnchadha et al., 2010).

The amygdala can be broadly subdivided into the basolateral amygdala (BLA) and the central amygdala (CeA). The BLA can be further divided anatomically into lateral (LA), basal (BA), and basomedial (BM) nuclei, while the CeA is made up of both lateral (CeL) and medial (CeM) nuclei. The major projection neurons of the BLA are glutamatergic principal neurons while the CeA is more "striatal-like" in that it is made up of mostly GABAergic projection neurons (Janak and Tye, 2015). Additionally, interposed between the BLA and CeA are groups of GABAergic neurons known as intercalated cell masses, that project to the BLA and CeA and serve an important inhibitory function by suppressing amygdala output (Asede et al., 2015a; Pinard et al., 2012). The general construct of information flow through the amygdala is that the LA receives input and forwards this to the BA, BM, and CeL, which in turn project to the CeM. The CeM is considered the major output nucleus of the amygdala, although this is an oversimplification, as the BLA and CeL also have direct projections to other brain regions, including the NAc (Janak and Tye, 2015).

The amygdala is well positioned anatomically to integrate converging afferent input from multiple brain areas (PFC, sensory cortex, thalamus, and VTA; **See Figure 3**), all of which are also activated by cue/context exposure (Cho et al., 2017; Ciccocioppo et al., 2001; Do-Monte et al., 2015; Peter et al., 2012). The LA has reciprocal connections with the cortex, including IL, PL, and primary sensory areas. The PL and IL send glutamatergic projections to LA principal neurons, but also synapse with LA GABAergic interneurons and intercalated neurons, which then inhibit LA neuronal output (An et al., 2017; Quirk and Mueller, 2008). The role of the amygdala in cue-dependent memory arises from the diversity of its inputs, as it receives afferent sensory information about CS and also interoceptive information about US. Together, CS-US pairings enhance excitatory synaptic plasticity. Fear conditioning, in particular, has repeatedly been shown to induce LTP within the LA. The enhanced synaptic strength of CS inputs allows future presentations of the CS to activate the same LA neurons that are activated by the US (footshock) alone, thereby initiating conditioned freezing responses (Schafe et al., 2001). The specific inputs responsible for carrying CS-relevant information have also begun to be delineated. Brain regions responsible for auditory perception synapse directly with LA principal neurons (**Figure 3**). Auditory cortical projections are carried by the external capsule (EC), while subcortical/thalamic auditory information is carried by the internal capsule (IC; LeDoux et al., 1984; Romanski and LeDoux, 1992). IC- and EC-BLA synapses have been identified as potential loci for cue-mediated behaviors, and plasticity at these synapses is involved in the formation and extinction of fear-associated memories. For example, following fear conditioning, increases in cue-elicited freezing are correlated with increased synaptic strength at both thalamo-amygdala (T-LA) and cortico-amygdala (C-LA) synapses, and these changes are reversed by fear extinction (Kim et al., 2007; Hong et al., 2009). These bidirectional changes were also observed

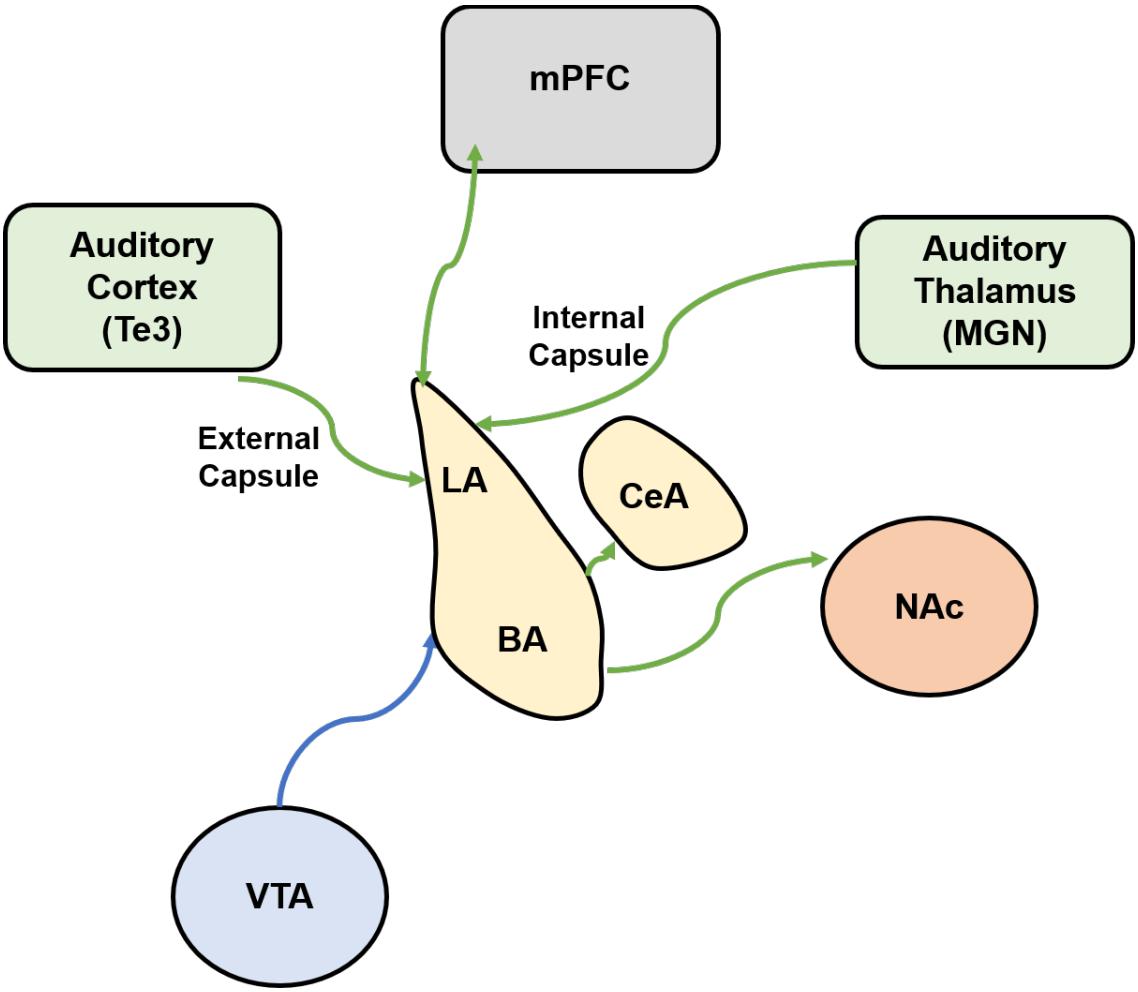

Figure 3. Afferent and efferent projections of the amygdala.

Simplified schematic representing the inputs and outputs of the amygdala. The amygdala receives sensory input from regions of thalamus and cortex from separate glutamatergic projections to lateral amygdala (LA) principal neurons. Auditory input is carried from the medial geniculate nucleus (MGN) by the internal capsule and from the auditory cortex (Te3) by the external capsule. The amygdala also has reciprocal connections with the mPFC; glutamatergic mPFC afferents synapse on LA principal neurons, but also on GABAergic interneurons and intercalated cell masses (not pictured). Bot the LA and BA received dopaminergic inputs from the ventral tegmental area (VTA) that can shape the response to glutamatergic inputs. The LA and BA integrate information and forward it to the central amygdala (CeA) and nucleus accumbens (NAc). The NAc functions as a sensorimotor gate which helps process incoming reward-related information and generate motor responses. The NAc also receives glutamatergic input from the mPFC and dopaminergic input from the VTA (Omitted for simplicity).

in a separate study that measured dendritic structural plasticity. Authors showed that fear conditioning increases BLA spine density while fear extinction reverses spine density back to control levels (Heinrichs et al., 2013). Optogenetic techniques have also been utilized to activate specific inputs to the LA during conditioned behaviors. In one such study, conditioned freezing responses and synaptic potentiation was evoked when a footshock US was paired with photoactivation of ChR2-expressing terminals from the medial geniculate nucleus (MGN; primary auditory thalamus) and/or primary auditory cortex (Te3). Subsequent induction of *in vivo* LTD in these pathways resulted in extinction of the fear-evoked response (Nabavi et al., 2014).

Interestingly, while aversive memories involve plasticity from both thalamic and cortical inputs, reward-associated learning appears to be a more complicated situation. As with fear-associated CS, LA neurons also develop enhanced excitatory responses to sensory cues paired with rewarding substances, like palatable food (Schoenbaum et al., 1998; Tye and Janak, 2007). In a series of experiments, Tye and colleagues trained rats to associate an auditory cue with a sucrose reward. Under normal circumstances, increases in AMPA:NMDA ratio were observed at T-LA synapses (IC pathway) but not C-LA synapses (EC pathway) (Tye et al., 2008). However, enhancing DA activity during cue-reward training by blocking DAT in the LA acted to prime C-LA synapses, resulting in enhanced synaptic activity at both T-LA and C-LA synapses (Tye et al., 2010). The implications of these results for understanding the acquisition of drug-cue learning remain unclear, although one might anticipate that since cocaine also blocks the DAT, pairing cocaine infusions with cues during cocaine SA might also enhance synaptic activity at both T-LA and C-LA synapses. The interplay between glutamatergic and dopaminergic activity is an important consideration, as activity of both neurotransmitters in the LA has been shown to

directly modulate aspects of synaptic activity during various types of Pavlovian conditioning (Feltenstein and See, 2007; Lee et al., 2005; Milton et al., 2008; Sanchez et al., 2010). For instance, application of the DA receptor antagonist, haloperidol, can block enhancements in neuronal excitability that are normally observed when a footshock is repeatedly paired with a particular odor (Rosenkranz and Grace, 2002). NMDAR antagonists also prevent the acquisition and extinction of several conditioned behaviors (Rodrigues et al., 2001; Sotres-Bayon et al., 2007; Zimmerman and Maren, 2010).

Finally, as was mentioned earlier, the initiation of drug-cue elicited behaviors is dependent on activity in the NAc. Recent studies have shown that the NAc receives direct cell-specific input from the BLA that may promote reward seeking (**Figure 3**). Optical stimulation of BLA-NAc glutamatergic fibers has been shown to be in and of itself reinforcing (Britt et al., 2012; Stuber et al., 2011). This positive reinforcement effect was blocked by intra-NAc injections of D1- but not D2-antagonists. Furthermore, optical inhibition of BLA-NAc projections blocked the ability of cocaine-paired cues to elicit reinstatement, and the same effect was observed when BLA-PL projections were inhibited (Stefanik et al., 2013). Together, the collective body of research gives rise to a potential circuit whereby the PL, IL, and LA function together to integrate subcortical information, and forward this processed information to the NAc core or shell, which either increases or limits drug-seeking, depending on the strength of the contextual association, the balance of afferent input, and the subtype of neurons that are activated. Although not directly tested, it is probable that drug-dependent learning would also involve LA-dependent synaptic changes that affect downstream communication to the NAc. Successful manipulation of the mechanisms that strengthen drug-associated memories may serve to disrupt the conditioned behaviors that promote relapse. Based on this premise, an emerging

line of research has been the use of pharmacological agents in the amygdala to target the molecules involved in the regulation of drug-associated memory reconsolidation and extinction.

1.2.3 Synaptic and molecular mechanisms underlying drug-associated memories

Early studies examining the role of DA transmission in reward-related behavior suggested that phasic DA release was responsible for encoding motivational significance. However, the diffuse projections of the VTA preclude DA, on its own, from encoding more detailed information, including specific details about an individual's experiences within an environment. In order for an individual to make specific predictions about a given stimulus, DA requires interaction with other circuits that are involved in encoding detailed information (Hyman et al., 2006; Robbins and Everitt, 2002). Therefore, associative learning processes (e.g., consolidation, reconsolidation, and extinction) require similar mechanisms that govern forms of excitatory neural plasticity, providing a conceptual framework for understanding how these memory processes function. As was mentioned above, LTP and LTD both require a specific temporal pattern of presynaptic glutamate release and postsynaptic depolarization. Similarly, for a CS to develop emotional significance and elicit a CR, CS-US pairings must occur in a time-specific manner, where the CS precedes the US by a short interval (Davis et al., 1989). The cellular processes that occur following cue retrieval (and lead to reconsolidation and/or extinction) also closely mirror the processes that occur following induction of LTP/LTD. For example, protein degradation (via polyubiquitination and proteasomal degradation) followed by the synthesis of new proteins is required for both LTP and reconsolidation (Artinian et al., 2008; Fonseca et al., 2006). Inhibiting protein degradation blocks destabilization and therefore prevents recall-induced

memory strengthening (Fukushima et al., 2014; Ren et al., 2013). The administration of protein synthesis inhibitors is also sufficient to block both the reconsolidation of fear after retrieval and the consolidation of extinction memories (Nader et al., 2000; Santini, 2004). The dependence on protein synthesis has been extended to drug-associated memories; inhibition of protein synthesis in the BLA prevents the reconsolidation of cocaine-associated cue and contextual memories (Fuchs et al., 2009; Lee et al., 2005). Similarly, inhibition of protein synthesis interferes with the induction and maintenance of both LTP and LTD (Barea-Rodríguez et al., 2000; Osten et al., 1996; Sajikumar and Frey, 2003).

Some of the most commonly investigated targets for the disruption of drug-associated memory are components of glutamatergic signaling (**Figure 4**). Activation of NMDAR are required for the reconsolidation of drug-cue memories, and therefore, NMDAR antagonists can interfere with reconsolidation. For example, intra-BLA infusion of the NMDAR antagonist D-APV prior to reconsolidation reduces subsequent cue-dependent drug-seeking behavior, which correlates with decreased expression of the immediate early gene, zif268 (Lee et al., 2005). Furthermore, systemic injections of the NMDAR antagonist MK-801 prior to reactivation also reduce cue-reinforced drug-seeking (Milton et al., 2008). Together, these studies suggest that NMDAR-dependent increases in BLA neural activity are necessary for drug-cue reconsolidation and the maintenance of drug-associated conditioned behaviors. Studies aimed at enhancing extinction have also targeted the glutamatergic system (**Figure 4**). Extinguishing an operant response for cocaine is associated with a reversal in cocaine-induced decreases in GluA1 and GluA2/3 expression in the NAc shell. Moreover, extinction is enhanced by viral overexpression of GluA1 and GluA2 in the NAc, demonstrating that extinction involves enhanced glutamatergic activity in the NAc shell (Self and Choi, 2004; Sutton et al., 2003). Antagonism of NMDAR in

this region results in disrupted extinction (Feltenstein and See, 2007; Fuchs et al., 2008). The involvement of gluamatergic activity during extinction has led to attempts to pharmacologically facilitate extinction via glutamate receptor agonists. D-Serine, an agonist at the glycine site of the NMDAR was shown to facilitate extinction, resulting in a decrease in cocaine-primed reinstatement (Kelamangalath et al., 2009). Similarly, N-acetylcysteine, given in combination with instrumental extinction training, produces long-lasting reductions in cue- and heroin-primed drug seeking (Zhou and Kalivas, 2008). Finally, D-cycloserine (DCS), a partial agonist at the NMDAR, has also been shown to augment the extinction of drug-associated memories. For example, the extinction of responding for self-administered cocaine was enhanced by DCS in both rats and monkeys, thereby reducing cocaine reacquisition (Nic Dhonnchadha et al., 2010). Additionally, DCS treatment following cue extinction in a novel context inhibits cue-induced renewal of drug-seeking, but only when injected systemically or directly to the NAc core (Torregrossa et al., 2010). The effects of DCS on the extinction of drug memories are similar to results observed in studies of fear memory extinction in both animal models and human clinical studies (Ledgerwood et al., 2004; Ressler et al., 2004). However, in clinical studies of human drug using populations, the success of DCS has been limited. A randomized, placebo-controlled study showed that DCS was unable to facilitate extinction, and instead, enhanced craving in cocaine-dependent individuals (Price et al., 2013). Moreover, in preclinical studies, DCS treatment can increase reinstatement of drug seeking. Increased cue-induced drug seeking after DCS treatment appears to occur following a brief re-exposure to the CS, suggesting that DCS may be strengthening the reconsolidation process as opposed to promoting extinction (Lee et al., 2009). Indeed, in this study, DCS was administered after re-exposure to 30 cue presentations, and studies from our lab indicate that 60 cue presentations only yield a mild reduction in

Pharmacological Compounds that Weaken Drug-Associated Memories			
Compound	**Effect**	**Behavioral Paradigm**	**Reference**
Propanolol (β2 adrenergic antagonist)	Inhibits Reconsolidation	Cocaine CPP, Morphine CPP (not Ethanol Conditioned Approach)	Bernardi et al., 2006 Robinson and Franklin, 2007 Milton et al., 2012
PKA Inhibitor	Inhibits Reconsolidation	Cocaine Cue- and Context-Induced Reinstatement	Arguello et al., 2014 Sanchez et al., 2010
PKMζ Inhibitor	Inhibits Reconsolidation	μ Opioid Agonist Conditioned Approach	Crespo et al., 2012
ERK1/2 Inhibitor	Inhibits Reconsolidation	Cocaine Context-Induced Reinstatement	Wells et al., 2013
mTOR/GSK-3 Inhibitor	Inhibits Reconsolidation	Cocaine CPP	Shi et al., 2014
Rac Inhibitor	Inhibits Reconsolidation	Cocaine CPP	Ding et al., 2013
D-Cycloserine (Partial NMDA agonist)	Facilitates Extinction (but can potentiate reconsolidation)	Cue-Induced Reinstatement	Torregrossa et al., 2010 Lee et al., 2009; Paolone et al., 2009
D-Serine (Partial NMDA agonist)	Facilitates Extinction	Cocaine-Primed Reinstatement	Kelamangalath et al., 2009
CDPPB (mGluR5 PAM)	Facilitates Extinction	Ethanol Cue-Induced Reinstatement Cocaine	Gass et al., 2014
CaMKII Inhibitor	Inhibits Reconsolidation Facilitates Extinction	Cocaine Cue-Induced Reinstatement	Rich et al., 2016

Figure 4. Pharmacological agents that weaken drug-associated memories.

List of pharmacological compounds that have been demonstrated to weaken drug-associated memories, either by inhibiting reconsolidation, facilitating extinction, or both. These manipulations are most often examined in the context of either reconsolidation or extinction, not both. Inhibition of CaMKII is the first manipulation that has been shown to inhibit reconsolidation *and* facilitate extinction. From (Rich and Torregrossa, 2017).

reinstatement, indicating that 30 was unlikely to sufficient produce extinction (Rich et al., 2016). Together, these studies highlight the complicated nature of reconsolidation and extinction following cue reactivation, and demonstrate that a combined approach, in which a single pharmacological compound can simultaneously enhance extinction and inhibit reconsolidation, may have a better likelihood of weakening drug-associated memory to prevent relapse.

Several intracellular signaling pathways that are important for the induction of LTP and LTD also mediate drug-memory reconsolidation. Chronic cocaine exposure persistently increases PKA activity (Lynch and Taylor, 2005; Nestler, 2004), and post-retrieval inhibition of PKA in the BLA reduces both cue-and context-induced, but not drug-primed reinstatement (Arguello et al., 2014; Sanchez et al., 2010). Inhibition of a constitutively active isoform of PKC, known as PKMζ blocks the development of cocaine sensitization, while reducing AMPAR membrane expression (Howell et al., 2014). Furthermore, direct inhibition of PKMζ in the NAc can disrupt the retrieval and reconsolidation of a drug-dependent conditioned approach behavior (Crespo et al., 2012). Additionally, the reconsolidation of cocaine-associated contextual memories depends on ERK (extracellular-regulated kinase) signaling. Intra-NAc core ERK inhibition decreases preference for a cocaine-conditioned context (Miller and Marshall, 2005), whereas intra-BLA ERK inhibition following reactivation results in less context-induced reinstatement (Wells et al., 2013). As with other kinases, ERK2 is involved in synaptic plasticity, including LTP in the BLA (Schafe et al., 2008), further demonstrating the importance for long-term synaptic alterations in the reconsolidation of drug-cue memories. Finally, the mammalian target of the rapamycin signaling (mTOR) pathway, heavily characterized for its involvement in the cell cycle and regulation of protein translation, has also been linked to synaptic plasticity and reconsolidation. The mTOR pathway has been linked to the insertion of AMPAR during the

maintenance phase of LTP in the hippocampus and mPFC (Man et al., 2003; Stoica et al., 2011). Due to its LTP-promoting effects, preventing the activation of mTOR has been linked to the inhibition of fear- and cocaine-associated memories (Gafford et al., 2011; Jobim et al., 2012; Shi et al., 2014).

Overall, because of the similarities between LTP/LTD and reconsolidation/extinction, many of the specific manipulations aimed at disrupting reconsolidation and/or facilitating extinction of drug-cue memories have targeted the same molecules that are involved in regulating mechanisms of LTP and LTD. The predominant process that occurs following memory retrieval (reconsolidation vs. extinction) appears to depend on the balance of intracellular signaling molecules and protein phosphorylation/dephosphorylation cascades. It should be noted that much of the research to this point has dealt with reconsolidation and extinction separately; there are very few, if any, studies that examine the effects of a single manipulation on both reconsolidation and extinction. This is an important limitation because, as is often the case, a pharmacological manipulation that is effective at enhancing extinction of drug-cue memories may fail to inhibit reconsolidation, leading to unintentional memory strengthening effects. A better approach is to identify cellular signaling events that simultaneous inhibit reconsolidation and enhance extinction.

1.2.4 Towards a bidirectional approach for targeting drug-cue memory

To determine if there was any overlap in the specific cellular events regulating extinction and reconsolidation, our lab recently conducted a study aimed at identifying specific phosphoproteins that are oppositely regulated by extinction and reconsolidation of a memory associated with self-

administered cocaine (Rich et al., 2016; Appendix A). Rats learned to associate self-administered cocaine infusions with an audiovisual cue and were later subjected to unreinforced presentations of the cue either 3 times to trigger reconsolidation or 120 times to trigger extinction. A phosphoproteomics analysis of the BLA identified a small number of signaling events that were oppositely regulated, including a novel phosphorylation event on calcium-calmodulin-dependent kinase II α (CaMKIIα) at Ser331. As described above, CaMKII is a well-characterized protein involved in various forms of memory and synaptic plasticity (Coultrap et al., 2014; Sanhueza & Lisman, 2013). Autophosphorylation of Thr286 has been implicated in numerous forms of learning, including those involved with drug-related memories (Easton et al., 2013, 2014). Not surprisingly, CaMKIIα dysfunction has been linked to neuropsychiatric disorders that involve changes to the number or size of dendritic spines, including schizophrenia, Angelman syndrome, and addiction (Müller et al., 2016a; Robison, 2014). Drugs of abuse may induce an upregulation of CaMKIIα, as evidenced by a reduction in amphetamine SA following NAc shell inhibition of CaMKIIα (Loweth et al., 2013). CaMKIIα has also been previously identified and studied as a potential key player in the regulation of synaptic memory associated with certain types of drug-associated stimuli (Sanchez et al., 2010, but see Arguello et al., 2014). To further characterize the effects of CaMKII in drug-cue memory, we tested the effects of intra-BLA CaMKII inhibition on cocaine-cue memory reconsolidation and extinction, and discovered that in both conditions, this pharmacological manipulation resulted in a reduction in cue-induced reinstatement, relative to controls. Whereas CaMKII mediates LTP via phosphorylation of GluA1 at S831, LTD can also be evoked by CaMKII-mediated phosphorylation of GluA1 at S567 (Coultrap et al., 2014). Thus, the effect of CaMKII inhibitors on reconsolidation and extinction might be explained by differential effects on LTP vs. LTD, respectively, suggesting

the existence of molecular mechanisms capable of simultaneously augmenting extinction and disrupting reconsolidation (Rich et al., 2016; See Appendix A).

Due to the involvement of CaMKIIα in the bidirectional control of drug-cue memories, it is possible that CaN (a 'negative regulator of CaMKIIα') may also be involved in extinction and reconsolidation. Whereas the inhibition of CaMKIIα may promote drug-memory amnestic effects, pharmacological agents that facilitate CaN activity would perhaps yield the same results. A series of studies by Gean and colleagues first identified a role for CaN in the regulation of fear-associated memories (Lin et al., 2003a; Lin et al., 2003b). Protein levels and enzymatic activity of CaN were increased by the extinction of a fear-associated memory. This was accompanied by the reversal of fear conditioning-induced phosphorylation of CaN substrates, including ERK. The administration of CaN inhibitors prevented this pattern of dephosphorylation and enhanced the resistance to fear extinction. A subsequent study showed that inhibitors of CaN blocked extinction-dependent decreases in CREB phosphorylation. These data suggest that CaN functions in a negative feedback loop and either directly or indirectly dephosphorylates specific substrates whose phosphorylation is required for memory consolidation. Several other studies have supported a role for CaN as a negative regulator of emotionally-salient memories (Baumgärtel et al., 2008; de la Fuente et al., 2014; Havekes et al., 2008).

As described above, the internal and external capsule are important signaling pathways that carry sensory information via thalamic and cortical routes, respectively, to the amygdala. Fear conditioning has been associated with potentiation of these synapses while fear extinction is associated with depotentiation. To model fear extinction, low-frequency stimulation (LFS) of the external capsule was used to elicit depotentiation in the LA following fear conditioning (Lin et

al., 2003a). LFS-induced depotentation was associated with an increase in CaN activity, and required NMDAR, LTCC, as well as CaN activation. Inhibitors of CaN prevented LFS-induced depotentiation in vitro and blocked LFS-induced fear extinction in vivo. Furthermore, an elegant study by Everitt and colleagues, investigated the molecular changes that occur during the shift from fear-memory reconsolidation to extinction by correlating results from biochemical assays with behavioral output after a various number of cue re-exposures (Merlo et al., 2014). Protein levels of calcineurin were shown to increase after 10 (but not fewer) presentations of a fear-associated CS, suggesting that CaN is specifically activated during extinction memory consolidation, and may be important during the shift between maintenance and loss of the fear-associated memory trace.

Finally, CaN has recently been identified in the destabilization of a methamphetamine (METH)-associated contextual memory (Yu et al., 2016). In this study, authors performed METH-CPP followed by treatment with the protein synthesis inhibitor, anisomycin, to disrupt reconsolidation. Memory destabilization was associated with a decrease in phosphorylation of GluA1 at Ser845, a decrease in spine density, and a decrease in AMPA:NMDA ratio. Blockade of NMDAR with MK801 or GluN2B-specific antagonists prevented destabilization-induced synaptic alterations. This is likely due to a block of the downstream phosphatase effects, as both PP1 and CaN inhibitors prevented the effects of anisomycin (Yu et al., 2016). This study indicates that destabilization of a METH-associated contextual memory occurs through a cascade of events that are CaN-dependent. Specifically, dephosphorylation of the peptide, Inhibitor-1, by CaN results in the activation of PP1, which dephosphorylates GluA1 at Ser845, resulting in the internalization of AMPAR and LTD (Mulkey et al., 1994). Together, there is strong evidence

that upregulating CaN activity may be an effective strategy for reducing the strength of drug-related memories, either via the blockade of reconsolidation or by the enhancement of extinction.

1.3 DISSERTATION AIMS

It has become clear that memory recall activates multiple processes that may have opposite effects on memory strength. That reconsolidation and extinction might be occurring simultaneously following memory recall makes it difficult to specifically target either reconsolidation or extinction via pharmacological manipulations. As described above, one strategy to overcome this difficulty is to develop a pharmacological agent that simultaneously disrupts reconsolidation and enhances extinction. A second strategy is to develop a methodological approach that directly and specifically manipulates neural circuits involved in the regulation of drug-cue memory. Studies within this dissertation examine afferent-specific synapses in the LA as potentially important for the bidirectional regulation of drug-cue memories and in mediating drug-seeking behaviors. Furthermore, these studies seek to uncover mechanisms by which drug-cue memories may be weakened to interfere with cue-evoked reinstatement. To this end, we use a combination of behavioral, physiological, optogenetics, and pharmacological approaches to (a) determine if drug-cue memory reconsolidation and extinction are encoded by LA synapses and (b) test whether circuit-specific induction of LTD in the LA and/or the activation of CaN is sufficient to interfere with drug-cue memories and prevent relapse-like behavior. The central hypothesis of this dissertation is that *thalamo-amygdala synapses are specifically involved in the consolidation, reconsolidation, and extinction of*

cocaine-cue memories, and that optical or pharmacological induction of depotentiation of these synapses is sufficient to attenuate cue-activated cocaine-seeking behavior. We test these hypotheses through the following experimental aims:

Aim 1: To determine whether thalamic and/or cortical LA synapses are potentiated by cocaine-cue pairings, strengthened by reconsolidation, and depotentiated by extinction.

Aim 2: To determine if MGN-LA synapses are specifically involved in regulating cocaine-cue memories, and whether optically-inducing LTD at this pathway is sufficient to reduce cocaine-seeking behavior.

Aim 3: To determine if cocaine-seeking behavior can be attenuated by pharmacological induction of LTD at thalamo-amygdala synapses during cocaine-cue memory processes.

2.0 PLASTICITY AT THALAMO-AMYGDALA SYANPSES REGULATES COCAINE-CUE MEMORY FORMATION AND EXTINCTION

Matthew T. Rich, Yanhua H. Huang, Mary M. Torregrossa; adapted from a version that is currently in submission at *Cell Reports*.

Repeated drug use has long-lasting effects on plasticity throughout the brain's reward and memory systems that promote future drug use. Environmental cues that are associated with drugs of abuse can elicit craving and relapse, but the neural circuits responsible have not been well delineated, thereby creating a major hurdle for the development of effective relapse-prevention therapies. In this study, we used a cocaine+cue self-administration paradigm followed by cue re-exposure to establish that the strength of the drug-cue association corresponds to the strength of synapses between the medial geniculate nucleus (MGN) of the thalamus and the lateral amygdala (LA). Furthermore, we demonstrate, via optogenetically-induced LTD of MGN-LA synapses, that reversing cocaine-induced potentiation of this pathway is sufficient to inhibit cue-induced relapse-like behavior.

2.1 INTRODUCTION

Chronic use of drugs like cocaine leads to the formation of long-term memories of the drug using experience and the environmental stimuli associated with that experience. Over time, exposure to just the drug-paired stimuli, or cues, is sufficient to induce physiological and psychological responses, such as craving, that drive continued use and relapse (Fuchs et al., 2008; Sinha and Li, 2007). Thus, one potential treatment strategy is to reduce the strength of drug-cue associations, so that the cycle of abstinence, craving, and relapse is blocked (Childress et al., 1986; Price et al., 2010). This may be possible by inducing extinction of the drug-cue association, a process whereby repeated cue exposure in the absence of the drug leads to a reduced expectation that the cue is predictive of drug use (Pedreira and Maldonado, 2003; Torregrossa and Taylor, 2013). However, a major limitation to enacting a memory-based treatment approach is that the precise neural correlates underlying drug-cue memories have not been well established.

In classical animal drug self-administration (SA) models, drug-cue associative memories form when an instrumental response (e.g., lever press) results in delivery of the drug (unconditioned stimulus; US)) together with a conditioned stimulus (CS; audiovisual cue). After repeated CS-US pairings, subsequent CS presentations by themselves can increase drug-seeking actions (lever presses). Cue-driven drug-seeking behaviors likely develop through cellular processes such as long-term potentiation (LTP) within neurons that are activated by the drug-cue experience (Cruz et al., 2014a; Shaham and Hope, 2005). On the other hand, extinction of drug-cue memories may reverse this plasticity and/or result in new LTP that inhibits the original memory, yet these possibilities have not been directly tested. Rather, prior studies have focused on neuroadaptations associated with the drug-seeking *action*, as opposed to the drug-associated

cue memories that drive craving and relapse. Moreover, pre-clinical studies of memory-based interventions, such as extinction learning, have largely focused on extinction of the lever press response, not of the drug-cue memory (Kalivas et al., 2005; Peters et al., 2008). In contrast, most clinical efforts aimed at extinguishing drug memories have focused on the cues that initiate the craving and relapse cycle. Thus, there is a large gap in the pre-clinical literature in identifying the locus of drug-cue memories, and the molecular mechanisms that regulate their formation and extinction remains unknown.

In contrast, numerous studies have investigated mechanisms regulating fear-conditioned memories, where manipulation of the cue, rather than the action, is the norm. These studies have shown that synaptic changes within thalamic and cortical inputs to the lateral amygdala (LA) underlie the formation and extinction of a conditioned fear memory (Hong et al., 2009; Kim et al., 2007a). Furthermore, Nabavi et al., (2014) used optogenetically induced long-term potentiation and depression (LTP or LTD) to demonstrate a causal link between synaptic modifications in the LA and the expression of a fear-associated memory. Given that, in addition to negatively valenced memories, the amygdala is also critical for the expression of memories related to positive affective value (Beyeler et al., 2016; Shabel and Janak, 2009), it may be that drug-cue associative memories are encoded in the amygdala, and can be targeted for treatment.

Here, we present the first evidence that cocaine cue-associated memory formation induces synaptic potentiation at medial geniculate nucleus (MGN) thalamo-amygdala, but not cortico-amygdala synapses, and that extinction of the cue memory, but not the lever pressing action, reverses this plasticity to reduce cue-induced relapse-like behavior. Furthermore, optogenetic LTD of MGN-LA synapses results in physiological and behavioral changes indicative of cue extinction. Together, these results identify a specific neural correlate and

cellular mechanism responsible for the acquisition and extinction of drug-cue memory, and present potential therapeutic approaches to prevent relapse.

2.2 METHODS

2.2.1 Animals

Naïve, adult male Sprague-Dawley rats (Envigo/Harlan), weighing 275-325 g on arrival, were used in all studies. All rats were housed in a temperature- and humidity-controlled room, in auto-ventilated racks with an automated watering system. Animals were housed in pairs, given *ad libitum* access to food and water, and maintained on a 12 h light-dark cycle. Prior to surgical procedures, rats were given at least 5 d to acclimate to the facility. Rats were food-deprived 24 h prior to the start of behavioral experiments and maintained at ~90% of their free-feeding body weight (~20 g of chow per day) for the duration of testing. All behavioral experiments were run during the light-cycle. Animals were allocated to groups following cocaine self-administration (SA) and, when applicable, instrumental extinction (IE), based on a matching procedure that ensured no significant differences between acquisition and IE behavior (See **Figures 8 and 11**). All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

2.2.2 Viral vector construct

Adeno-associated virus serotype 5 (AAV5) vectors were constructed to deliver oChIEF, a variant of the blue-light sensitive opsin channelrhodopsin (ChR2). oCHIEF is a mammalian codon version of ChIEF, with stronger expression in mammalian cells and an additional N-terminal amino acid residue that can respond to both low and high frequency stimulations (Nabavi et al., 2014; Lin et al., 2013). oChIEF was flanked downstream by the fluorescent marker tdTomato and expression of oChIEF was driven by the neuron-specific synapsin (hSyn) promoter (Lin et al., 2013). The construct was donated by Dr. Roger Tsien and processed for packaging and purification by the Duke Viral Vector Core.

2.2.3 Drugs

Cocaine hydrochloride (generously provided by the Drug Supply Program of the National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in sterile 0.9% saline (2 mg/ml) and filter-sterilized for SA.

2.2.4 Rodent intravenous catheterization

Rats were fully anesthetized with ketamine hydrochloride (87.5-100 mg/kg, i.m.) and xylazine hydrochloride (5 mg/kg, i.m.) and then received an analgesic (Carprofen, 5 mg/kg, s.c.) and 5 ml of Lactated Ringer's (s.c.) prior to surgery. Betadine and 70% ethanol were applied to all incision sites. All rats received a chronic indwelling intravenous (i.v.) catheter as described

previously (Rich et al., 2016). Catheters were implanted into the right jugular vein, then fed subcutaneously to the midscapular region, where they exited through a round incision. After surgery, rats recovered on a heating pad. Rats were then individually housed and given at least 7 d to recover before behavioral training. Rimadyl (5 mg/kg; s.c.) was administered for the first two days after surgery and catheters were kept patent by daily infusions of sterile saline containing gentamicin (5 mg/ml) and heparin (30 USP/ml).

2.2.5 Virus delivery and optic fiber implantation

For experiments involving viral infection of MGN neurons, rats were placed in a stereotaxic frame immediately following catheter surgery. They were given a small injection (~0.2-0.3 ml) of lidocaine (Henry Schein) to the scalp as a local anesthetic. A 26-gauge stainless steel injection cannula connected to a Hamilton syringe was used to bilaterally inject 1 μL of concentrated AAV solution into the medial portion of MGN (in mm from bregma, anterior and posterior (AP): –5.4; medial and lateral (ML): ±3.0; dorsal and ventral (DV): –6.6) through a pump (Harvard Apparatus) at a flow rate of 0.1 μL/min. Cannula were left in place for 5 min after infusions were complete before being slowly withdrawn. For experiments involving *in vivo* optogenetic control of MGN-LA terminals, two 200-μm optic fibers (0.5 NA, Thor Labs) were implanted (See Sparta et al., 2012) at the dorsal portion of the lateral amygdala (in mm from bregma, AP: -3.0; ML: ±5.1; DV: -7.9 mm). Fibers were lowered at a rate of 2 mm/min, then secured to the skull with screws, Loctite instant adhesive (Henkel Corp) and OrthoJet dental cement (Lang Dental).

2.2.6 Rodent cocaine or saline self-administration

Rats were trained to SA cocaine in standard operant conditioning chambers (MedAssociates), as described previously (Rich et al., 2016). Rats administered saline (0.9%) or cocaine (2 mg/ml) during daily sessions for 1 h, on a fixed ratio 1 (FR1) schedule of reinforcement with a 10 s timeout. The designated active lever (counterbalanced across left and right levers) produced a cocaine or saline infusion paired with a 10 s compound light and tone cue. Pump durations were adjusted daily according to body weight in order to deliver the correct dose of drug (1.0 mg/kg of body weight per infusion). Responses on the other, inactive, lever were recorded, but had no programmed consequences. Rats underwent training for at least 10 d and until they administered at least 8 infusions per day over 3 consecutive days. Cocaine self-administering rats that did not meet acquisition criteria by 20 d were excluded from the study; saline self-administering rats were advanced regardless, as most do not reach acquisition criteria. The program was controlled by and data were collected using MedPC IV (MedAssociates).

2.2.7 Instrumental lever extinction

After successful acquisition of SA, rats underwent IE for at least 7 d. During these daily 1 h sessions, responses on both the active and inactive levers were recorded but had no programmed consequences. IE continued until extinction criteria had been met (an average of < 25 lever presses on the last two days of extinction). Throughout IE, rats received no cocaine or cocaine-associated cue reinforcement, thus reducing responses to a stable, low rate. This reduces the motivational value of other cues in the SA context, so that subsequent reinstatement testing or

physiological assessment specifically isolates the memory for the discrete cue associated with cocaine infusion.

2.2.8 Pavlovian cue re-exposure

Cue re-exposure occurred as described previously (Rich et al., 2016). Briefly, rats were returned to the SA context 24 h after the final day of SA (or IE) and lasted for 1 hr. During this session, rats that had undergone cocaine SA received noncontingent presentations of the previously drug-paired cues: either 0, 3, 60, or 120. A separate group of cocaine-trained rats were left in their home cages undisturbed as a control for re-exposure to the training context. Saline-trained animals were returned to the training context and did not undergo cue re-exposure (0 cue presentations). During re-exposure sessions, the cocaine-associated cue was presented for 10 s, with each presentation separated by 30 s.

2.2.9 Cue-induced reinstatement

24 h after cue re-exposure, cue-induced reinstatement was assessed during a 1 h session that took place in the original SA context. A lever press on the active lever produced a 10-s presentation of the cocaine-associated cue on an FR1 schedule, but no drug reinforcement. Lever presses on the inactive lever were recorded but had no programmed consequences. In the optical LTD experiment, to measure spontaneous recovery of drug-seeking, rats underwent a second cue-induced reinstatement test 7 d later.

2.2.10 *Ex vivo* slice preparation

Slices were prepared as described previously (Dong et al., 2006; Huang et al., 2008b), with slight modifications, and using methods designed to improve neuronal health in adult rodents (Ting et al., 2014). Briefly, 24 h after cue re-exposure sessions, rats were deeply anesthetized with isoflurane. Rats were then briefly perfused with ice-cold cutting solution containing (in mM): 92 N-methyl-d-glucamine (NMDG), 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$, saturated with carbogen (95% O2/5% CO2), pH adjusted to 7.4 with HCl. Rats were then decapitated and brains removed. Acute coronal slices of the amygdala (250 μm thick) were obtained (normally 4–6 slices were obtained from each rat) using a VT1200S vibratome (Leica, Weltzar, Germany) in 4 °C cutting solution. Slices were placed in a holding chamber filled with the same cutting solution, and incubated at 37°C for 10-15 min before being transferred to a beaker of HEPES-based holding solution containing (in mM): 86 NaCl, 2.5 KCl, 1.2 NaH2PO4, 35 NaHCO3, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 1 MgCl2, and 2 CaCl2, saturated with carbogen. Slices were allowed to recover for >30 min at room temperature before experimentation.

2.2.11 *Ex vivo* electrophysiological recordings

Slices were transferred to an Olympus BX51WI upright microscope equipped with gradient contrast infrared optics. The LA was identified using a 4X objective and this region was then magnified for identification of neurons with a 40X water immersion lens. Whole-cell recordings

were obtained from individual principal neurons in the dorsal LA using glass pipettes (3–5 MΩ). Principal neurons are identified by morphology, and in voltage clamp, typically show low levels of spontaneous activity. For a subset of cells, principal neurons could be confirmed in current clamp by injecting current pulses to elicit action potentials, as described elsewhere (Kim et al., 2007a; See **Figure 5A**). Voltage-clamp experiments used pipettes filled with a cesium-based internal solution [in mM, cesium methanesulfonate 108, CsCl 15, CsEGTA 0.4, TEA-Cl 5, HEPES 20, Mg-ATP 2.5, Na-GTP 0.25, QX-314-Cl 1, sodium phosphocreatine 7.5, and L-glutathione 1, and pH to 7.3 with CsOH] and current-clamp experiments used pipettes filled with a potassium-based internal solution [in mM, potassium methanesulfonate 108, KCl 20, K-EGTA 0.4, HEPES 10, Mg-ATP 2.5, Na-GTP 0.25, sodium phosphocreatine 7.5, L-glutathione 1, MgCl2 2, and pH to 7.3 with KOH]. During recordings, slices were superfused with aCSF that was heated to 31–33 °C by passing the solution through a feedback-controlled in-line heater (Warner, CT) before entering the chamber. External perfusion consisted of a modified artificial cerebrospinal fluid (ACSF), containing, in mM NaCl 119, KCl 2.5, NaHCO3 26, NaH2PO4 1.2, glucose 12.5, HEPES 5, MgSO4 1, CaCl2 2, saturated with 95% O2/5% CO2. Neurons were voltage-clamped at -70 mV. For experiments involving electrical stimulation, a concentric bipolar stimulating electrode (FHC, Bowdoin, ME) was placed over axon fibers emerging from the internal capsule (putative thalamic afferents) or external capsule (putative sensory cortical afferents). Projections to the LA were stimulated using 0.1 ms pulses at predetermined series of intensities (10-35 μA) using an isolated current stimulator (A-M instruments; Digitimer Ltd, Hertfordshire, England), and the evoked excitatory postsynaptic currents (EPSCs) were recorded. For paired pulse delivery, each pulse was separated by a 50 ms interpulse interval. AMPAR currents were elicited at E_{Rev}-70 mV holding potential and NMDAR currents were elicited at a

E_{Rev}+40 mV holding potential. NMDAR amplitude was operationally defined as the amplitude of the current 35 ms after the peak of the AMPAR current; at this time point, AMPAR-mediated currents have subsided (Huang et al., 2008). To elicit action potential firing, in current clamp mode, depolarizing current pulses of -100 to +200 pA (20 pA steps, 1 s duration) were delivered. For experiments involving optical stimulation, AAV-infected MGN projections were identified using fluorescence and then stimulated using a blue light (473 nm) DPSS laser (IkeCool), that was generated using the Clampex software (Molecular Devices) and a pulse generator (A-M Systems). Collimated laser light was coupled to a fluorescent port of the Olympus BX51WI microscope, allowing the blue laser light to illuminate the slice through the objective, placed immediately above the cell. Optical stimulations of 1 ms duration were used for paired-pulse or AMPA:NMDA ratio measurements. Neurons receiving input from AAV-infected MGN neurons exhibited reliable EPSCs in response to stimulation (See **Figure 5B**). Likewise, under current-clamp conditions, AAV-infected MGN neurons generated action potentials in response to various frequencies of blue-light stimulations (See **Figure 5C**). *Ex vivo* LTD experiments were performed in current-clamp mode, with the bridge balanced routinely. Optically-evoked EPSPs were recorded at 0.1 Hz for 10 minutes prior to LTD induction [900 2-ms pulses of blue light, at 1 Hz (15 min induction protocol)]. Following LTD induction, EPSPs were continuously recorded at 0.1 Hz for the next 60 minutes. For all experiments, series resistance was 10–25 MΩ, uncompensated, and monitored continuously during recording. Cells with a change in series resistance beyond 20% were not accepted for data analysis. Synaptic currents were recorded with a MultiClamp 700B amplifier (Molecular Devices), filtered at 3 kHz, amplified 5 times, and then digitized at 20 kHz. Picrotoxin (100 μM; dissolved in DMSO) was included in the bath solution to inhibit $GABA_A$ receptor-mediated currents in all experiments.

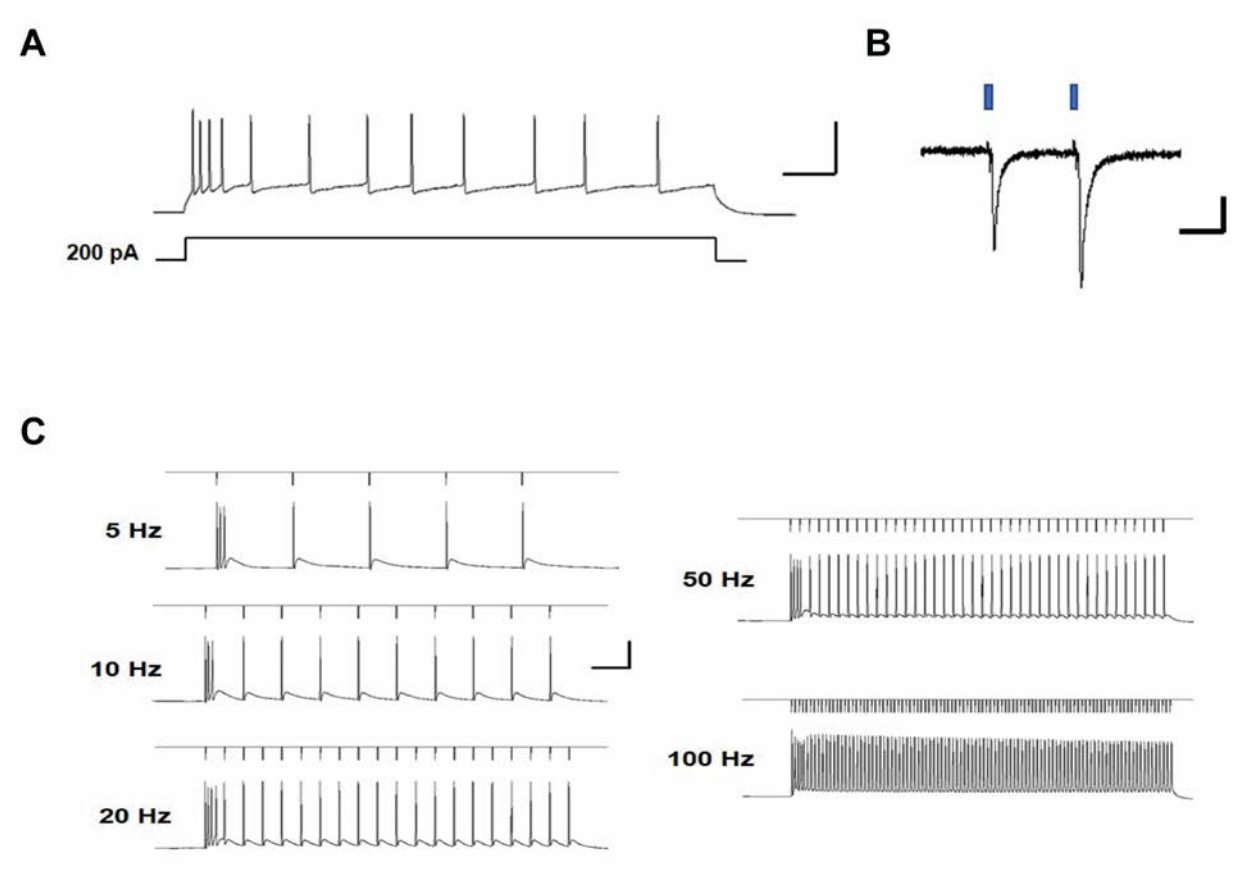

Figure 5. Sample electrophysiological recordings from LA and MGN neurons.

(**A**) Sample current clamp recording from LA neuron. Injection of a prolonged depolarizing current (0.2 nA, 1 s) demonstrating action potential firing that shows spike frequency adaptation typical of principal neurons (*See Kim et al., 2007a*). Scale bars: 100 ms, 40 mV. (**B**) Sample voltage clamp recording from LA neuron receiving projections from AAV-oChIEF-infected MGN neurons. EPSCs were elicited by two brief (1 ms) pulses of blue light (473-nm) separated by 50 ms. Scale bars: 25 ms, 50 pA. (**C**) Sample current clamp recordings from AAV-oChIEF-infected MGN neurons. Action potentials were elicited by blue light stimulation (5-100 Hz), demonstrating the capacity for MGN-infected neurons to respond to both low and high frequency stimulation. Scale bars: 100 ms, 40 mV.

2.2.12 *In vivo* optogenetic procedures

Rats were transferred to a clean standard housing cage. Bilateral optic fiber implants were connected to an optic fiber patch cord, which was connected to a 473-nm blue laser diode (IkeCool) via a rotary joint (Prismatix). The light intensity through the optical fiber, which was measured by a light sensor (S130A; Thor Labs), was adjusted to ~5-10mW. Rats were allowed to explore the environment for 3 min prior to LTD induction. LTD was induced using the paradigm described above (900 2-ms pulses of light delivered at 1 Hz). After induction rats remained in the cage for 3 min, before being placed back in their home cage. Control rats had a sham optic fiber patch cord attached to the head-mounted optic fiber for the same duration as the LTD induction. 24 hours after *in vivo* optogenetic stimulation, rats were assessed for drug-seeking in a standard cue-induced reinstatement session (See above).

2.2.13 Staining, fluorescence, and imaging

Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). Rats were perfused through the aorta with 1X PBS for 5 min followed by 4% paraformaldehyde in 1X PBS, pH 7.4 for 10 min. The brains were extracted, postfixed in 4% paraformaldehyde for 24 h, and transferred to 30% sucrose solution. Brains were sectioned at 50 μm using a cryostat (Leica). Slices containing the LA or MGN were mounted onto glass slides, and cover-slipped with Fluoroshield with DAPI (for nuclear identification) mounting media (Sigma-Aldrich). Slices were imaged using an Olympus BX61VS epifluorescent slide-scanning microscope to verify AAV-oChIEF-tdTomato expression in the MGN and its projections to the LA (See **Figure 6**).

Additionally, position of the optic fiber over the LA was verified. Rats lacking expression of AAV in MGN or LA and those in which the optic fiber was not correctly positioned were removed from the study.

2.2.14 Data Acquisition and Statistical Analysis

All statistical analyses were performed using GraphPadPrism for Windows and results are expressed as mean ± SEM. Rats were distributed into groups based on a matching procedure that ensured that each group had no statistical differences in their cocaine infusions acquired over days, or differences in instrumental extinction behavior. For behavioral experiments, reinstatement tests were analyzed by two-way ANOVA with repeated measures, with the within-subjects factor being responding on the last day of instrumental extinction versus reinstatement responding and the between-subjects factor being cue re-exposure condition. For electrophysiological experiments, data were coded such that experimenters were not aware of treatment groups when performing data analysis, and then decoded for final results. Data were analyzed offline using ClampFit 10.3. For experiments in which the end points were from individual cells, such as EPSCs, we used the averaged value of a parameter from all cells recorded from an animal to represent the parameter of this animal. For electrical stimulation experiments, EPSC amplitude was calculated at each stimulation intensity and compared between groups using two-way ANOVA with repeated measures, with the within-subjects factor being stimulation intensity and the between-subjects factor being cue re-exposure condition. For correlation analyses, Pearson's correlation coefficients were calculated, with number of infusions as the independent variable and EPSC amplitude as the dependent variable. Paired pulse ratio

(PPR) was calculated as the ratio of the peak current of the second EPSC to the first EPSC. AMPAR current was calculated as the peak current at Erev -70mV and NMDAR current was calculated as the peak current at Erev+40 mV, 35 ms after peak AMPAR current. For AMPA, NMDA, and PPR, comparisons were made using one-way ANOVA. All data points were an average of 10 trials. For optical stimulation experiments, EPSC amplitude, PPR, and AMPA:NMDA ratios were calculated as described above and compared using one-way ANOVA or unpaired t-test. For LTD experiments, peak EPSP amplitude and EPSP rise slope were calculated for every trial and six consecutive trials were averaged together for each data point. For comparisons of pre- and post-LTD comparisons, data points across the last 7 minutes of baseline were compared to the last 7 minutes of post-LTD recordings with a paired t-test. Each experiment was replicated in at least 5-6 rats (1-5 cells were recorded from each rat) for electrophysiological analysis and at least 6 rats for behavioral tests. For all analyses, significant effects were further analyzed by Tukey's or Bonferroni's post hoc tests, with significance set at $P < 0.05$. All data were determined to be normally distributed using the Shapiro-Wilk test, and Bartlett's test was used to determine that there were no significant differences in the estimated variance between groups. Statistical parameters for each analysis can be found in the corresponding figure legends.

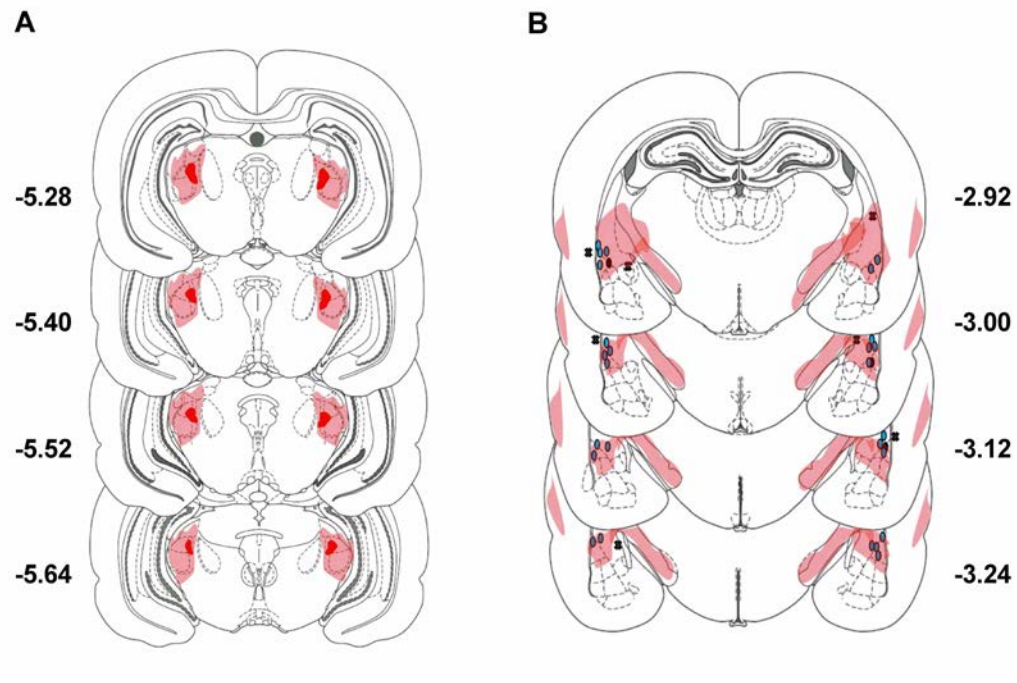

Figure 6. Histological verification of AAV injection and optic fiber placements.

(**A**) Schematic showing injection of AAV-oChIEF-tdTomato throughout the anterior-posterior extent of the MGN (For Figures 13; n = 33 rats and 14; n = 13 rats). Dark red shading shows representation of smallest acceptable virus spread, and light pink shading shows representation of largest acceptable spread. Inclusion required dual hemisphere viral expression. (**B**) Schematic showing spread of AAV-oChIEF-tdTomato (corresponding to Figures 13 and 14) and optic fiber placements (corresponding to Figure 14) throughout the anterior-posterior extent of the LA. Light pink shading shows representation of AAV-infected MGN-projecting axons. There is robust expression through the internal capsule targeting the LA. Notably, there is also expression in auditory temporal cortex, which receives dense projections from the MGN. Blue circles correspond to successful optic fiber placement in both hemispheres. Black circles correspond to successful optic fiber placement in only one hemisphere. Black "X" corresponds to unsuccessful fiber placement. To be included in final analysis, rats required viral expression in the LA as well as successful placement of fibers in both hemispheres. *Coordinates are in mm, posterior from bregma.*

2.3 RESULTS

2.3.1 Thalamo-amygdala synaptic modifications regulate cocaine-cue memories

To determine how the strength of drug-cue associations impacts drug-seeking behavior, rats were trained on a fixed ratio 1 (FR1) schedule of reinforcement, during which a single active lever press was required to receive an infusion of cocaine or saline (Sal), paired with an audiovisual CS (**Figure 7A**). Cocaine- but not saline-trained animals demonstrate reliable acquisition of SA (**Figure 7B**). After the completion of at least 10 daily training sessions, and once acquisition criteria were met, rats underwent instrumental extinction (IE) for ~7 days, where lever pressing produced no consequences, and was thus reduced. 24 h following the last IE session, rats underwent 'cue re-exposure sessions' during which they received passive presentations of the CS (0, 3, 60, or 120 times) in the absence of cocaine reinforcement. 24 h later, the capacity of the CS to promote cocaine-seeking behavior was determined during a cue-induced reinstatement session. Increasing the number of CS presentations during cue re-exposure resulted in a progressive reduction in cue-elicited drug seeking (**Figure 7C**). Compared to non-re-exposed controls (0 CS presentations), 120 CS presentations significantly attenuated reinstatement, confirming previous studies demonstrating that relapse-promoting, drug-cue associations that form during cocaine SA can be extinguished by sufficient cue re-exposure in the absence of drug reinforcement (Torregrossa et al., 2013).

In separate groups of animals, we next tested if drug-cue associations were regulated by specific synaptic modifications in the LA by performing *ex vivo* electrophysiological recordings in rats trained to SA cocaine or saline, followed by cue exposure either 24 h after the last SA

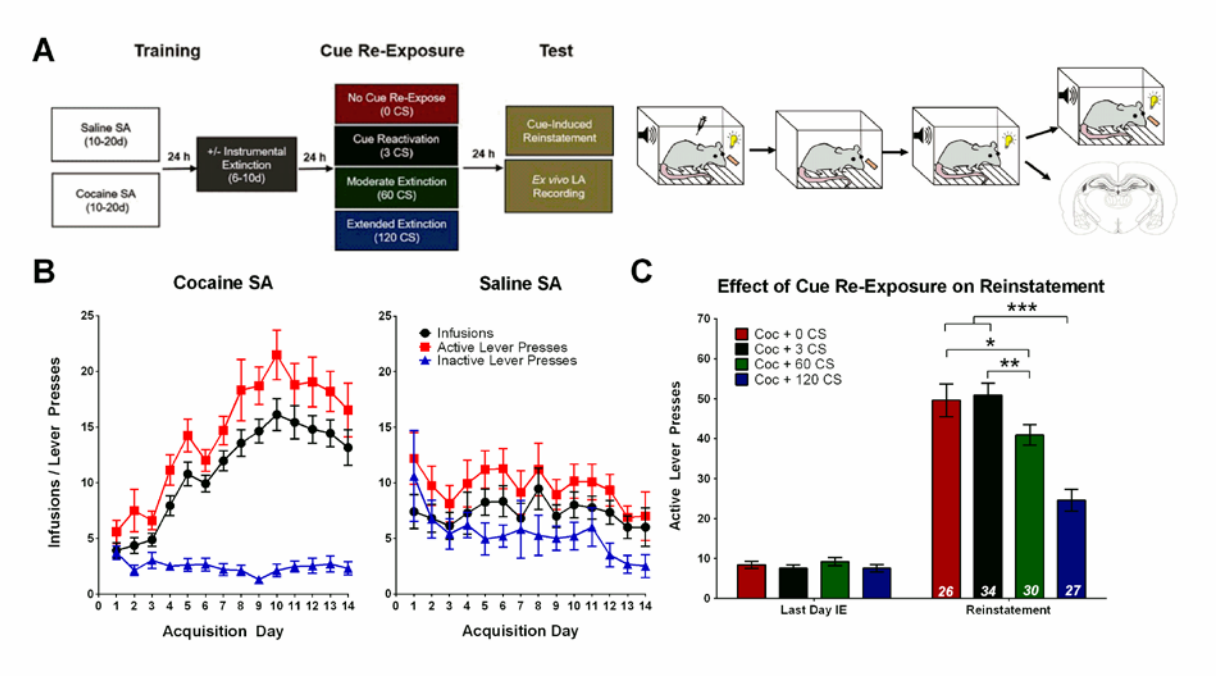

Figure 7. Drug-cue memory manipulations alter cue-induced reinstatement.

(**A**) Experimental timeline and schematic demonstrating timepoints of each experimental phase and color-coded treatment groups. SA, self-administration; IE, instrumental extinction; CS, conditioned stimuli; LA, lateral amygdala. (**B**) Cocaine SA (Left, n = 50), but not saline SA (Right, n = 15) animals exhibit an increasing number of cocaine infusions throughout acquisition. (**C**) Effect of cue re-exposure on cue-induced reinstatement. All groups made significantly more active lever presses during reinstatement compared to the last day of IE; however, reinstatement is reduced by extended cue extinction (120 CS). Two-way ANOVA, main effect of group ($F(3,112) = 12.56$, $P < .001$) and a day x group interaction ($F(3, 112) = 14.69$, $P < .001$); post hoc analysis: *p < .05, **p < .01, ***p < .001. Error bars equal mean ±SEM, n in italics, *number of rats (number of neurons).*

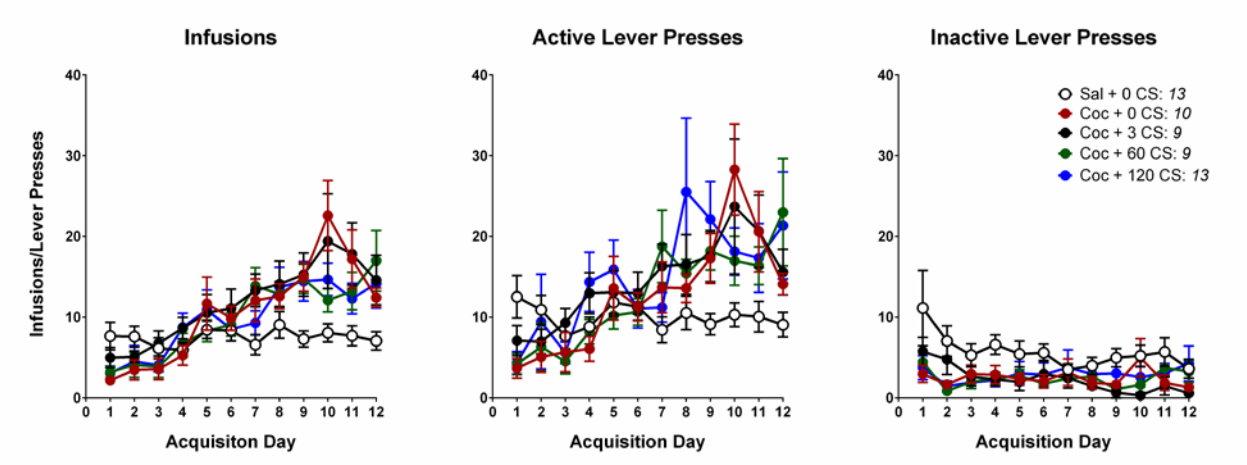

Figure 8. No group differences in acquisition of cocaine self-administration.

Acquisition data for rats from electrophysiological experiments. There were no differences in infusions earned (Left: $F_{(4,49)} = 1.92$, $P > .05$), active lever presses (Middle: $F_{(4,49)} = 1.46$, $P > .05$), or inactive lever presses (Right: $F_{(4,49)} = 2.19$, $P > .05$) between any cocaine SA animals (all two-way ANOVA; n in italics, *number of rats*), but there are differences between cocaine and saline SA animals.

session or after IE sessions (described below). Importantly, the timing of recordings corresponded to the time when cue-induced reinstatement behavioral tests were conducted (**Figure 7A**). Additionally, rats were assigned to groups based on a random matching procedure to ensure no significant differences between groups for the number of infusions earned, active lever presses, or inactive lever presses during acquisition (**Figure 8).** Previous studies involving auditory fear conditioning revealed that the strength of thalamic and cortical synapses in the LA correspond to the strength of the fear memory (Hong et al., 2009; Kim et al., 2007a), suggesting that similar synaptic modifications may underlie drug-cue associations. To test this, electrically-evoked EPSCs were recorded from LA principal neurons by stimulating either internal capsule (IC: putative thalamic afferents or external capsule (EC: putative cortical afferents (Doron and

Ledoux, 2000; Hong et al., 2009; Kim et al., 2007a) (**Figures 9A and 12A**). We tested a series of stimulation intensities to generate an input-output relationship, whereby EPSC amplitude is increased with larger stimulation intensity. These relationships have commonly been used to assess synaptic changes following extinction of conditioned fear memories (Hong et al., 2009; Kim et al., 2007a). Relative to saline-trained controls, cocaine-trained non-CS re-exposed animals showed significantly increased EPSC amplitudes at thalamo-amygdala (T-LA) synapses at given current injections, suggesting that the formation of drug-cue associations potentiates this synapse (**Figure 9B**). To determine if context re-exposure alone could account for changes in T-LA synaptic strength, a control group of animals remained in their home cage instead of undergoing cue re-exposure. Although context re-exposed animals show slightly higher EPSC amplitudes than the home-cage controls, these differences were not significant (**Figure 10**). This reveals that synaptic strengthening is likely due to the repeated pairing of discrete and contextual cues in combination with cocaine SA and is not dependent on re-exposure to the drug-related context alone.

We next assessed how the number of CS presentations during cue re-exposure impacted T-LA synaptic strength and again discovered a progressive effect of CS re-exposure (**Figure 9B**). Compared to 0 CS animals, EPSC amplitude was slightly, although non-significantly increased in animals that underwent brief CS re-exposure (3 CS), suggesting that brief cue memory reactivation in the absence of cocaine does not disrupt the original drug-cue association, and may tend to strengthen the association through reconsolidation processes. However, EPSC amplitude was attenuated by increasing the number of cue presentations (60 and 120 CS). Animals that underwent 120 CS re-exposure exhibited average EPSC amplitudes similar to that

of saline-controls. This shows that long-term cue re-exposure depotentiates T-LA synapses, thereby reversing the synaptic changes that occur after Coc+cue SA.

We next asked whether T-LA synaptic strength was directly affected by the strength of the cocaine-cue association (**Figure 9C**). We discovered a positive correlation between the total number of US-CS pairings received during SA and the average EPSC amplitude for animals that received no cue re-exposure. Conversely, in animals that underwent either 60 or 120 CS re-exposure (cue memory extinction), there was no correlation between these two factors. These data suggest that increased cocaine-cue pairings is associated with more strongly potentiated T-LA synapses. However, sufficient cue re-exposure in the absence of drug reinforcement weakens T-LA synapses independent of prior drug-cue experience.

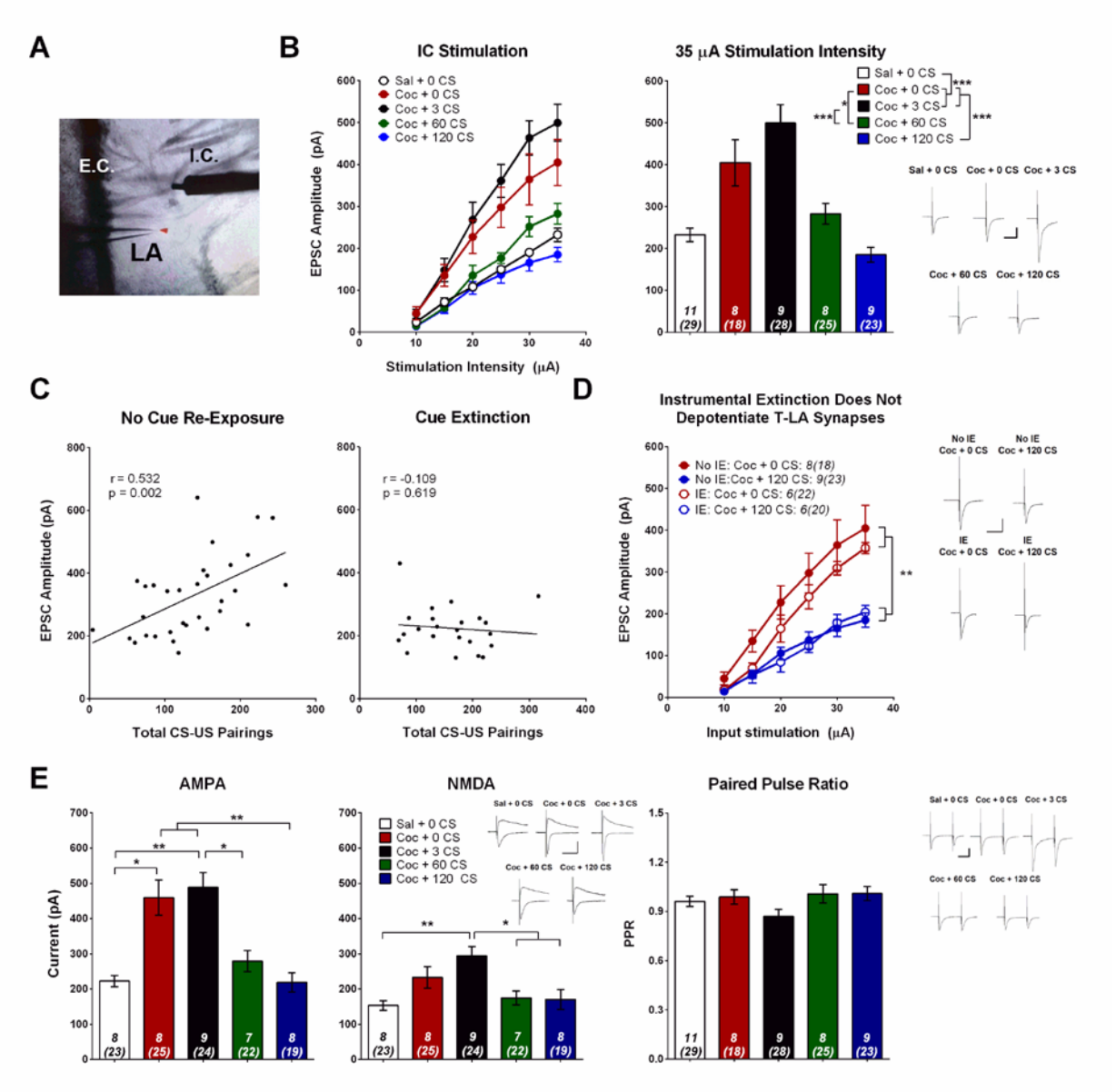

Figure 9. Cocaine-cue manipulations drive thalamo-amygdala synaptic modifications.

(**A**) Image of LA coronal section, demonstrating placement of stimulating electrode above IC fibers and position of patch pipette. EPSCs were evoked from LA principal neurons by stimulating the IC (putative T-LA synapses). IC, internal capsule; EC, external capsule. (**B**) Cocaine SA and drug-cue manipulations alter T-LA synaptic strength. Left, Average EPSC amplitude for each group at various stimulation intensities. Right, Average EPSC amplitude at the highest stimulation intensity (35 μA). Cocaine SA increases EPSC amplitude relative to Sal SA. Brief cue re-exposure (3 CS) does not further alter EPSC amplitude, but moderate (60 CS) and extended (120 CS) cue extinction

reverses the cocaine-cue-induced potentiation. Two-way ANOVA, main effect of group ($F_{(4,40)}$ = 13.54, P < .001) and a stim. intensity x group interaction ($F_{(20,200)}$ = 8.96, P < .001); post hoc anlaysis: *p < .05, ***p < .001. *Inset: Sample average EPSC traces evoked at -70 mV*. (**C**) Left, EPSC amplitude (35 μA stimulation intensity) was strongly correlated with the number of CS-US pairings received during acquisition for cocaine- and saline SA animals that did not receive cue re-exposure; r(30) = 0.532, **p < .01, n = 32. Right, EPSC amplitude was not correlated with number of CS-US pairings for animals that received cue extinction (60 or 120 CS); r(21) = -0.109, p = .619, n = 23. (**D**) Instrumental extinction does not alter T-LA synaptic strength. EPSC amplitude was significantly higher for rats that received no cue re-exposure (0 CS) compared to rats that received extended extinction (120 CS), independent of IE. Two-way ANOVA, main effect of group ($F_{(3,24)}$ = 8.38, P < .001) and a stim intensity x group interaction ($F_{(15,120)}$ = 4.87, P < .001); post hoc analysis: **p < .01, ***p < .001. *Inset: Sample average EPSC traces from each group evoked at -70 mV*. (**E**) Changes in T-LA synapses are driven by postsynaptic changes in AMPAR. Cocaine-cue memory manipulations significantly affect AMPAR (Left: $F_{(4,34)}$ = 12.70, P < .001) and NMDAR current (Middle: $F_{(4,34)}$ = 5.77, P = .001) but do not alter PPR (Right: $F_{(4,40)}$ = 1.81, P > .05); all one-way ANOVA; post hoc analysis: *p < .05, **p < .01. *Inset: Sample average EPSC traces evoked at Erev -70 mV (AMPA, PPR) and Erev +40 mV (NMDA)*. Error bars equal mean ±SEM, n in italics, *number of rats (number of neurons)*. All scale bars: 50 ms, 200 pA

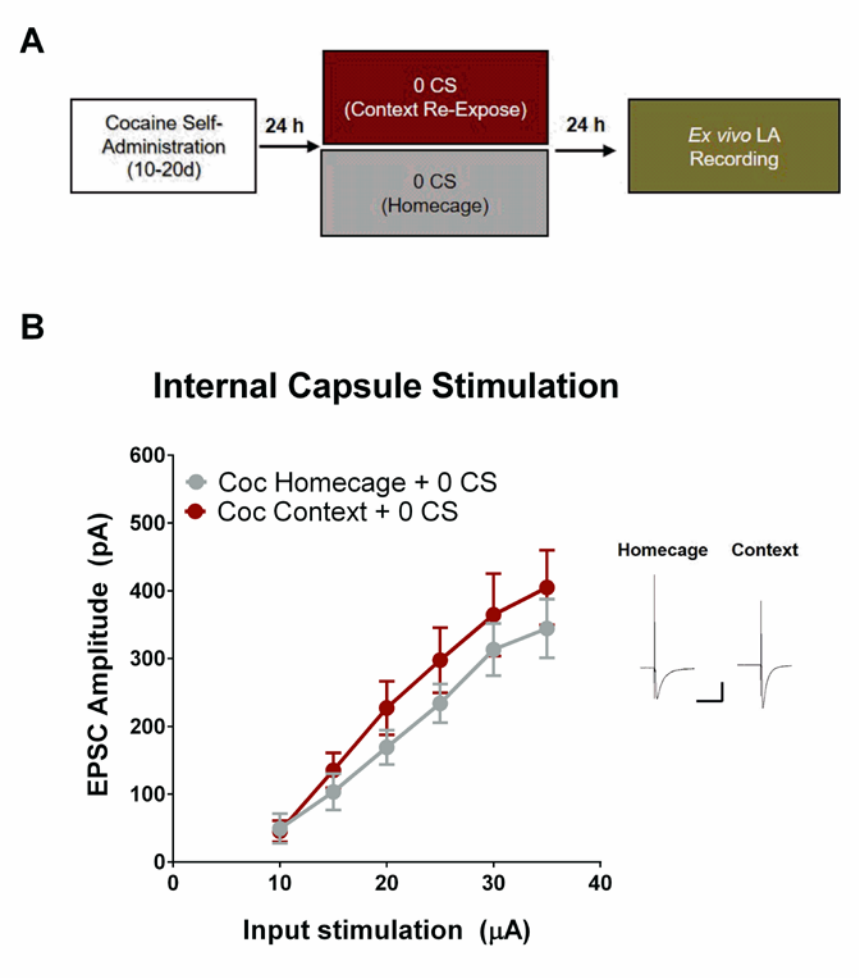

Figure 10. Potentiation of thalamo-amygdala synapses following drug-cue learning is not context-dependent. (**A**) Experimental timeline. Rats self-administered cocaine for 10-20 d. 24 h following the last day of acquisition, one group of rats was returned to the operant chamber, but received 0 CS presentations, while a second group of rats remained undisturbed in their home cage. The following day, rats were euthanized and slices were prepared for electrophysiological recordings. T-LA EPSCs were elicited by stimulating fibers from the internal capsule. (**B**) No difference in EPSC amplitude between the two groups. Average EPSC amplitude for the two groups at various stimulation intensities. Context re-exposed animals show slightly higher, yet nonsignificant EPSC amplitude than home cage controls (Two-way ANOVA, $F_{(1,12)} = 0.84$, $P > .05$; n in italics, *number of rats (number of neurons)*). Scale bars: 50 ms, 200 pA.

As described above, previous studies have found that extinction of a drug-seeking action is associated with synaptic changes throughout the brain's reward circuitry (Kalivas et al., 2005; Park et al., 2002). To determine whether T-LA synapses would be differentially regulated by whether or not the instrumental extinction (IE) phase of the experiment was included, a separate group of rats underwent cocaine SA followed by 1-7 days of IE. During IE, animals had the opportunity to make unreinforced responses (no cocaine infusion, no CS presentation) on both the active and inactive levers. During the first day of IE rats demonstrate a high tendency to press the active lever (**Figure 11B**), likely in anticipation of a cocaine infusion. However, rats quickly adapt to the new association, resulting in extinction of the lever pressing response. Interestingly, IE alone was insufficient to depotentiate T-LA synapses (**Figure 9D**). Cocaine-trained rats that underwent IE followed by 0 CS re-exposure had EPSC amplitudes similar to rats that did not undergo IE. Similarly, rats that underwent IE plus 120 CS re-exposure exhibited depotentiated T-LA synapses, as did the group that underwent 120 CS re-exposure in the absence of IE. These differences are not due to pre-existing group differences, as rats were again grouped to ensure no differences in SA or IE behavior (**Figure 11**). Together, these data demonstrate that T-LA synapses are regulated explicitly by the strength of *drug-cue* associations and not by memories of the drug-taking action.

We next tested whether changes in the T-LA synaptic strength may be due to postsynaptic changes in glutamatergic receptors, by examining whether AMPAR and NMDAR currents changed as a result of cocaine SA and subsequent cue re-exposure. We determined that the observed synaptic changes were driven by changes in AMPAR, not NMDAR (**Figure 9E**). AMPAR current was enhanced by cocaine SA, and maintained by brief cue re-exposure;

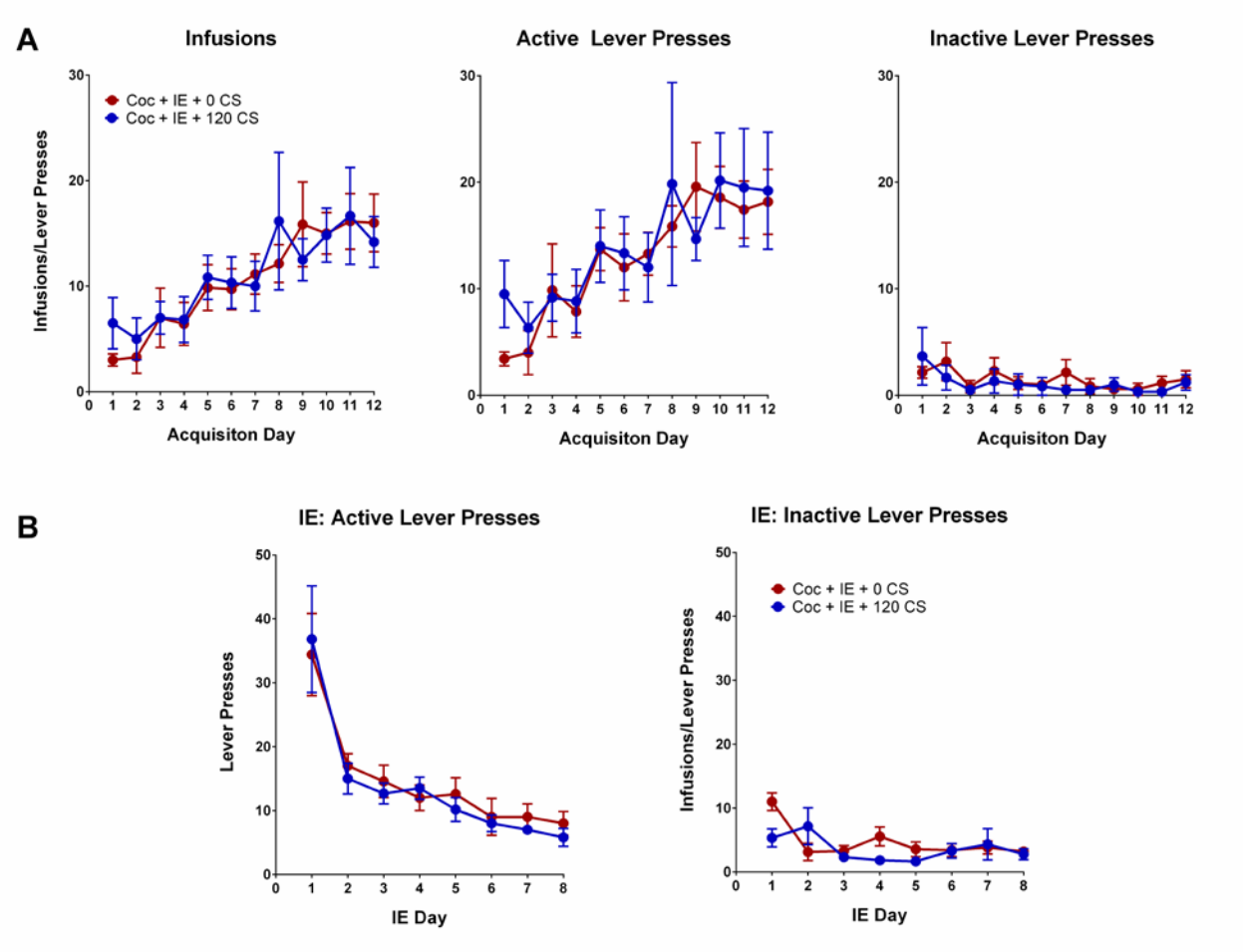

Figure 11. No group differences in acquisition of self-administration or instrumental extinction.

(**A**) Comparison of acquisition data for rats that underwent instrumental extinction (IE) prior to drug-cue re-exposure sessions. Following IE, rats received either no cue re-exposure (0 CS) or extended cue extinction (120 CS). During SA, there were no differences in infusions earned (Left: $F_{(1,11)} = 0.02$, $P > .05$), active lever presses (Middle: $F_{(1,11)} = 0.10$, $P > .05$), or inactive lever presses (Right: $F_{(1,11)} = 0.45$, $P > .05$) between the two groups (all two-way ANOVA; n in italics, *number of rats*). (**B**) Comparison of IE data. There were no differences in active lever presses (Middle: $F_{(1,11)} = 0.07$, $P > .05$), or inactive lever presses (Right: $F_{(1,11)} = 1.99$, $P > .05$) between the two groups (all two-way ANOVA).

however, it was decreased by cue extinction. Interestingly, changes in NMDAR may be a contributing factor to the potential memory-strengthening effects of brief cue reactivation, as 3-CS re-exposed animals had significantly higher AMPAR and NMDAR current relative to saline-trained controls and 120-CS re-exposed animals (**Figure 9E**). To rule out a presynaptic mechanism, such as changes in neurotransmitter release probability, we also compared paired pulse ratio (PPR), and found no significant differences between groups (**Figure 9E**). Together, these data suggest that during cocaine-cue memory formation, T-LA synapses are potentiated due to increased AMPAR transmission, which may promote drug-seeking behavior. Sufficient cue re-exposure in the absence of drug reinforcement reverses these changes, leading to an extinction of the drug-cue memory, and a decreased ability of the CS to promote drug-seeking.

Finally, we sought to determine if cortico-amygdala (C-LA) synapses also encode the strength of drug-cue associations. To test this, we performed similar electrophysiological recordings from LA principal neurons, while stimulating EC afferents (**Figure 12A**). In contrast to T-LA synapses, the EPSC amplitude input-output relationship was not affected at C-LA synapses by cocaine SA or CS re-exposure (**Figure 12B**). Also, unlike T-LA synapses, AMPAR EPSC amplitude at C-LA synapses did not correlate with the number of CS-US pairings received during training. (**Figure 12C**). These results suggest that cortical afferents to the amygdala are not correlated with cocaine-cue learning. Nevertheless, instrumental extinction depotentiated AMPAR EPSCs at C-LA synapses, independent of cue extinction (**Figure 12D**). EPSC amplitude at C-LA synapses was significantly decreased after IE both in rats that received no cue re-exposure (Coc + 0 CS) and those that received extended cue re-exposure (Coc + 120 CS). Therefore, cortical input to the amygdala appears to be regulated not by *drug*-cue associations, but by memories of the drug-taking action.

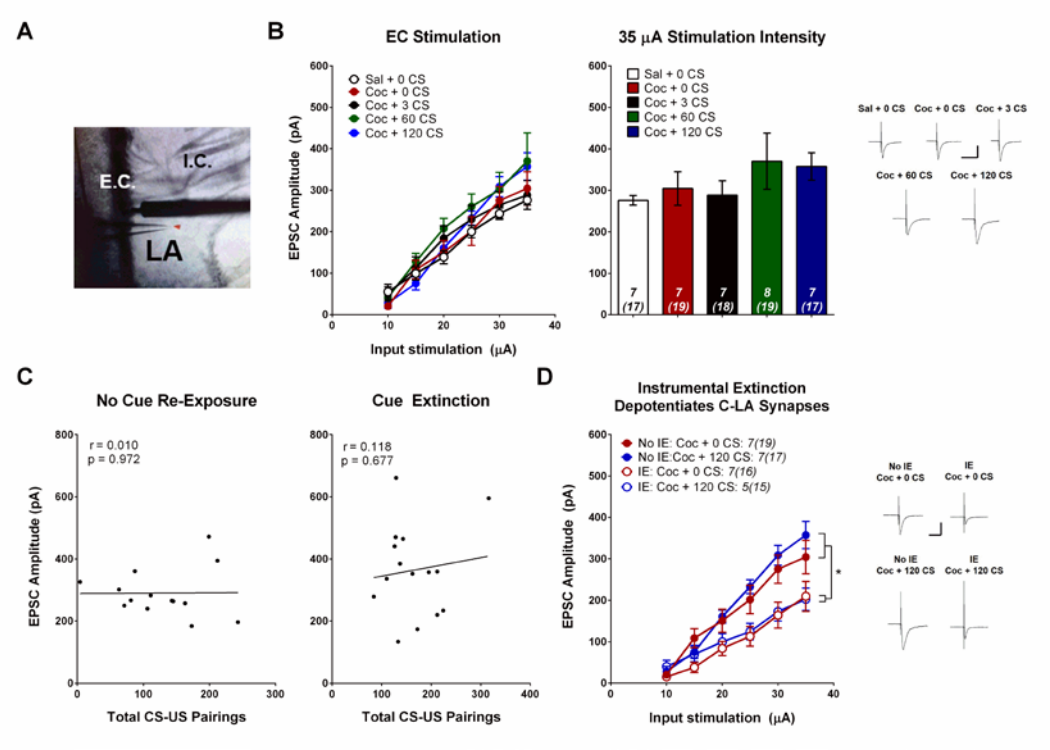

Figure 12. Cortico-amygdala synapses are altered by instrumental extinction, but not by drug-cue memory manipulation.

(**A**) Image of LA coronal section, demonstrating placement of stimulating electrode above EC fibers and position of patch pipette. EPSCs were evoked from LA principal neurons by stimulating the EC (putative C-LA synapses). IC, internal capsule; EC, external capsule. All scale bars: 50 ms, 200 pA. (**B**) Cocaine SA and cue-memory manipulations have no effect on C-LA synaptic strength. Left, Average EPSC amplitude for each group at various stimulation intensities. Right, Average EPSC amplitude at the highest stimulation intensity, 35 μA. Two-way ANOVA, ($F_{(4,31)}$ = 0.854, *P* > .05). *Inset: Sample average EPSC traces evoked at -70 mV*. (**C**) EPSC amplitude (35 μA stim intensity) was not correlated with the number of CS-US pairings received during acquisition for cocaine- and saline-SA animals that did not receive cue re-exposure (Left; r(12) = 0.010, p = .972, n = 14) or for those that received cue extinction (Right; r(13) = 0.118, p = .677, n = 15). (**D**) C-LA synapses are depotentiated by IE. EPSC amplitude was significantly lower for all rats that received IE, independent of cue re-exposure. Two-way ANOVA, main effect of group ($F_{(3,22)}$ = 5.11, *P* = .008) and a stim intensity x group interaction ($F_{(15,110)}$ = 3.69, *P* < .001); post hoc analysis: **p < .01, ***p < .001. *Inset: Sample average EPSC traces evoked at -70 mV*. Error bars equal mean ±SEM, n in italics, *number of rats (number of neurons)*. All scale bars: 50 ms, 200 pA

2.3.2 MGN-LA synapses are altered by cocaine-cue associations

Projections from multiple thalamic nuclei innervate the amygdala (LeDoux et al., 1990; Nabavi et al., 2014; Vertes et al., 2015), and studies involving auditory fear conditioning suggest that connections between the MGN and LA are particularly important for mediating auditory cue-specific memories. To test whether projections from the MGN to the LA are strengthened by cocaine-cue associations, we expressed a variant of channelrhodopsin in MGN neurons (AAV5.hSyn.oChIEF.tdTomato) (Lin et al., 2013; Nabavi et al., 2014). Approximately 2 weeks after viral infusions, rats underwent a similar procedure as described above (**Figure 13A**), where they were trained to SA cocaine or saline, followed by either 0 or 120 CS re-exposures. 24 h after cue re-exposure sessions, *ex vivo* slices of the amygdala were prepared and light-evoked (473-nm) EPSCs were recorded from LA principal neurons (**Figure 13B,C)**. Optogenetic stimulation of MGN terminals within the LA revealed increased EPSC amplitude in cocaine-trained non-CS re-exposed animals relative to saline-trained controls, whereas 120 CS re-exposure resulted in a reversal of this potentiation **(Figure 13C)**. Again, the changes in synaptic strength appear to be primarily mediated by postsynaptic changes in AMPAR. Compared to cocaine-trained non-CS re-exposed animals, those that underwent extended cue re-exposure had significantly reduced AMPAR current, but there were no group differences for either NMDAR current or PPR (**Figure 13D**). These results suggest that drug-cue associations are mediated by dynamic changes in AMPAR at MGN-LA synapses.

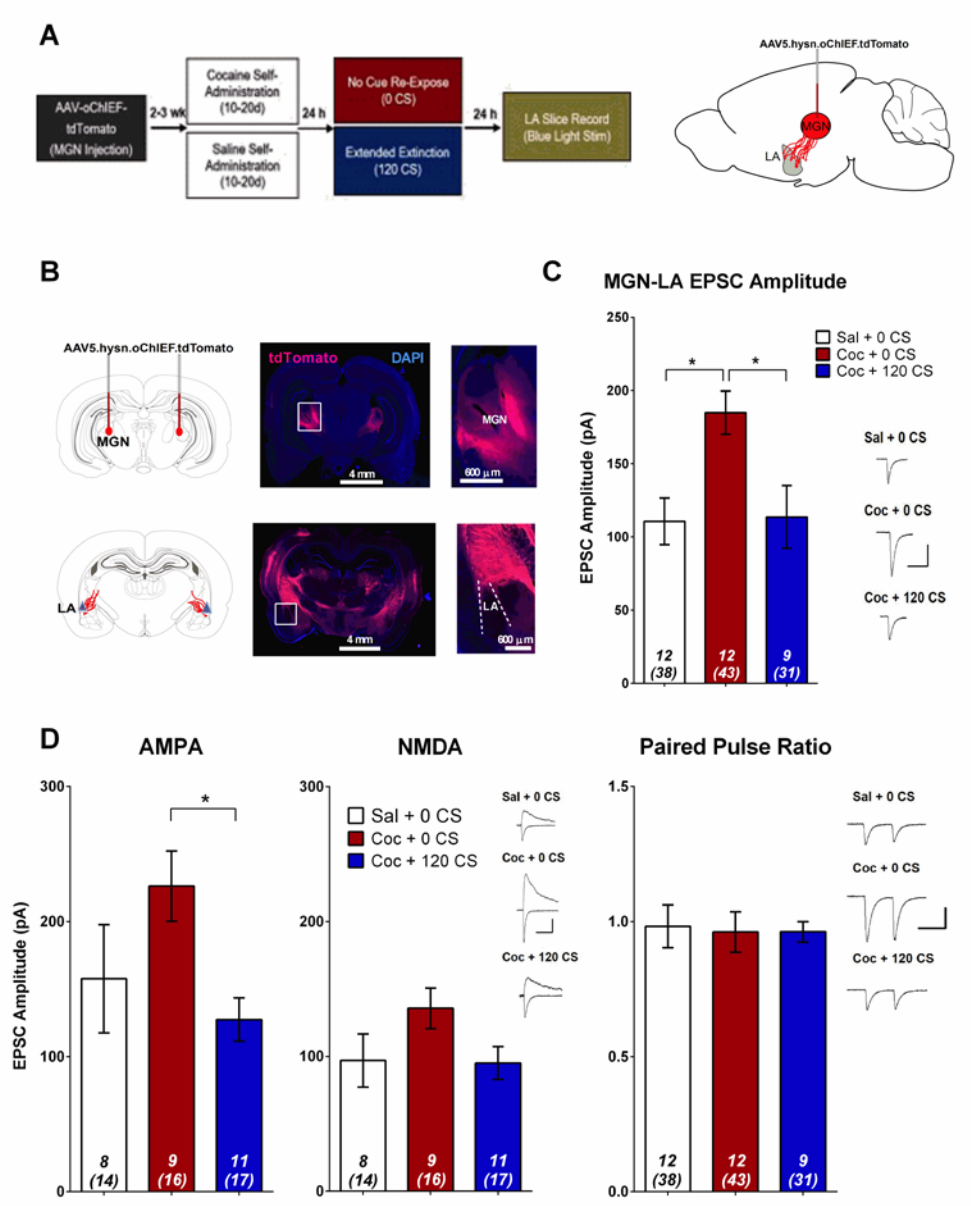

Figure 13. MGN-LA synapses regulate cocaine-cue associations.

(**A**) Diagram and schematic demonstrating the experimental timeline and injection of oChIEF-expressing AAV5 targeting the MGN. Scale bars in traces: 50 ms, 100 pA. (**B**) Diagram and images demonstrating the position in the MGN at which AAV-oChIEF was stereotaxically injected (Top). Injection of virus resulted in labeling of MGN axon terminals that synapses at the LA (Bottom). (**C**) EPSCs were optically-evoked from LA principal neurons by stimulating AAV-infected MGN axon terminals with 0.1 ms pulses of blue light (473-nm). Cocaine-trained animals that were not re-exposed to cues had significantly higher EPSC amplitude relative to saline-trained controls and

cocaine-trained 120-CS re-exposed animals. One-way ANOVA, ($F_{(2,25)}$ = 6.87, *P* = .004); post hoc analysis: *p < .05, one-way ANOVA). *Inset: Sample average EPSC traces evoked at -70 mV*. (**D**) Plasticity at MGN-LA synapse is due to postsynaptic changes in AMPAR and NMDAR, but not due to presynaptic changes. Cocaine-cue memory manipulations significantly affect AMPAR current (Left: $F_{(2,25)}$ = 3.72, *P* = .039) but not NMDAR current (Middle: $F_{(2,25)}$ = 2.22, *P* > .05) or PPR (Right: $F_{(2,30)}$ = 0.03, *P* > .05); all one-way ANOVA; post hoc analysis: *p < .05. *Inset: Sample average EPSC traces evoked at Erev -70 mV (AMPA, PPR) and Erev +40 mV (NMDA)*. Error bars equal mean ±SEM, n in italics, *number of rats (number of neurons)*. Scale bars: 50 ms, 200 pA.

2.3.3 *In vivo* optogenetic induction of LTD at MGN-LA synapses attenuates relapse-like behavior

We next determined if we could mimic extinction of drug-cue memories via inducing circuit-specific LTD at MGN-LA synapses. To do this, we first demonstrated the capacity to optically induce LTD at MGN-LA synapses. Rats were injected with AAV-oChiEF into the MGN and were trained to SA cocaine or saline, followed by either 0 or 120 CS re-exposure. 24 h later, *ex vivo* electrophysiological recordings were performed. In current-clamp configuration, LA-projecting MGN afferents were stimulated with blue light to optically-evoke excitatory postsynaptic potentials (EPSPs). Following LTD induction (1 Hz, 15 min), a sustained suppression of EPSP slope and amplitude was reliably observed in cocaine-trained 0 CS re-exposed animals (**Figure 14A,B, and 15A**). However, LTD was not observed in saline-trained animals (**Figure 14A,B**), nor cocaine-trained 120 CS re-exposed animals (**Figure 14A,B**), presumably because of an occlusion effect. These results suggest not only that you can induce

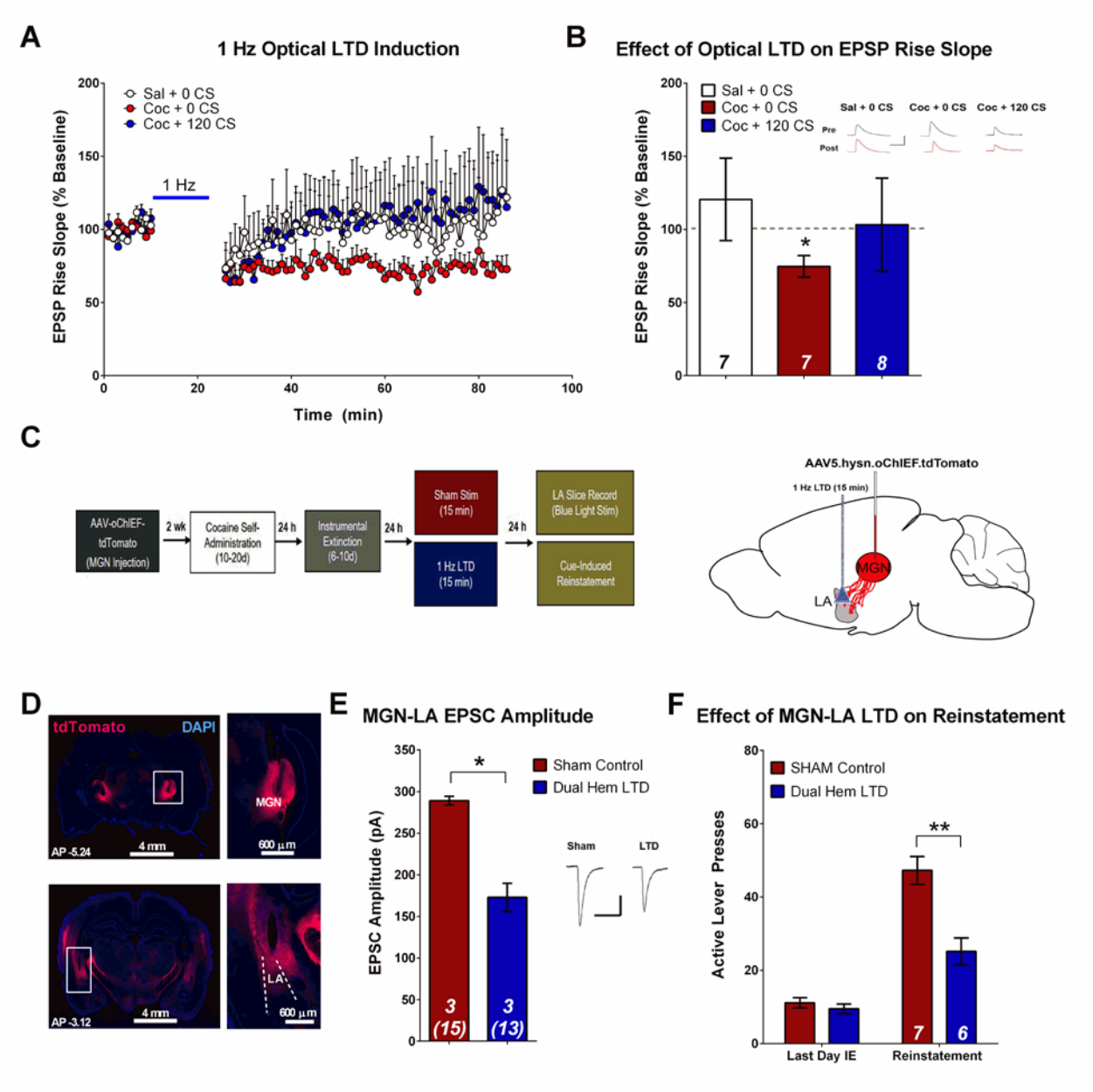

Figure 14. ***In vivo*** **optical LTD of MGN-LA circuit inhibits cue-induced reinstatement.**

(**A**) Scatter plots demonstrating effect of *ex vivo* optical LTD induction on EPSP rise slope at MGN-LA terminals. 15 min. of 1 Hz optical stimulation induced a sustained reduction in EPSP rise slope only in cocaine-trained non-CS re-exposed animals. (**B**) Quantification of A. Bar graph demonstrating effect of 1 Hz stimulation on EPSP rise slope as a percent change from baseline. LTD induction significantly decreased EPSP rise slope relative to baseline only in cocaine-trained non-CS re-exposed animals (Paired t-test, $t_{(6)} = 3.34$, $^*P = .016$, n in bars, number of neurons.

Inset: Sample average EPSP traces across groups during baseline (Pre, black) and 1 hour after LTD induction (Post, red). Scale bars: 100 ms, 10 mV. (**C**) Experimental timeline and schematic for testing the effect of *in vivo* LTD of MGN-LA synapses on cue-induced reinstatement. oChIEF-expressing AAV5 was injected into the MGN and optical fibers were implanted at the dorsal tip of the LA. (**D**) Diagram and images demonstrating the position of virus injection in the MGN (Top) and placement of optic fibers in the LA (Bottom). (**E**) *In vivo* dual hemisphere LTD of MGN-LA synapses attenuates EPSC amplitude relative to SHAM-controls (Unpaired t-test, $t_{(4)} = 6.60$, $^{**}P = .003$). *Inset: Sample average EPSC traces across groups.* Scale bars: 50 ms, 100 pA. (**F**) *In vivo* dual hemisphere LTD of MGN-LA synapses attenuates reinstatement. There were no differences in active lever pressing between groups during the last day of IE. All groups show pronounced reinstatement to drug-associated cues, but there is a significant reduction in active lever presses during reinstatement in rats that previously underwent optical LTD relative to SHAM controls. Two-way ANOVA, main effect of group ($F_{(1,11)} = 18.16$, $P = .001$) and a day x group interaction ($F_{(1,11)} = 12.76$, $P = .004$); post hoc analysis: $^{**}p < .01$.

LTD in cocaine-trained rats, but that an LTD-like process is induced at MGN-LA synapses by cue-memory extinction.

Finally, we tested if *in vivo* optogenetic LTD induction was sufficient to block cue-induced cocaine-seeking. Rats were again injected with the AAV-oChIEF targeting the MGN and optic fibers were implanted targeting the dorsal portion of the LA (**Figure 14C,D**). Following SA and IE, rather than undergoing cue re-exposure, rats received *in vivo* low frequency optical LTD (1 Hz, 15 min 473-nm light stim) of MGN-LA terminals. A second group of rats was treated similarly, but did not receive laser stimulation (SHAM-controls). 24 h later, either *ex vivo* optical recordings or cue-induced reinstatement was performed. Animals that were exposed to optical LTD had significantly reduced MGN-LA EPSC amplitude relative to SHAM-

controls (**Figure 14E**), confirming induction of LTD by our stimulation protocol. Furthermore, LTD-exposed rats also made significantly fewer lever responses during reinstatement compared to SHAM-controls (**Figure 14F**). Rats subsequently underwent an additional reinstatement test 7 days later, and LTD-exposed rats maintained a low level of responding that was lower than SHAM-controls (**Figure 15B**). These data show that induction of LTD at MGN-LA synapses is sufficient to reduce drug-seeking behavior, in a manner similar to drug-cue extinction, and that this reduction can persist across multiple reinstatement tests. Together, these results indicate that drug-cue memories may be malleable to circuit-specific optogenetic manipulations, presenting the possibility for novel therapeutic approaches.

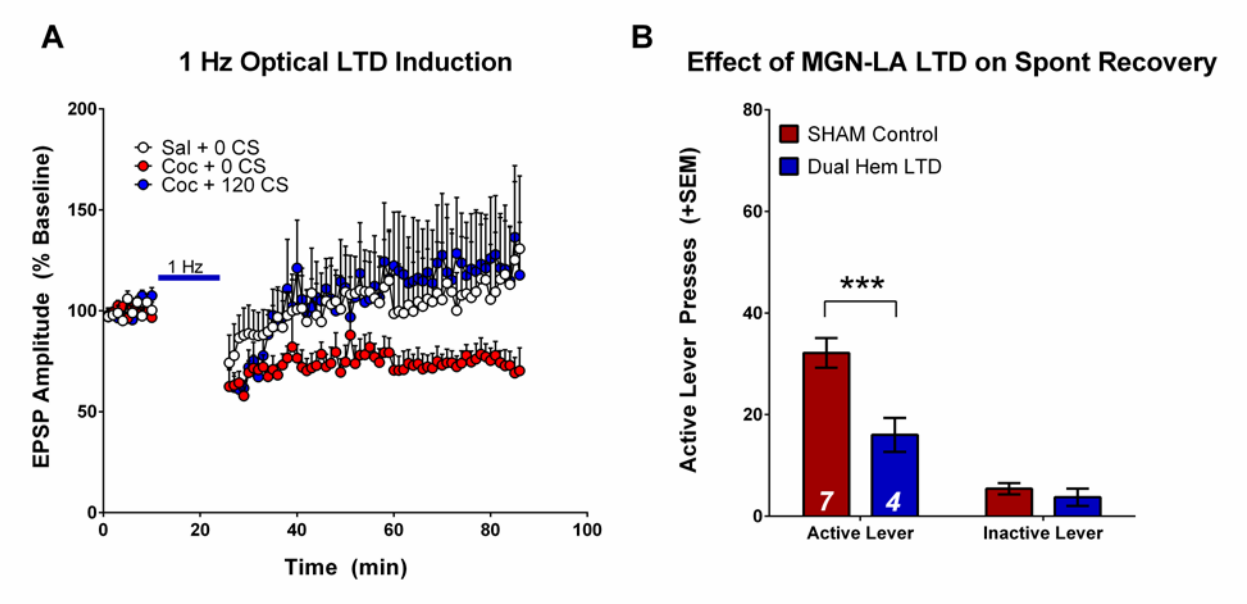

Figure 15. Further characterization and behavioral effects of MGN-LA optical LTD.

(**A**) Scatter plots demonstrating effect of *ex vivo* optical LTD induction on EPSP peak amplitude at MGN-LA terminals. 15 min. of 1 Hz blue light stimulation induced a sustained reduction in EPSP amplitude only in cocaine-trained non-CS re-exposed animals, with no effect on saline-trained or cocaine-trained 120-CS re-exposed animals; n in bars, number of neurons. (**B**) *In vivo* dual hemisphere LTD of MGN-LA synapses effects spontaneous recovery. 7 days after initial cue-induced reinstatement, rats underwent a second reinstatement test, revealing a significant reduction in active lever pressing in animals that previously underwent MGN-LA LTD relative to SHAM controls. Two-way ANOVA, main effect of group ($F_{(1,18)}$ = 12.43, P = .002), significant interaction ($F_{(1,18)}$ = 7.03, P = .010); post hoc analysis, ***p < .001; n in bars, number of rats.

2.4 DISCUSSION

We have previously shown that re-exposure to cocaine-associated discrete cues in the absence of cocaine reinforcement can attenuate cue-elicited drug-seeking (Rich et al., 2016). In this study, we extend our findings and demonstrate that sensory input from the thalamus, but not cortex, is critical for regulating cue-driven drug-seeking behaviors. Specifically, T-LA, but not C-LA synapses are potentiated by the repeated pairing of cocaine with a discrete audiovisual cue (**Figures 9B and 12B**). Importantly, this drug-cue association is long-lasting, as animals that underwent >7 days of instrumental extinction, still showed T-LA synaptic potentiation, which promotes reinstatement to the cue (**Figures 7C and 9D)**. Conversely, re-exposure to the cue in the absence of drug reinforcement dose-dependently depotentiates T-LA synapses, thereby inhibiting relapse-like behavior (**Figure 7C and 9B**). Furthermore, *in vivo* optogenetic induction of LTD in the MGN-LA pathway of cocaine-trained animals was sufficient to prevent reinstatement (**Figure 14F**). While optical LTD of this pathway has previously been demonstrated to inhibit a fear-associated memory (Nabavi et al., 2014), our work provides the first direct evidence of circuit-specific neuroadaptations associated with drug-cue-specific memories that promote craving and relapse.

In our study, rats that underwent brief cue re-exposure (3 CS) still exhibited the drug-cue conditioning-induced potentiation of T-LA synapses and remained vulnerable to cue-induced reinstatement (**Figures 7C and 9B**). Brief cue re-exposure has been proposed to reactivate the drug-cue memory, and initiate reconsolidation, during which time the memory is vulnerable to disruption via pharmacological manipulation (Arguello et al., 2014; Rich et al., 2016; Sanchez et al., 2010). Reconsolidation is thought to strengthen or at least maintain memory, and our study

reveals a slight, yet nonsignificant increase in T-LA EPSC amplitude relative to cocaine-trained non-CS re-exposed animals. The lack of a statistical significance does not rule out a memory-strengthening effect of brief cue re-exposure. For example, the destabilization and restabilization of memory that occurs after reactivation is likely to involve postsynaptic signaling, which may explain the significant effect at NMDAR (**Figure 9E**). It is important to note, however, that our recordings occur 24 hours after reactivation, a timepoint at which the reactivated memory is thought to be restabilized. Other circuits within the brain are also impacted by reconsolidation (Hafenbreidel et al., 2017; Liang et al., 2017; Liu et al., 2017), and depending on the way memory is distributed throughout the brain, there may be a cumulative effect that leads to persistence of the drug-cue memory. Future experiments should examine a timepoint within the reconsolidation window (<6 hours after reactivation) to determine if there is evidence of destabilization, which would indicate that reconsolidation does indeed occur following brief cue reactivation. Furthermore, it would be useful for future experiments to examine the effect of cue re-exposure on saline self-administering rats. We would predict that because these animals do not form a strong association between saline and the conditioned stimuli, that there would be no differences between cue re-exposed and non-re-exposed animals.

The importance of experience-driven synaptic plasticity within specific amygdala circuits has been well documented for the regulation of other types of associative learning. For example, auditory fear conditioning potentiates both the T-LA and C-LA pathway, resulting in increased freezing in the presence of the auditory cue. Cue extinction training reverses the synaptic changes and blocks the expression of fear (Hong et al., 2009; Kim et al., 2007a). The importance of the amygdala during reward-based learning has also been demonstrated (Fuchs et al., 2006; Robbins et al., 2008), but the specific afferent inputs involved have not been well examined,

especially in the context of drugs of abuse. Tye and colleagues showed that sucrose-reward learning rapidly strengthens T-LA synapses, but enhancement of C-LA synapses required elevated dopamine, which was achieved by blocking the dopamine transporter (DAT) (Tye et al., 2008, 2010). In our study, despite the DAT-blocking effects of Coc, we did not see training-induced C-LA potentiation; however, procedural differences including timing of *ex vivo* recordings and absence of sucrose reward may explain this seeming discrepancy. Here, we show that T-LA synapse are indeed strengthened by the formation of a drug-cue association, but that these alterations are reversible, depending on the amount of unreinforced cue re-exposure (**Figure 9B**). Moderate cue re-exposure (60 CS) slightly reduced, while extended cue re-exposure (120 CS) fully reversed the cocaine-cue-associated potentiation. Recent evidence supports dose-dependent synaptic effects of cue re-exposure. The extinction of a fear-associated memory has been shown to consist of two separate phases, each with distinct mechanisms (An et al., 2017). Early fear extinction (following a single extinction session; 20 CS presentations) promotes the inhibition of the original fear memory through mechanisms including enhanced inhibitory tone in LA neurons, enhanced CS-evoked activity in the mPFC, and enhanced synaptic efficacy of amygdala intercalated neurons (Amano et al., 2010; Chhatwal, 2005; Lin et al., 2009; Milad and Quirk, 2002). Late fear extinction (following multiple extinction sessions; 60 CS presentations) involves erasure of the fear memory, which consists of depotentation of T-LA synapses. Thus, our results are consistent with the data from these multiple fear extinction session studies. However, we do find that this large number of CS presentations (120) is necessary to observe consistent reductions in the reinstatement of drug seeking, which is different from the fear conditioning literature.

The lack of group effects on C-LA synapses (**Figure 12B**) may speak to the importance of bottom-up signaling during reward-motivated behaviors that is less dependent on cognitive influence. Previous studies have demonstrated the influence of top-down circuits in extinction, however, these studies primarily involve extinction of the drug-taking action (Augur et al., 2016; Stefanik et al., 2016). Interestingly, we *did* observe synaptic depotentiation of C-LA synapses in response to instrumental extinction (**Figure 12D**), suggesting a role for this circuit in learning to inhibit the drug-taking action when the drug is unavailable. These findings do not rule out any cortical influence on drug-cue memory extinction, as the C-LA pathway mostly relays input from auditory, (Te2/Te3), visual, and perirhinal cortex (Mcdonald, 1998), while other cortical areas project via separate pathways. For example, mPFC afferents synapse on LA interneurons, which then locally inhibit LA principal neurons (An et al., 2017). However, in our study we only recorded from principal neurons, and the inclusion of picrotoxin in the bath solution blocks any contribution from this potentially important inhibitory circuit.

Our study identifies postsynaptic glutamatergic signaling as a mechanism responsible for regulating drug-cue memory (**Figures 9E and 13D**). Future studies should continue to expand upon the mechanisms and circuits involved. Calcium signaling and intracellular kinases and phosphatases are important for cue-associated memories (Merlo et al., 2014; Rich et al., 2016), and these downstream signaling mechanisms are likely involved in synaptic modifications following drug-cue re-exposure; for example, the internalization of AMPAR, which is necessary for the extinction of acquired fear (Dalton et al., 2008). Additionally, mGluR1 and mGluR2 receptors in the amygdala are critical for depotentiation and the extinction of fear-associated memories (Hong et al., 2009; Kim et al., 2007b). Although our study focuses on auditory thalamic inputs to the LA, the mediodorsal and paraventricular nuclei of the thalamus also

project to the mPFC, nucleus accumbens, and the amygdala (Do-Monte et al., 2017; Vertes et al., 2015). These circuits have been implicated in fear conditioning (Penzo et al., 2015), incubation of drug seeking (Li et al., 2015; Lu et al., 2005), and the regulation of reward seeking when an anticipated reward is omitted (Do-Monte et al., 2017), and therefore could also influence drug-cue memory. Finally, because drugs of abuse act on the brain's reward system, the role of dopaminergic inputs from the VTA in modulating LA afferent input should also be examined. Activation of DA receptors was previously shown to enhance Te3-evoked responses in the LA (Rosenkranz and Grace, 2001). Similarly, elevated dopamine during cue-sucrose learning was sufficient to enhance cortico-amygdala plasticity when measured 30 minutes after training (Tye et al., 2010), so dopamine likely influences plasticity during reconsolidation and extinction of a drug-cue memory as well.

The use of optogenetics in this study helps overcome the inherent limitations of *ex* vivo recordings that involve electrical stimulation. While our electrical stimulation experiments are informative, stimulation of the internal capsule in this manner, may cause unintentional activation of neurons that do not project via the internal capsule. Optical stimulation of LA-projecting MGN neurons yields a higher degree of specificity. However, there remains several important controls that should be tested in future experiments. In order to show that MGN neurons are specifically involved in the regulation of drug-cue memory, other nuclei from the thalamus, such as the medial dorsal thalamus, which also projects to subregions of amygdala, should also be examined. It would also be beneficial to include a control group of animals that is optically stimulated at a frequency that would not be predicted to induce LTD. This is a stronger control than the SHAM group because these animals would still be exposed to light stimulation. Finally, another alternative control is a group of animals that is injected with a control virus

targeted to the MGN that is unresponsive to blue light stimulation. These animals could then receive the same 1 Hz LTD induction protocol, but since they do not express channelrhodopsin, there should be no effect of stimulation.

One limitation of cue extinction as a therapy is that drug-seeking often spontaneously returns after a period of abstinence (Peters et al., 2008; Rescorla, 2004). Interestingly, in our study, extended cue extinction was sufficient to reduce relapse-like behavior, even in animals that had extensive drug-cue experience (**Figure 9C**), suggesting that long-term cue re-exposure in the absence of drug reinforcement may be effective at preventing relapse even in chronic drug abusers. Optical induction of LTD in the MGN-LA pathway, which is occluded by prior cue extinction, results in similar decreases in reinstatement (**Figure 14**), suggesting that cue extinction occurs via a persistent depotentiation of T-LA synapses. Furthermore, this reduction was maintained across multiple reinstatement tests, suggesting decreased spontaneous recovery (**Figure 15B**). Together, these results support the idea that circuit-specific neuroadaptations can support the long-term inhibition or erasure of a drug-cue memory and offer an important consideration for future treatments.

3.0 CALCINEURIN MODULATES NEUROPLASTIC CHANGES IN THE AMYGDALA TO SUPPORT THE INHIBITION OF DRUG-ASSOCIATED MEMORIES

Interfering with memory reconsolidation or inducing memory extinction are two therapeutic approaches for weakening maladaptive memories in disorders such as PTSD and addiction. Both extinction and reconsolidation are regulated by various intracellular protein kinases and phosphatases, and interfering with these signaling molecules can alter memory strength. The calcium dependent protein phosphatase, calcineurin, is one such molecule that has been implicated in both the consolidation and extinction of fear memories. However, the role of calcineurin in regulating cocaine-cue associative memories has not been investigated. Previous studies have indicated that the lateral amygdala (LA) is a critical locus for cocaine-cue memory reconsolidation and extinction. Furthermore, in Chapter 2, we demonstrated that thalamo-amygdala (T-LA), but not cortico-amygdala (C-LA) synapses are involved in the regulation of cocaine-cue memories. We therefore tested the effects of LA administration of an activator of calcineurin, chlorogenic acid (CGA), on both behavioral and electrophysiological indices of cocaine cue memory extinction and reconsolidation. Rats were trained to self-administer cocaine paired with an audiovisual cue. The cue memory was then either briefly reactivated, extinguished, or not manipulated followed immediately by LA infusion of CGA. Rats were tested 24 hrs later for cue-induced reinstatement, or LA slices were prepared for

electrophysiological recordings. We found that CGA infusions in the LA following cue extinction or reconsolidation caused a significant reduction in both EPSC amplitude at thalamic inputs to the LA and cue-induced reinstatement relative to vehicle-infused controls, suggesting that calcineurin can affect drug-cue memory reconsolidation and extinction by altering T-LA synaptic strength. Therefore, calcineurin may represent a novel target for disrupting cocaine-associated memories to reduce relapse.

3.1 INTRODUCTION

Addiction is characterized by a progressive switch from occasional, casual drug use to a more frequent, habitual pattern of use (Everitt and Robbins, 2005). With repeated drug use, originally neutral environmental contexts and cues become conditioned by the drug and develop emotional significance based on the drug-using experience (Fuchs et al., 2009; Kalivas, 2009). Re-exposure to the cues, even in the absence of the drug itself, can activate memories of prior drug use that initiate feelings of craving and promote relapse (Parvaz et al., 2016). Reducing the strength of drug-associated memories may therefore help establish long-term abstinence. Memory weakening can be accomplished in one of two manners: either interfering with drug-cue memory reconsolidation or by promoting drug-cue memory extinction (Bossert et al., 2013; Torregrossa et al., 2011). Reconsolidation is the process of restabilization of a memory after retrieval. The reconsolidation process involves a cascade of intracellular signaling events and requires protein synthesis. Thus, during this period of lability, the administration of certain pharmacological agents can prevent memory re-stabilization, thereby weakening or even erasing the memory

(Tronson and Taylor, 2007). While reconsolidation is triggered by brief re-exposure to conditioned stimuli, the extinction process involves the repeated presentation of a conditioned stimulus in the absence of the expected outcome (e.g., foot shock or drug reinforcement), which leads to the formation of a new association that the cue is no longer predictive of the outcome. Thus, if drug-related cues are repeatedly presented in the absence of drug reinforcement, this extinction memory can inhibit craving and relapse upon subsequent cue re-exposure. (Holmes and Quirk, 2010; Nader et al., 2000; Nic Dhonnchadha et al., 2010).

The lateral amygdala (LA) has been established as a locus for the encoding and storage of emotionally-salient memories. The LA receives afferent input from sensory thalamic and cortical sources, as well as inputs from the ventral tegmental area (VTA) and medial prefrontal cortex (mPFC), and therefore functions to integrate information during cue-associated learning (Janak and Tye, 2015; Maren, 2016). The LA is activated during both fear- and drug-associated conditioning and during subsequent re-exposure to fear- and drug-related cues (Ciccocioppo et al., 2001; Neisewander et al., 2000; Schafe et al., 2001). Recent work has begun to highlight specific synaptic changes within the LA during the reconsolidation and extinction of cue-associated memories. Auditory thalamic and cortical synapses (T-LA and C-LA, respectively) are potentiated during fear conditioning, and depotentiated following cue extinction (Hong et al., 2009; Kim et al., 2007a). However, during reward-associated learning a different pattern emerges where T-LA, but not C-LA synapses are preferentially involved (Tye et al., 2008; Rich et al., unpublished; See Chapter 2).

Extinction and reconsolidation both involve similar neural mechanisms, and may be simultaneously activated during cue re-exposure, so timing and selection of the appropriate molecular target for pharmacological manipulation are important considerations. Previous

attempts to enhance cue extinction training have inadvertently promoted reconsolidation, leading to a strengthened drug-cue memory (Hofmann et al., 2012; Price et al., 2013). Due to these unintentional effects, it is necessary to identify agents that can bidirectionally regulate drug-cue memory. We recently found that inhibition of CaMKII in the amygdala can indeed bidirectionally interfere with a drug-associated memory (Rich et al., 2016). Intra-amygdala infusion of CaMKII inhibitors immediately after either drug-cue memory reactivation or extinction caused a reduction in cue-induced reinstatement relative to vehicle-infused controls. In this study, we investigate the involvement of calcineurin (CaN), a calcium-dependent phosphatase in drug-cue memory extinction and reconsolidation. CaN is considered a negative regulator of CaMKII, and so it is possible that upregulating CaN activity would have the same effects on drug-cue memory and relapse as inhibiting CaMKII activity. Inhibition of CaN in the hippocampus was previously shown to enhance reconsolidation of a contextual fear memory (de la Fuente et al., 2014). Additionally, the shift from maintenance to inhibition of an auditory fear memory by cue re-exposure is correlated with increased protein levels and enzymatic activity of CaN in the amygdala (Lin et al., 2003a, 2003b; Merlo et al., 2014), suggesting that increasing CaN activity at the time of memory retrieval may have amnestic effects. The goal of the present study was to determine if calcineurin activation would both interfere with the reconsolidation of, and enhance the extinction of, a memory associated with self-administered cocaine. Based on the prior physiological evidence that T-LA synapses are strengthened by cue-dependent learning and weakened by cue extinction (Kim et al., 2007a; Tye et al., 2008) we also investigated whether upregulation of CaN in the LA would alter T-LA synaptic plasticity. Our physiological and behavioral results demonstrate a causal role for CaN activity in the inhibition of drug-cue

memory reconsolidation and the activation of memory extinction. Therefore, CaN represents a potential therapeutic target for pharmaco-behavioral relapse prevention therapies.

3.2 METHODS

3.2.1 Subjects

Naïve, adult male Sprague-Dawley rats (Envigo/Harlan), weighing 275-325 g on arrival, were used for all experiments. Rats were housed in a temperature- and humidity-controlled room, in auto-ventilated racks with an automated watering system. Animals were housed in pairs, given *ad libitum* access to food and water, and maintained on a 12/12 light-dark cycle. Prior to surgical procedures, rats were given at least 5 days to acclimate to the facility. Rats were food-deprived 24 h prior to the start of behavioral experiments and maintained at ~90% of their free-feeding body weight (~20 g of chow per day) for the duration of testing. All behavioral experiments were run during the light-cycle. In addition, all procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

3.2.2 Self-administration test chambers

Rats were trained to self-administer cocaine in standard operant conditioning chambers (MedAssociates), described previously (Rich et al., 2016). Experiments were counterbalanced across one of two chamber designs. The inclusion of two chambers allows the reconsolidation

group of rats to undergo reactivation in a novel context. The first type of chamber consisted of bar floors and the second type of chamber had grid floors. All chambers contained two retractable levers on one wall of the chamber, a tone-generator, stimulus-light above each lever, house light, and infusion pump. Operant boxes were kept in sound-attenuating chambers equipped with a fan for background noise.

3.2.3 Drugs

Cocaine hydrochloride (generously provided by the National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in sterile 0.9% saline (2 mg/ml) and filter-sterilized for self-administration. Chlorogenic acid (CGA, Fisher Scientific) was dissolved in 1X PBS to a concentation of 200 ng/µl. CaN inhibitor, FK506 (Sigma) was dissolved in DMSO to a concentration of 10 µg/µl.

3.2.4 Surgical procedures

Surgeries were performed as previously described (Rich et al., 2016). Briefly, rats were anesthetized with ketamine hydrochloride (87.5 mg/kg; i.m.; Henry Schein) and xylazine hydrochloride (5 mg/kg; i.m.; Henry Schein) and then received an analgesic (Rimadyl, 5 mg/kg; s.c.; Henry Schein) and 5 ml of Lactated Ringer's (s.c.). Betadine and 70% ethanol were applied to all incision sites. All rats were implanted with a chronic indwelling intravenous (i.v.) catheter (CamCaths) into the right jugular vein that was fed subcutaneously to the midscapular region, where they exited through a round incision. For experiments involving intracranial infusions, rats

were immediately placed into a stereotaxic instrument (Stoelting) and implanted with bilateral stainless-steel guide cannulae (22 gauge; Plastics One) targeting the LA (AP –3.0 mm, ML ±5.1 mm, DV –7.9 mm, relative to bregma; Paxinos and Watson, 1998). For experiments involving intracranial infusions prior to electrophysiological recordings, guide cannulae were implanted more dorsal (DV: -7.2 mm) to prevent damage to axon fibers from the internal capsule which pass through the lateral amygdala. Guide cannulae were secured to the skull with 3 miniature screws and dental acrylic resin. Rats were then placed on a heating pad for recovery. After surgery, rats were individually housed, and given at least 7 days to recover before the start of behavioral training. Carprofen (5 mg/kg; s.c.) was administered for the first two days after surgery. Catheters were kept patent by daily infusions of sterile saline containing gentamicin (5 mg/ml) and heparin (30 USP/ml).

3.2.5 Self-administration procedures

Rats administered saline or cocaine during daily sessions for 1 h, on a fixed ratio 1 (FR1) schedule of reinforcement with a 10 s timeout. The designated active lever (counterbalanced across left and right levers) produced a cocaine infusion paired with a 10 s tone-light compound cue. Pump durations were adjusted daily according to body weight in order to deliver the correct dose of drug (1.0 mg/kg/infusion). Responses on the inactive lever were recorded, but had no programmed consequences. Rats underwent training for at least 10 d and until they administered at least 8 infusions/day over 3 consecutive days. Rats that did not meet acquisition criteria by 20 d were excluded from the study. The program was controlled by and data were collected using MedPC (MedAssociates).

3.2.6 Instrumental lever extinction

For behavioral experiments only, after successful acquisition of self-administration, rats underwent instrumental lever extinction (IE) for at least 6 d. These sessions lasted for 1 h and continued until extinction criteria had been met (an average of < 25 lever presses on the last two days of extinction). Throughout IE, rats received no cocaine or cocaine-associated cue reinforcement. IE was conducted to reduce responding to a stable, low rate to later assess cue-induced reinstatement. In addition, IE reduces the motivational value of other cues in the self-administration context, such as the levers, so that subsequent testing specifically isolates the memory for the discrete cue associated with cocaine infusion.

3.2.7 Pavlovian cue re-exposure procedures

Cue re-exposure was conducted in the SA context. The session occurred 24 h after the final day of SA or, when included, IE, and lasted for 1 hr. During this session, rats received noncontingent presentations of the previously drug-paired cues: either 0 (no extinction), 3 (reactivation/reconsolidation), or 60 (extinction) times.

3.2.8 Intracranial infusions

Immediately following cue re-exposure sessions, CGA or vehicle was administered in a volume of 0.5 µl/hemisphere. CGA was given at a dose of 100 ng/hemisphere. CGA has not been commonly used for infusion experiments, but this concentration is higher than the dose used *in*

vitro to achieve maximal CaN activity (Tong et al., 2007). For FK506 experiments, rats received one of four infusion cocktails: Veh + Veh, CGA + Veh, Veh + FK506, or CGA + FK506. FK506 was given at a dose of 5 μg/side, which is a dose that has previously been administered via intra-hippocampal microinfusions to inhibit CaN activity and enhance reconsolidation of a contextual fear memory (de la Fuente et al., 2014). Infusions were given by removing dummy cannuale and inserting injection cannulae (28 gauge; Plastics One) that extended 1 mm beyond the guide cannulae. The injectors were connected to Hamilton syringes controlled by a syringe pump via polyethylene tubing. Infusions were given over the course of 2 min and injectors were left in the cannulae for an additional 1 min to allow for drug diffusion.

3.2.9 Cue-induced reinstatement

24 h after cue memory manipulations, cue-induced reinstatement was assessed during a 1 h session that took place in the SA context. A lever press on the active lever produced a 10 sec presentation of the cocaine-associated cue on an FR1 schedule, but presses were not reinforced with cocaine. Lever presses on the inactive lever were recorded but had no programmed consequences.

3.2.10 Prepartion of *ex vivo* amygdala slices

Slices were prepared as described previously (Dong et al., 2006; Huang et al., 2008a), with slight modifications, with methods designed to improve neuronal health in adult rodents (Ting et al., 2014). Briefly, 24 h after cue re-exposure sessions, rats were deeply anesthetized with isoflurane.

Rats were then briefly perfused with ice-cold cutting solution containing (in mM): 92 N-methyl-d-glucamine (NMDG), 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$, saturated with carbogen (95% O_2/5% CO_2), pH adjusted to 7.4 with HCl. Rats were then decapitated and brains removed. Acute coronal slices of the amygdala (250 μm thick) were obtained (normally 4–6 slices were obtained from each rat) using a VT1200S vibratome (Leica, Germany) in 4 °C cutting solution. Slices were placed in a holding chamber filled with the same cutting solution and incubated at 37°C for 10-15 min before being transferred to a beaker of HEPES-based holding solution containing (in mM): 86 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 35 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 1 $MgCl_2$, and 2 $CaCl_2$, saturated with carbogen. Slices were allowed to recover for >30 min at room temperature before experimentation.

3.2.11 *Ex vivo* electrophysiology

Whole-cell recordings were obtained from individual principal neurons in the dorsal LA using glass pipettes (3–5 MΩ). Voltage-clamp experiments used pipettes filled with a cesium-based internal solution [in mM, cesium methanesulfonate 108, CsCl 15, CsEGTA 0.4, TEA-Cl 5, HEPES 20, Mg-ATP 2.5, Na-GTP 0.25, QX-314-Cl 1, sodium phosphocreatine 7.5, and L-glutathione 1, at pH 7.3] and current-clamp experiments used pipettes filled with a potassium-based internal solution [in mM, potassium methanesulfonate 108, KCl 20, K-EGTA 0.4, HEPES 10, Mg-ATP 2.5, Na-GTP 0.25, sodium phosphocreatine 7.5, L-glutathione 1, $MgCl_2$ 2, at pH 7.3]. During recordings, slices were superfused with aCSF that was heated to 31–33 °C by

passing the solution through a feedback-controlled in-line heater (Warner, CT) before entering the chamber. External perfusion consisted of a modified artificial cerebrospinal fluid (ACSF), containing, in mM NaCl 119, KCl 2.5, NaHCO3 26, NaH2PO4 1.2, glucose 12.5, HEPES 5, MgSO4 1, CaCl2 2, saturated with 95% O2/5% CO2. Neurons were voltage-clamped at -70 mV. To stimulate putative thalamic afferents, a concentric bipolar stimulating electrode (FHC, Bowdoin, ME) was placed over axon fibers emerging from the internal capsule. Evoked excitatory postsynaptic currents (EPSCs) were recorded by stimulating LA projections using 0.1 ms pulses at a predetermined series of intensities (10-35 μA) from an isolated current stimulator (A-M instruments; Digitimer Ltd, Hertfordshire, England. For paired pulse recordings each pulse was separated by a 50 ms interpulse interval. AMPAR currents were elicited at E_{Rev}-70 mV holding potential and NMDAR currents were elicited at a E_{Rev}+40 mV holding potential. NMDAR amplitude was operationally defined as the amplitude of the current 35 ms after the peak of the AMPAR current; at this time point, AMPAR-mediated currents have subsided (Huang et al., 2008a). For all experiments, series resistance was 10–25 MΩ, uncompensated, and monitored continuously during recording. Cells with a change in series resistance beyond 20% were not accepted for data analysis. Synaptic currents were recorded with a MultiClamp 700B amplifier, filtered at 3 kHz, amplified 5 times, and then digitized at 20 kHz. Picrotoxin (100 μM) was included to inhibit $GABA_A$ receptor-mediated currents in all experiments.

3.2.12 Histological analysis

After the completion of behavioral experiments, rats with intracranial cannulae were sacrificed via decapitation. Brains were dissected and placed in 10% formalin for at least 3 d then

transferred to 30% sucrose for at least 3 d. Brains were then frozen and sectioned coronally through the BLA on a cryostat. Sections were taken at 50 µm and placed on slides for visualization of infusion placements (**Figure 16**). The investigator was blind to treatment group when analyzing histology, and animals with infusions outside of the BLA were removed from the main analysis.

3.2.13 Statistical analyses

All statistical analyses were performed using GraphPadPrism for Windows and results are expressed as mean ± SEM. Rats were distributed into groups based on a matching procedure that ensured that each group had no statistical differences in their cocaine infusions acquired over days, or differences in lever extinction behavior. For behavioral experiments, reinstatement tests were analyzed by two-way ANOVA with repeated measures, with the between-subjects factor being responding on the last day of lever extinction versus reinstatement responding and the within-subjects factor being CGA vs. Veh. For electrophysiological experiments, data were coded such that experimenters were not aware of treatment groups when performing data analysis, and then decoded for final results. Data were analyzed offline using ClampFit 10.3. For experiments in which the end points were from individual cells, such as EPSCs, we used the averaged value of a parameter from all cells recorded from an animal to represent the parameter of this animal. For electrical stimulation experiments EPSC amplitude was calculated at each stimulation intensity and compared between groups using two-way ANOVA with repeated measures. Paired pulse ratio (PPR) was calculated as the ratio of the peak current of the second EPSC to the peak current of the first EPSC. AMPA:NMDA ratio was calculated as the ratio of

peak current at -60mV to the current at +40 mV, 35 ms after stimulus. For, PPR, AMPA:NMDA ratio, AMPAR, and NMDAR current analyses, comparisons were made using unpaired t-tests. All data points are an average of 10 trials. Each experiment was replicated in at least 5-6 rats (1-5 cells were recorded from each rat) for electrophysiological analysis and at least 7 rats for behavioral tests. For all analyses, significant effects were further analyzed by Tukey's or Bonferroni's *post hoc* tests, with significance set at $P < 0.05$. All data were determined to be normally distributed using the Shapiro-Wilk test, and Bartlett's test was used to determine that there were no significant differences in the estimated variance between groups.

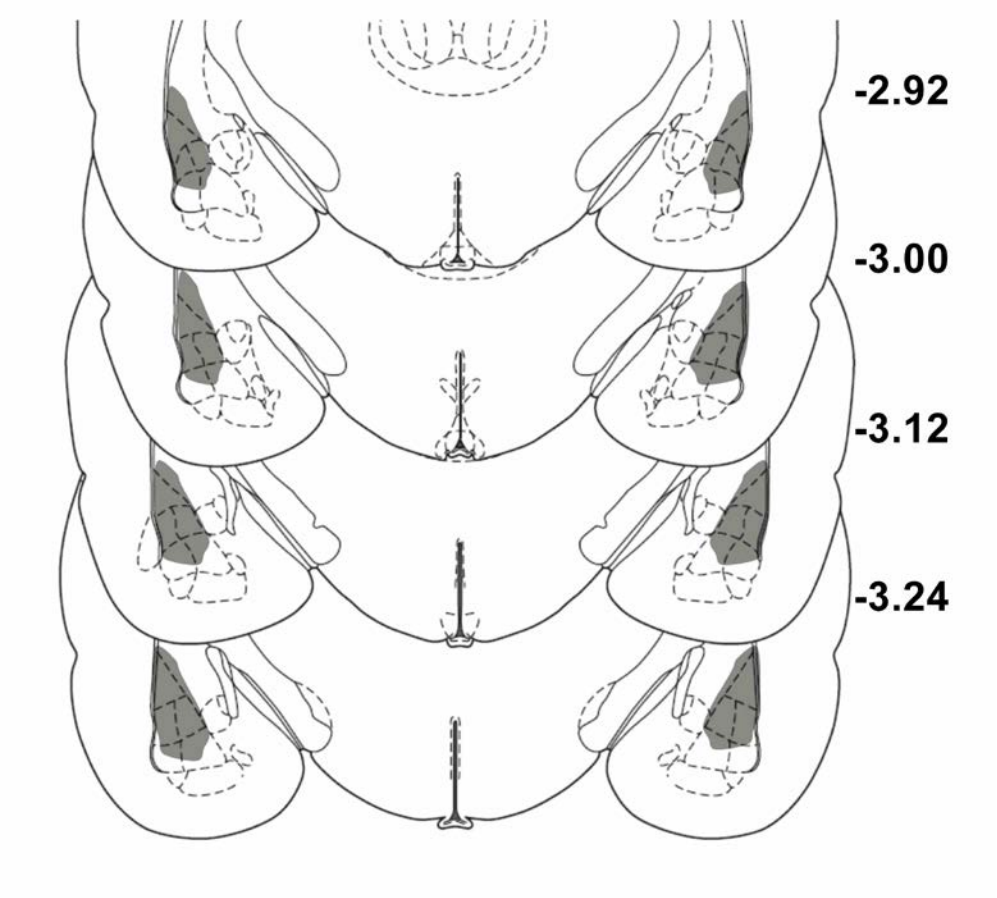

Figure 16. Histological verification of LA guide cannulae implants.

Schematic showing guide cannulae placements throughout the anterior-posterior extent of the LA. The shaded area represents region of acceptable cannulae placement. Rats that received cannulae implants outside of these regions were excluded from analysis. Coordinates are in mm, posterior from bregma.

3.3 RESULTS

To determine if CaN in the LA was involved in the regulation of cocaine-cue memory we used an approach that combined *in vivo* pharmacological activation of CaN with specific cue memory manipulations followed by behavioral and electrophysiological assessments. Rats were trained to self-administer (SA) cocaine on a fixed ratio (FR1) schedule of reinforcement, so that a single press on a predesignated active lever resulted in an intravenous cocaine infusion together with presentation of an audiovisual cue (conditioned stimulus, CS). Following SA, for reinstatement experiments, rats underwent instrumental lever extinction (IE) (**Figure 17A,B**). During IE, rats were allowed to perform operant responses, however, presses on the active lever were no longer reinforced by either cocaine or CS presentation. While this extinguishes the operant response, the cocaine-cue association remains, so that future presentations of the CS can promote reinstatement of the lever pressing response (Torregrossa and Taylor, 2013). For electrophysiology experiments, IE was omitted because our previous experiments show that thalamo-amgydala (T-LA) synaptic plasticity is not mediated by IE (Rich et al., unpublished; See Chapter 2). Rats were next assigned to one of three memory manipulation groups and exposed to passive presentations of CS: no reactivation (0 CS), cue reactivation (3 CS) or cue extinction (60 CS) (**Figure 17A,B**). Immediately following these sessions, rats received intra-LA microinfusions of the CaN activator, chlorogenic acid (CGA; 100 ng/hemisphere) (Tong et al., 2007), or vehicle (Veh) to determine what effect CaN activation has on cocaine-cue memory processes. Finally, 24 hours later, rats either received a cue-induced reinstatement test or *ex vivo*

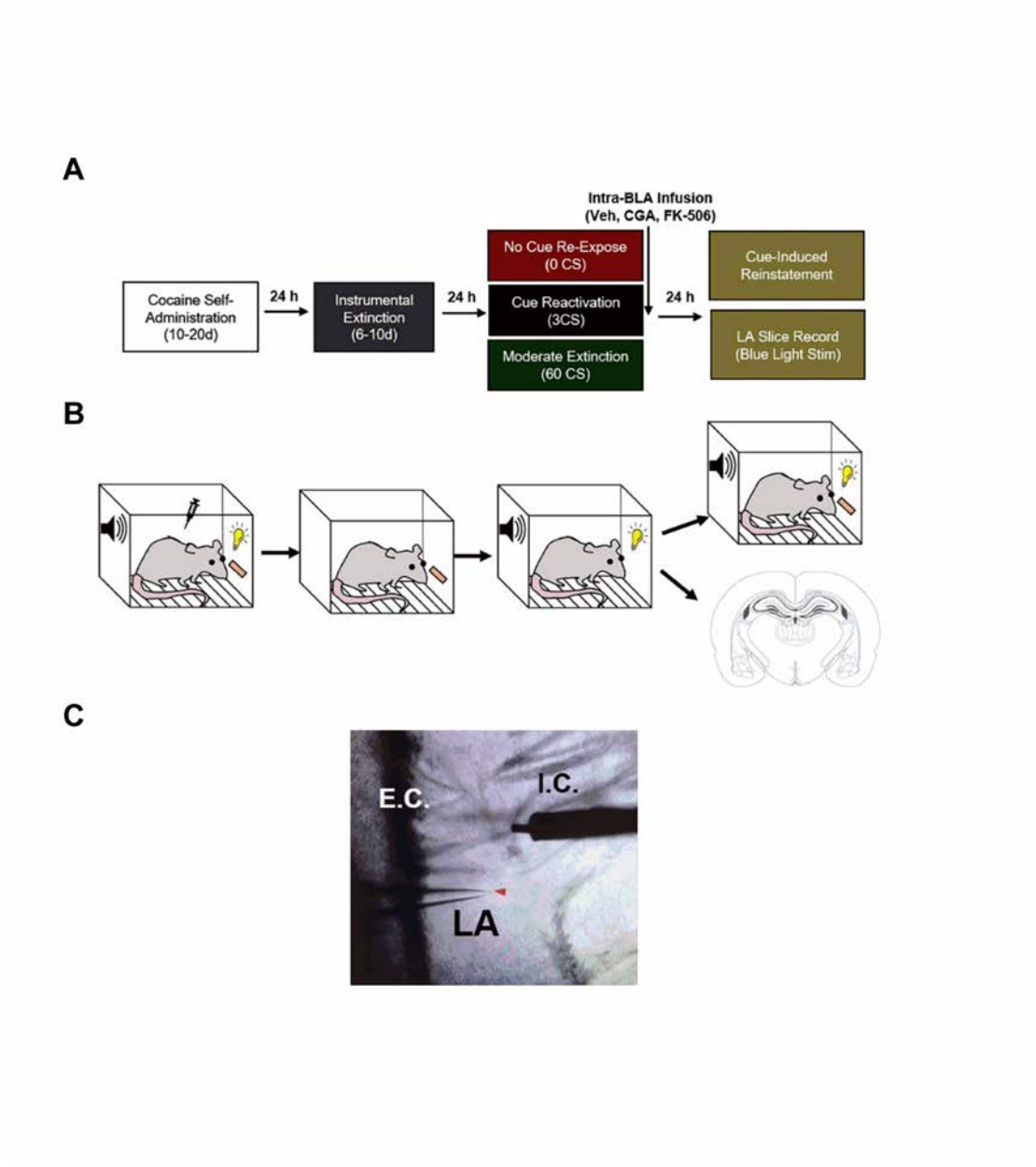

Figure 17. Experimental design.

Schematic demonstrating (**A**) timeline of each experimental phase and color-coded treatment groups (**B**) cartoon of operant chamber with visual depiction of each experimental stage, and (**C**) image of LA coronal section, with representative placement of stimulating electrode above IC fibers and position of patch pipette. EPSCs were evoked from LA principal neurons by stimulating the IC (putative T-LA synapses). SA, self-administration; IE, instrumental extinction; CS, conditioned stimuli; IC, internal capsule; EC, external capsule.

slices of the LA were prepared for electrophysiological recordings (**Figure 17A,B**). For reinstatement, rats returned to operant boxes and received response-contingent presentations of CS, but not cocaine. A high degree of lever pressing during reinstatement is suggestive of an intact drug-cue association, while low levels of lever pressing indicate that the drug-cue association has been disrupted. For electrophysiological recordings, whole-cell voltage clamp recordings were performed from LA principal neurons. EPSCs were evoked by stimulating internal capsule fibers to target T-LA synapses (**Figure 17C**). Using a predetermined series of stimulation intensities, we generated EPSC input-output relationships. We also compared measures of pre- and postsynaptic plasticity. This strategy enables a direct comparison between T-LA synaptic activity and cocaine-cue memory and allows for the assessment of the role of CaN-induced plasticity in mediating relapse-like behavior.

3.3.1 Effect of Intra-LA CaN Activation in the Absence of Drug-Cue Memory Retrieval

We first determined the effects of LA CaN activation in the absence of drug-cue memory retrieval (0 CS re-exposure). Previous studies have shown that amnestic agents are ineffective at inhibiting memories if they are not reactivated or retrieved; this prevents the memory from entering a destabilized, labile state, and so it cannot be interfered with pharmacologically (Rich et al., 2016; Tronson et al., 2006). Therefore, we predicted that CGA-induced activation of CaN would have no effect on reinstatement or T-LA synaptic plasticity in the absence of retrieval. For both experiments, rats were split into groups based on a random matching procedure that ensured no training differences between groups. There were no significant differences or interactions with day of training for active lever presses during SA or IE (**Figure 18A,B**; both two-way

ANOVA; $P > 0.05$). We found that treatment with CGA following the no-reactivation control procedure had no significant effect on active lever responding during cue-induced reinstatement, indicating that pharmacological manipulation of CaN signaling has no effect on cocaine seeking in the absence of cue memory retrieval (**Figure 18C**; two-way ANOVA: $P > 0.05$).

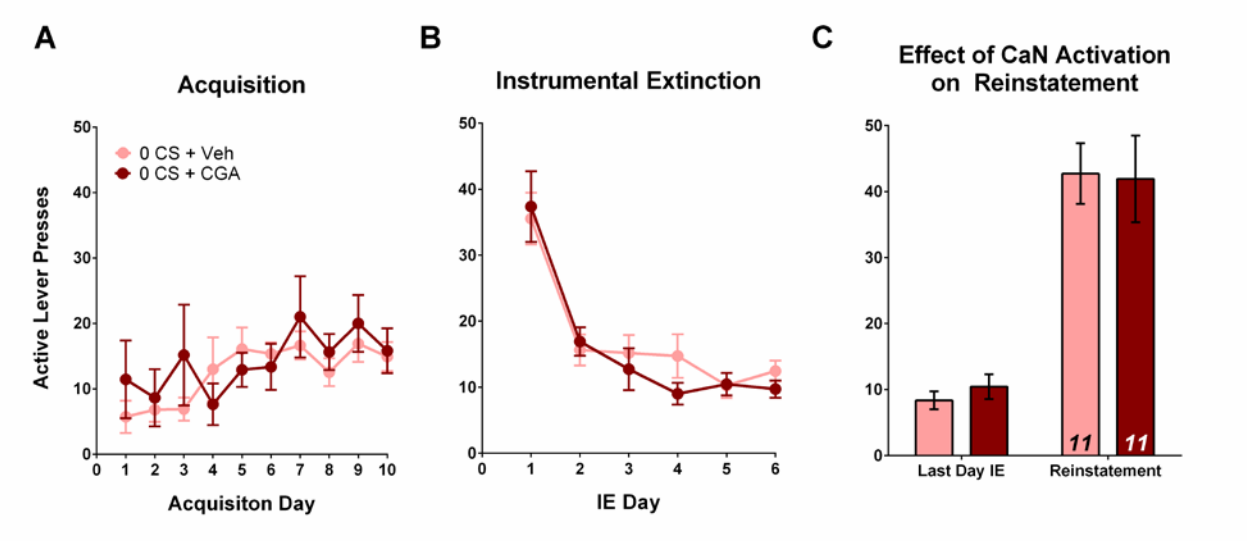

Figure 18. Calcineurin activation has no effect on cue-induced reinstatement in the absence of memory retrieval.

(**A,B**) Mean number of active lever presses per day during self-administration (SA) and instrumental extinction (IE). There were no significant differences in active lever presses during (**A**) SA (Two-way ANOVA, $F_{(1,20)} = 0.343$, $P > 0.05$) or (**B**) IE (Two-way ANOVA, $F_{(1,20)} = 0.286$, $P > 0.05$). Following IE, rats were placed back in training context and received a no-reactivation (0 CS) session followed immediately by infusion with either CGA or Veh. (**C**) Mean number of active lever presses during the last day of instrumental extinction compared to cue-induced reinstatement. CGA activation following 0 CS has no effect on reinstatement (two-way ANOVA, $F_{(1,20)} = 0.021$, $P > 0.05$). Data are expressed as mean ± SEM; n in bars, number of rats.

We next assessed if intra-LA infusion of CGA in the absence of memory retrieval would alter T-LA synaptic strength, and as expected, found no differences between CGA- and Veh-treated animals for any measure of synaptic plasticity. EPSC input-output relationships did not vary between groups (**Figure 19A**). At the highest stimulation intensity (35 μA), CGA- and Veh-groups had average EPSC amplitudes of 375 and 376 pA, respectively, which is consistent with our previous studies for animals in the no cue re-exposure condition (Rich et al., unpublished; See Chapter 2). Similarly, we saw no differences in PPR, AMPA:NMDA, AMPAR current, or NMDAR current (**Figure 19B-E**; all unpaired t-test, $P > 0.05$). Together, these data suggest that in the absence of memory retrieval, attempted activation of CaN by CGA produces no alterations in T-LA pre- or postsynaptic plasticity, corresponding to the lack of difference in cue-elicited drug-seeking.

3.3.2 Effect of Intra-LA CaN Activation during Drug-Cue Memory Reconsolidation

We next investigated whether activating CaN in the LA immediately following drug-cue memory reactivation (3 CS) would alter subsequent cue-induced drug-seeking. CaN activity promotes LTD-like synaptic modifications (Baumgartel and Mansuy, 2012; Mulkey et al., 1994); because reconsolidation is regulated by opposing LTP-like mechanisms, we hypothesized that activating CaN following cue reactivation would interfere with reconsolidation. The resulting disruption of the cocaine-cue association would suppress the conditioned response (lever pressing) to subsequent cue presentations, thereby attenuating reinstatement. Again, rats were split into groups so that there were no significant differences or interactions with day of training for active lever presses during SA or IE (**Figure 20A,B**; both two-way ANOVA; $P > 0.05$). We

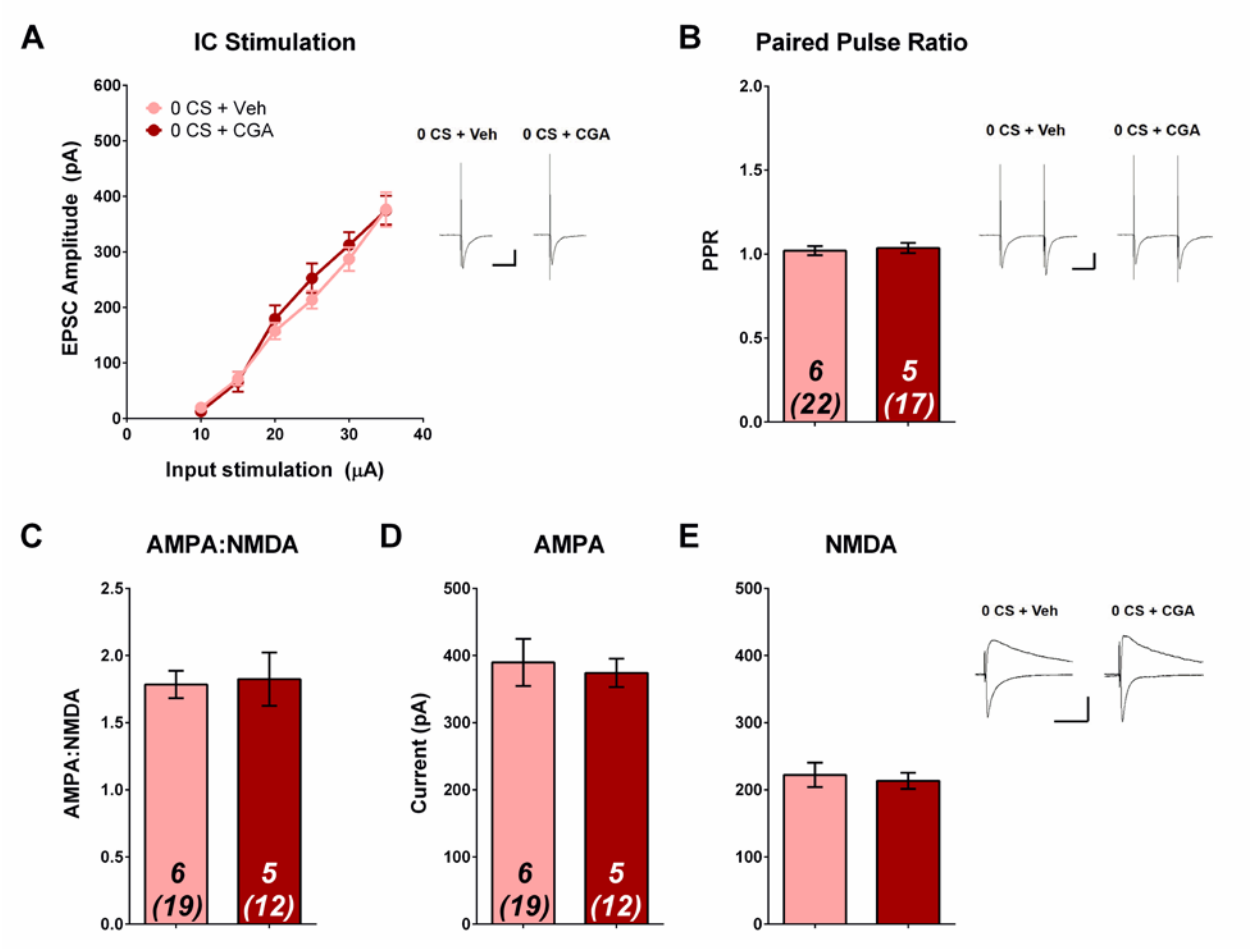

Figure 19. Calcineurin activation has no effect on T-LA plasticity in the absence of memory retrieval.

Following self-administration, rats received a no-reactivation (0 CS) session followed immediately by infusion with either CGA or Veh. 24 h later, *ex vivo* recordings were performed in the LA. (**A**) Input-output curve demonstrating average evoked EPSC amplitude across various stimulation intensities. No significant differences in EPSC amplitude between groups were found at any of the stimulation intensities (Two-way ANOVA, $F_{(1,9)} = 0.356$, $P > 0.05$). (**B**) No significant differences in PPR between groups (Unpaired t-test, $t_{(9)} = 0.387$, $P > 0.05$). (c-e) No significant differences between groups for (**C**) AMPA:NMDA (Unpaired t-test, $t_{(9)} = 0.188$, $P > 0.05$), (**D**) AMPAR current (Unpaired t-test, $t_{(9)} = 0.363$, $P > 0.05$), or (**E**) NMDAR current (Unpaired t-test, $t_{(9)} = 0.388$, $P > 0.05$). Data are expressed as mean ± SEM; n in bars, number of rats (number of neurons). *Insets: Sample average EPSC traces.* Scale bars: 50 ms, 200 pA.

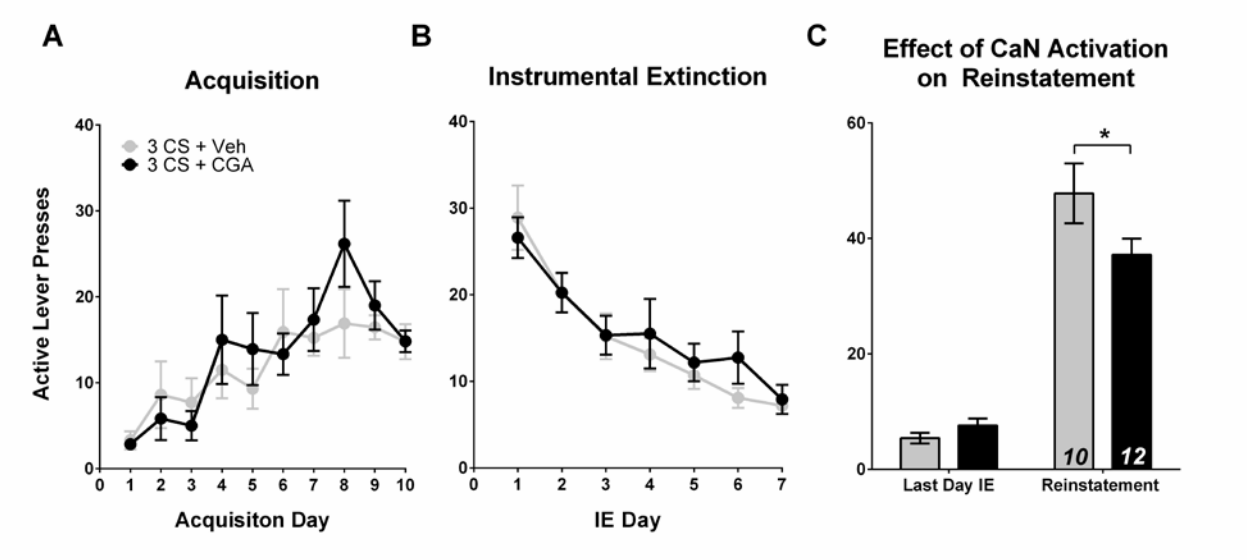

Figure 20. Calcineurin activation following cue reactivation inhibits reconsolidation and attenuates subsequent cue-induced reinstatement.

(**A,B**) Mean number of active lever presses per day during self-administration (SA) and instrumental extinction (IE). There were no significant differences in active lever presses during (**A**) SA (Two-way ANOVA, $F_{(1,20)} = 0.542$, $P > 0.05$) or (**B**) IE (Two-way ANOVA, $F_{(1,20)} = 0.152$, $P > 0.05$). Following IE, rats were placed back in training context and received a brief cue-reactivation (3 CS) session followed immediately by infusion with either CGA or Veh. (**C**) Mean number of active lever presses during the last day of instrumental extinction compared to cue-induced reinstatement. CGA activation following 0 CS reduces active lever responding during reinstatement. Two-way ANOVA, significant group x day interaction ($F_{(1,20)} = 4.79$, $P = 0.041$); post hoc analysis: *$p < 0.05$. Data are expressed as mean ± SEM; n in bars, number of rats

found that treatment with CGA following cue reactivation did indeed reduce active lever responding during cue-induced reinstatement, indicating that pharmacological manipulation of CaN signaling disrupts reconsolidation (**Figure 20C**; two-way ANOVA; significant group x day interaction; $F_{(1,20)} = 4.79$, $P = 0.041$).

We next determined if the suppression in cue-induced reinstatement was caused by CaN-dependent alterations at T-LA synapses. Unlike the non-reactivation condition, there were differences in measures of synaptic plasticity between CGA- and Veh-treated animals. First, we found that CGA-induced activation of CaN caused a reduction in the EPSC input-output relationship at T-LA synapses (**Figure 21A**; two-way ANOVA, significant group x stimulation intensity interaction, $F_{(5,60)} = 2.39$, $P = 0.048$, $P = 0.048$). At the highest stimulation intensity (35 μA), Veh-treated animals had an average EPSC amplitude of 497 pA, while CGA-treated animals had an average EPSC amplitude of 405 pA. These data suggest a synaptic basis for the disruption in reconsolidation produced by CGA that suppressed drug-seeking behavior. Next, we further examined measures of synaptic plasticity to determine if a pre- or postsynaptic mechanism could explain the reduced synaptic strength. We again saw no group differences in PPR (**Figure 21B**; unpaired t-test, $P > 0.05$). However, AMPA:NMDA was reduced by CGA infusion at a trend level (**Figure 21C**; unpaired t-test, $t_{(12)} = 2.16$, $P = 0.052$). Upon further examination, it became apparent that this reduction was driven by changes in AMPAR, as average AMPAR current was significantly reduced by CGA (**Figure 21E**; unpaired t-test, $t_{(12)} = 3.02$, $P = 0.011$) while there was no difference in NMDAR current (**Figure 21E**; unpaired t-test, $P > 0.05$). Together, these data suggest that following drug-cue memory reactivation, CGA infusion leads to postsynaptic reductions in AMPAR that interfere with reconsolidation.

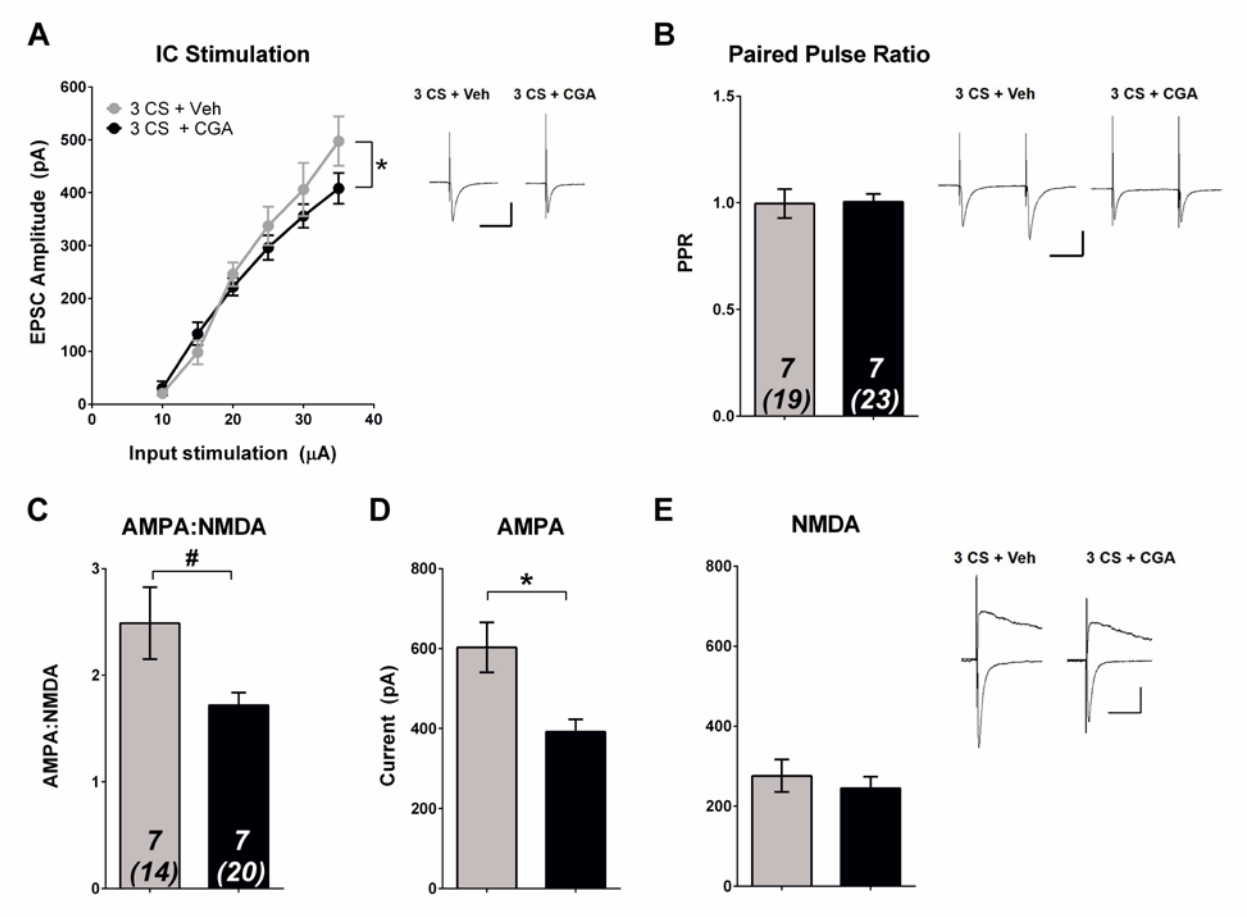

Figure 21. Calcineurin activation during reconsolidation alters synaptic plasticity through a postsynaptic reduction in AMPAR current.

Following self-administration, rats received a brief cue reactivation (3 CS) session followed immediately by infusion with either CGA or Veh. 24 h later, *ex vivo* recordings were performed in the LA. (**A**) Input-output curve demonstrating average evoked EPSC amplitude across various stimulation intensities. Intra-LA CaN activation by CGA alters EPSC amplitude. Two-way ANOVA, significant group x stim intensity interaction ($F_{(5,60)}$ = 2.39, *P* = 0.048). (**B**) No significant differences in PPR between groups (Unpaired t-test, $t_{(12)}$ = 0.111, *P* > 0.05). (**C**) CaN activation by CGA altered AMPA:NMDA at a trend level (Unpaired t-test, $t_{(12)}$ = 2.16, *P* = 0.052) (**D**) CaN activation by CGA significantly reduced AMPAR current (Unpaired t-test, $t_{(12)}$ = 3.02, *P* = 0.011), but had no effect on (**E**) NMDAR current (Unpaired t-test, $t_{(12)}$ = 0.623, *P* > 0.05). Data are expressed as mean ± SEM; n in bars, number of rats (number of neurons). *p < .05; #p < 0.10. *Insets: Sample average EPSC traces.* Scale bars: 50 ms, 200 pA.

3.3.3 Effect of Intra-LA CaN Activation during Drug-Cue Memory Extinction

Finally, we tested the effects of CGA-induced CaN activation following drug-cue memory extinction (60 CS). We hypothesized that we would observe CGA-induced enhancement of extinction. Specifically, we predicted that cue extinction is mediated by CaN activity, so an upregulation of CaN activity would further suppress the original drug-cue association, leading to an even more pronounced attenuation in active lever pressing during cue-induced reinstatement. Further, we predicted that these reductions would again be mediated by postsynaptic changes in AMPAR. As with the other experiments, rats were again split into groups so that there were no significant differences or interactions with day of training for active lever presses during SA or IE (**Figure 22A,B**; both two-way ANOVA; $P > 0.05$). As with reconsolidation, we found that CGA-induced CaN activation following cue extinction did indeed attenuate reinstatement relative to Veh-treated animals (**Figure 22C**; two-way ANOVA; $F_{(1,17)} = 6.89$, $P = 0.018$), suggesting that upregulating CaN activity enhances cue extinction. To rule out any nonspecific effects of CGA, we next performed an experiment in which we combined intra-LA CGA infusions with a known inhibitor of CaN, FK-506 (de la Fuente et al., 2014). Rats again received cue extinction, but this time received one of four infusions (Veh-Veh, CGA-Veh, Veh-FK-506, CGA-FK-506) after the session, and were tested for reinstatement the following day. Results revealed that active lever responding during reinstatement depended on the compound that was infused (**Figure 22D**; two-way ANOVA, main effect of group; $F_{(3,33)} = 3.04$, $P = 0.043$). CGA-infusion alone resulted in a reduction in reinstatement, replicating our findings from the from **Figure 22C**. Additionally, we found that the CGA-induced reductions in reinstatement were

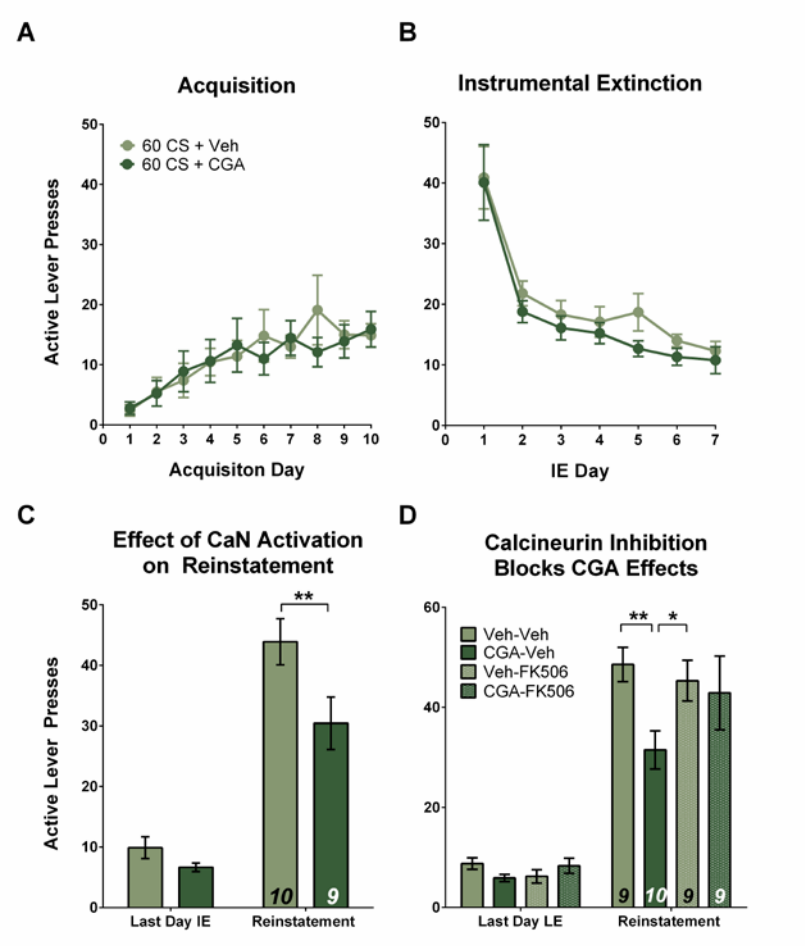

Figure 22. Calcineurin activation enhances cue extinction causing a reduction in subsequent cue-induced reinstatement.

(**A,B**) Mean number of active lever presses per day during self-administration (SA) and instrumental extinction (IE). There were no significant differences in active lever presses during (**A**) SA (Two-way ANOVA, $F_{(1,17)} = 0.179$, $P > 0.05$) or (**B**) IE (Two-way ANOVA, $F_{(1,17)} = 1.35$, $P > 0.05$). Following IE, rats were placed back in training context and received a cue-extinction (60 CS) session followed immediately by infusion with either CGA or Veh. (**C**) Mean number of active lever presses during the last day of instrumental extinction compared to cue-induced reinstatement. CGA activation following 0 CS reduces active lever responding during reinstatement. Two-way ANOVA, main effect of group ($F_{(1,17)} = 6.89$, $P = 0.018$) (**D**) Effect of CGA on extinction is blocked by co-infusion of CaN inhibitor, FK-506. Two-way ANOVA, main effect of group ($F_{(3,33)} = 3.04$, $P = 0.043$) post hoc analysis: *p < 0.05, **p < .01. Data are expressed as mean ± SEM; n in bars, number of rats.

blocked by the addition of FK-506, suggesting that CGA does indeed exert its effects through activation of CaN, and that this upregulated activity is blocked by FK-506.

We then determined if the CaN-dependent enhancements in drug-cue memory extinction were due to T-LA synaptic alterations. We found that CGA-induced activation of CaN during cue extinction caused a reduction in the EPSC input-output relationship at T-LA synapses (**Figure 23A**; two-way ANOVA, significant group x stimulation intensity interaction, $F_{(5,65)}$ = 2.64, P = 0.031, P = 0.031). Interestingly, at the highest stimulation intensity (35 μA), both CGA- and Veh-infused animals had noticeably lower EPSC amplitudes than the 0 CS or 3 CS experiments. Veh-treated animals had an average EPSC amplitude of 311 pA; however, CGA-treated animals were significantly lower than Veh-animals, with an average EPSC amplitude of just 239 pA. These data are consistent with previous results, in which 60 CS re-exposure reduces T-LA plasticity (Rich et al., unpublished, see Chapter 2). These data suggest a T-LA synaptic mechanism for both extincton learning and the enhancement in extinction by CGA that suppressed relapse-like behavior. Next, we further examined measures of synaptic plasticity to determine if a pre- or postsynaptic mechanisms could explain the reduced synaptic strength. Surprisingly, CGA-induced activation of CaN during cue extinction increased PPR (**Figure 23B**; unpaired t-test, $t_{(13)}$ = 2.75P = 0.031). Additionally, AMPA:NMDA was reduced by CGA infusion at a trend level (**Figure 23C**; unpaired t-test, $t_{(12)}$ = 1.99, P = 0.070), but further examination determined that this reduction was not driven by specific changes in AMPAR or NMDAR, as neither AMPAR current (**Figure 23D**; unpaired t-test, P > 0.05) nor NMDAR current (**Figure 23E**; unpaired t-test, P > 0.05) were significantly altered by CGA infusion. Together these data suggest that a presynaptic signaling mechanism (ie. change in glutamate release) is primarily responsible for CaN-induced reductions in synaptic strength that correlate

with enhanced drug-cue memory extinction. Additionally, there may be a combination of effects at AMPAR and NMDAR that together lead to slight changes at the postsynaptic membrane that contribute to reduced excitatory synaptic strength.

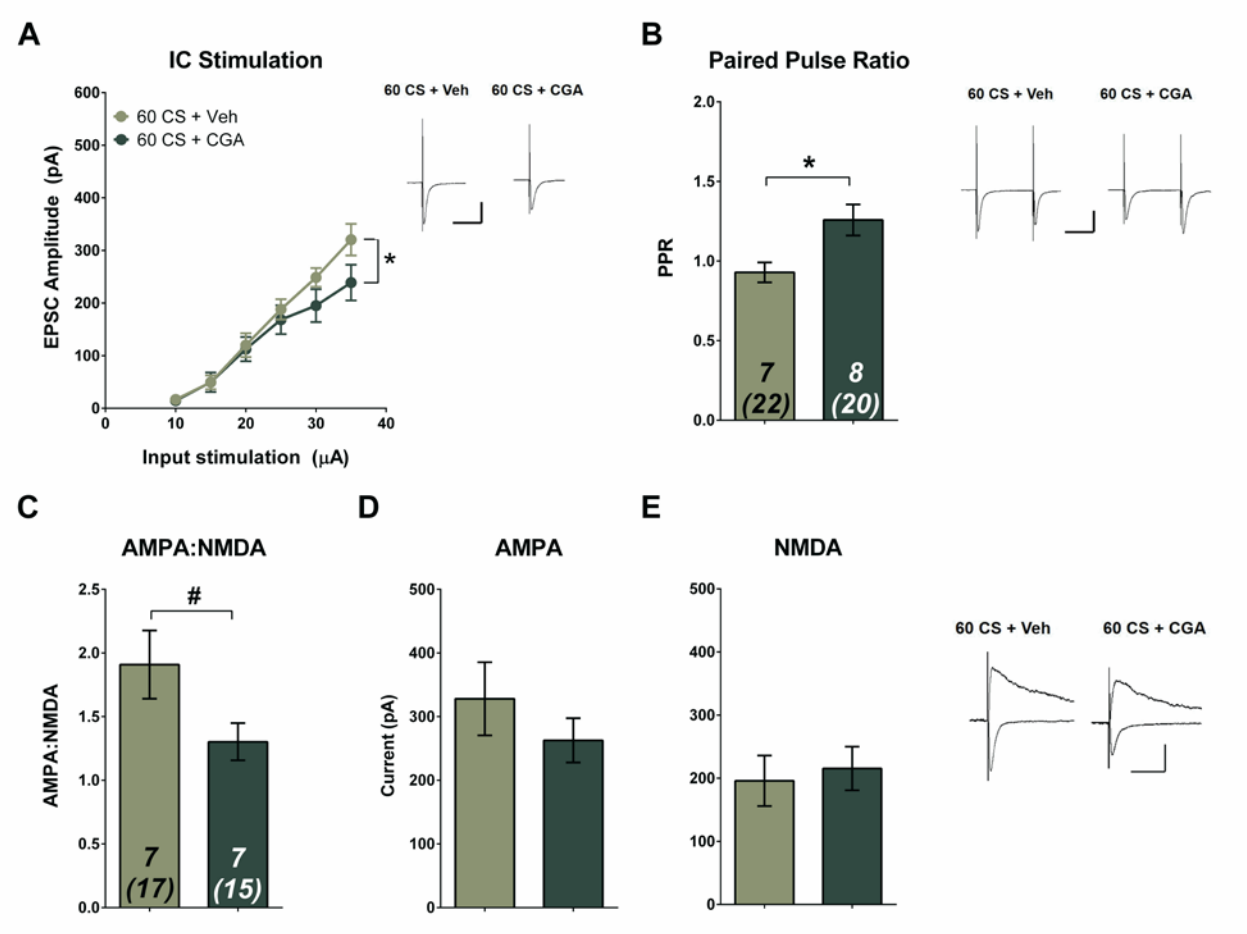

Figure 23. Calcineurin activation during cue extinction alters synaptic plasticity through a presynaptic mechanism.

Following self-administration, rats received a cue-extinction (60 CS) session followed immediately by infusion with either CGA or Veh. 24 h later, *ex vivo* recordings were performed in the LA. (**A**) Input-output curve demonstrating average evoked EPSC amplitude across various stimulation intensities. Intra-LA CaN activation by CGA alters EPSC amplitude. Two-way ANOVA, significant group x stim intensity interaction ($F_{(5,65)}$ = 2.64, P = 0.031). (**B**) CGA infusion significantly increases PPR (Unpaired t-test, $t_{(13)}$ = 2.75, P = 0.12). CaN activation by CGA altered (**C**) AMPA:NMDA at a trend level (Unpaired t-test, $t_{(12)}$ = 1.99, p = 0.070), but had no effect on (**D**) AMPAR current (Unpaired t-test, $t_{(12)}$ = 0.972, P > 0.05) or (**E**) NMDAR current (Unpaired t-test, $t_{(12)}$ = 0.368, P > 0.05). Data are expressed as mean ± SEM; n in bars, number of rats (number of neurons). *p < .05; #p < 0.10. *Insets: Sample average EPSC traces.* Scale bars: 50 ms, 200 pA.

3.4 DISCUSSION

The present study identifies a causal relationship between CaN-induced activity at T-LA synapses and the suppression of cue-elicited cocaine-seeking. Specifically, activation of CaN, during either reconsolidation or extinction of a memory associated with cocaine self-adminsitration led to synaptic modifications that resulted in a reduction in cue-induced reinstatement (**Figures 20C and 22C)**, effectively disrupting the drug-associated memory. These results are consistent with both a partial inhibition of reconsolidation of the original cocaine-cue memory and an enhancement in the new cocaine-cue extinction memory. Importantly, in the absence of memory retrieval, CaN activation had no memory-interfering effects, confirming that the memory trace must be reactivated in order to achieve pharmacologically-induced disruption (**Figure 18C**). We have previously found similar behavioral effects following intra-BLA infusion of CaMKII inhibitors (Rich et al., 2016; See Appendix A), suggesting that CaN and CaMKII within the BLA have opposing actions on drug-associated memory processes. We have also shown that T-LA synapses are an important locus for drug-cue memory, and that these synapses are differentially regulated by consolidation/reconsolidation and extinction (See Chapter 2). However, to our knowledge this study is the first report of pharmacologically-induced changes in synaptic activity that are directly associated with changes in the conditioned response to drug-associated cues. Upregulation of CaN activity following cue retrieval induces postsynaptic changes, perhaps driven by activity at AMPAR that prevents the memory-maintenance/strengthening effects of reconsolidation and results in less reinstatement (**Figure 21**). Similarly, upregulation of CaN activity following cue extinction also induces synaptic changes, although this may be a combination of pre- and postsynaptic effects; for instance, a

decreased glutamate release probability along with AMPAR and NMDAR-mediated effects (**Figure 23**). Together, these synaptic changes appear to enhance the consolidation of drug-cue extinction, which further reduces the propensity of cues to trigger relapse-like behaviors.

3.4.1 Targeting memory processes as a strategy for relapse prevention

Interfering with memories that trigger drug-seeking in addicted individuals is hypothesized to assist in the maintenance of long-term abstinence, however, this strategy has had limited success in preventing relapse (Koob and Volkow, 2010). Clinical studies have attempted to use cue extinction as a means to interfere with drug-related memories. In these experiments, individuals were re-exposed to multimodal cues in an effort to decrease subsequent craving and relapse when the drug-associated cues were re-encountered (Price et al., 2013). This idea is taken from research on fear-associated memories, including PTSD, where cue exposure therapy has been quite successful at limiting the detrimental effects of maladaptive memories in both animals and humans (Hofmann et al., 2012; Monfils et al., 2009; Ressler et al., 2004). Unfortunately, unlike fear memories, extinction of drug-associated memories has not met with clinical success. However, it may be possible to combine behavioral therapies, like extinction, with pharmacological treatments. Indeed, this approach has been demonstrated to have the highest success rates (Carroll and Onken, 2005); however, it is becoming clear from preclinical studies that the best medications would be those that both block drug-cue memory reconsolidation and augment extinction learning (Cleva et al., 2010; Rich et al., 2016; Sorg, 2012; Torregrossa and Taylor, 2016). Our current study investigating CaN activation further validates this theory. A bidirectional effect on memory overcomes previous limitations of pharmacological agents, such

as glutamatergic agonists like DCS (Lee et al., 2009; Price et al., 2013). The efficacy of DCS is dependent upon effective extinction training, and in the absence of memory extinction, DCS can cause unintentional, reconsolidation-associated memory-strengthening effects, limiting its usefulness as an adjunctive treatment (Lee et al., 2009). As we have demonstrated with CaMKII inhibition (Rich et al., 2016), activating CaN signaling pathways in conjunction with exposure therapy may therefore be a viable treatment strategy, as memory weakening occurs under both memory reactivation and extinction conditions.

In the current study, we demonstrate potential therapeutic effects of CaN activation following 3 (reconsolidation) or 60 (extinction) presentations of cocaine-associated cues. In our previous work we have shown that 120 presentations of cues during re-exposure results in an even more pronounced AMPAR-associated depotentiation at T-LA synapses that correlates with a further suppression of cue-induced reinstatement. That the number of unreinforced cue presentations is a critical determinant of extinction efficacy is not altogether a novel premise (Price et al., 2013; Unrod et al., 2014). However, we did not examine the effects of CaN activation following this extended extinction duration because we previously failed to observe pharmacological enhancement via CaMKII inhibtion, due to a potential floor effect (Rich et al., 2016). Still, it would be interesting for future studies to examine what, if any, changes occur at T-LA synapses following the activation of CaN immediately after more extensive extinction. Furthermore, it would be interesting to see if the combination of extinction and CaN activation together prevents either context-dependent renewal or spontaneous recovery of drug-seeking, both of which have proven to limit the therapeutic potential of cue re-exposure.

3.4.2 Mechanisms of calcineurin action at the synapse

CaN is a Ca^{2+}/calmodulin-dependent phosphatase that has a high affinity for Ca^{2+} and can therefore be activated by a relatively low concentration of intracellular Ca^{2+}. When synaptic activity is sparse (ie. low frequency LTD induction), the primary means of Ca^{2+} entry is through L-type voltage-gated calcium channels (LTCC), which allow for a sustained, but low increase in Ca^{2+} that results in activation of CaN (Bi and Poo, 1998; Ghosh et al., 2017). CaN is a heterodimer composed of a catalytic A subunit and a regulatory B subunit (Mumby and Walter, 1993). Sequential conformational changes following binding of Ca2+ and calmodulin to CaN_B activates the phosphatase via displacement of the CaN_A autoinhibitory domain (Shen et al., 2008). CaN then directly dephosphorylates GluA1 at Ser845, which prevents kinase-induced membrane insertion of AMPAR (Beattie et al., 2000; Man et al., 2007; Roche et al., 1996). CaN also initiates a phosphatase cascade (via protein phosphatase 1 and protein phosphatase 2A) that ultimately leads to the inactivation of LTP-promoting kinases as well as downstream actions at AMPAR and other synaptic proteins (Baumgartel and Mansuy, 2012; Mulkey et al., 1994). CaN-induced internalization of AMPAR causes changes in spine morphology, decreased spine density and decreased AMPA:NMDA (Beattie et al., 2000; Lu et al., 2000; Sanderson et al., 2016). These changes are consistent with our present results in which CaN activation following drug-cue memory reactivation and extinction has effects on plasticity through a postsynaptic mechanism, including alterations in AMPAR (**Figures 21 and 23**).

CaN has also been shown to have effects at the presynaptic membrane. For example, in the cortex, CaN is enriched at presynaptic terminals (Shields et al., 1985) and application of CaN inhibitors increases both the rate of spontaneous action potential firing and the frequency of

EPSCs (Victor et al., 1995). These data are suggestive of an inhibitory role of CaN in glutamatergic transmission and is consistent with the increase in PPR (decrease in release probability) that we observe following the activation of CaN during cue extinction (**Figure 23B**). Previous studies involving fear conditioning have found that activation of presynaptic group II metabotropic glutamate receptors (mGluRII) reduces T-LA synaptic transmission and reduces the expression of conditioned fear (Heinbockel and Pape, 2000; Lin et al., 2005). However, it should be noted that our previous experiments involving drug-cue memory extinction did not uncover any presynaptic-based changes in T-LA synaptic plasticity. Therefore, the implications of CaN-induced presynaptic plasticity, as well as the precise mechanisms by which this occurs, remain to be determined.

3.4.3 Role for calcineurin in cue-dependent memory processes

CaN has long been established as a negative regulator of emotionally-salient memories (Baumgärtel et al., 2008; Havekes et al., 2008; de la Fuente et al., 2014). CaN was first identified to play a role in the regulation of fear-associated memories, specifically the establishment of fear extinction. Fear extinction was shown to evoke increases in protein levels and enzymatic activity of CaN within the amygdala that were accompanied by the reversal of fear conditioning-induced protein phosphorylation (Lin et al., 2003a, 2003b). These effects were blocked by the administration of CaN inhibitors, suggesting that CaN either directly or indirectly dephosphorylates specific substrates whose phosphorylation is required for the consolidation of fear-associated memories. These investigators next attempted to model fear extinction via low-frequency stimulation (LFS) of amygdala-projecting sensory cortical afferents, a manipulation

that elicited depotentiation in the LA following fear conditioning (Lin et al., 2003b). As with cue extinction, LFS-induced depotentiation was associated with an increase in CaN activity that was dependent on NMDAR and LTCC. Administration of CaN inhibitors blocked LFS-induced depotentiation *in vitro* and LFS-induced fear extinction *in vivo*. The fear extinction-associated effects of CaN appear to be dependent on the amount or duration of cue re-exposure. Everitt and colleagues demonstrated that protein levels of calcineurin increase after 10 (but not fewer) presentations of a fear-associated CS, suggesting that CaN is likely important for the switch between maintenance and loss of fear memory (Merlo et al., 2014).

Finally, there is evidence that CaN is also involved in the regulation of drug-related memories, specifically the destabilization of a contextual memory associated with methamphetamine (METH) (Yu et al., 2016). In this study, authors performed conditioned place preference for METH and used a protein synthesis inhibitor (anisomycin) to disrupt the reconsolidation of the contextual memory. Destabilization was confirmed by observations of , reduced phosphorylation of GluA1 at Ser845, decreased spine density, and smaller AMPA:NMDA. When authors combined anisomycin treatment with inhibitors of either CaN or protein phosphatase 1, the destabilization-associated deficits were prevented, suggesting that destabilization of drug-related contextual memory occurs through a CaN-dependent dephosphorylation cascade that results in AMPAR internalization and LTD (Mulkey et al., 1994; Yu et al., 2016). Together with the results from the present study, there is strong evidence that upregulating CaN activity may be an effective strategy for reducing the strength of drug-related memories, either via the blockade of reconsolidation or by the enhancement of extinction.

4.0 GENERAL DISCUSSION

The work presented in this dissertation focused on cellular and molecular mechanisms involved in the regulation of a memory associated with self-administered cocaine. We investigated whether specific afferent inputs to the LA were involved in the formation, maintenance, and modification of a cocaine-cue associative memory We also investigated whether modification of LA synapses (either through behavioral cue re-exposure, optogenetics, or site-specific infusion of a pharmacological agent), could suppress future drug-seeking behavior. Our primary conclusions are that cocaine-cue memories are encoded, at least in part, by the strengthening of thalamo-amygdala (specifically MGN), but not cortico-amygdala, synapses. Brief cue reactivation initiates a reconsolidation event that maintains the cocaine-cue association and promotes drug-seeking behavior. More extensive cue re-exposure, in the absence of cocaine reinforcement, extinguishes the cocaine-cue association by engaging cellular mechanisms that depotentiate T-LA synapses, thereby attenuating cue-elicited drug-seeking. Finally, we propose that cocaine-cue memories can be interfered with by promoting synaptic activity that mirrors cue extinction, either through optically-induced LTD of MGN-LA synapses (**Chapter 2**), or by administration of pharmacological agents that either activate the CaN signaling pathway (**Chapter 3**) or inhibit the CaMKIIα signaling pathway (See **Appendix A**). In this discussion, we will describe how our findings lead toward a better understanding of drug-related memories and

addiction in general, as well as potential therapeutic implications of a circuit- and pharmacology-based approach for relapse prevention.

4.1 UNDERSTANDING THE CELLULAR MECHANISMS OF RECONSOLIDATION AND EXTINCTION

The results of this dissertation support the notion that drug-cue memory reconsolidation and extinction are separate and competing processes that likely co-occur following memory reactivation. We also demonstrate the involvement of competing neural mechanisms: cellular events that enhance synaptic strength promote reconsolidation and prevent extinction while cellular events that weaken synaptic strength support extinction and inhibit reconsolidation. Moreover, we have uncovered an opposing intracellular phosphatase/kinase pathway (CaN/CaMKII) that bidirectionally regulates the two processes. Activating CaN signaling and/or inhibiting CaMKII signaling simultaneously blocks reconsolidation and promotes extinction. Pharmacological manipulation of this molecular pathway overcomes limitations of previous pharmacotherapies that, in attempting to enhance extinction, inadvertently enhanced reconsolidation, thus unintentionally strengthening the memory.

The amount of cue re-exposure appears to be a major determinant in the outcome of the memory, although this is not surprising when one considers the differences between LTP and LTD induction. Whereas LTP occurs following brief, phasic bouts of synaptic activity that lead to large increases in intracellular Ca^{2+}, LTD occurs following synaptic activity that induces only moderate increases in Ca^{2+}. Similarly, reconsolidation occurs following brief cue re-exposure (3

CS presentations), while extinction is induced after extensive cue re-exposure (60-120 CS presentations). Presumably, this is due to similar mechanistic differences, such that brief reactivation results in large Ca^{2+} increases, kinase activation, and AMPAR trafficking to the membrane, and longer exposure results in smaller, but sustained Ca^{2+} increases that preferentially activate phosphatases, leading to AMPAR internalization (Baumgartel and Mansuy, 2012; Mulkey et al., 1994). Future experiments could examine differences in Ca^{2+} transients during reconsolidation vs. extinction using *in vivo* Ca^{2+} imaging (Grienberger and Konnerth, 2012; Resendez et al., 2016). Injection of a GCaMP calcium indicator under control of the CaMKII promoter would enable viral expression in the LA that can then allow Ca^{2+} imaging in real time through an implanted microendoscope. These techniques can even be combined with traditional optogenetic approaches to add additional circuit-specific control over behavior.

In our experiments, we often fail to demonstrate a "strengthening" of the cocaine-cue association following brief cue reactivation, as assessed by behavioral or physiological measures. However, this does not necessarily correspond to a lack of reconsolidation. In fact, several studies have consistently shown that cue reactivation fails to alter behavior or induce synaptic potentiation. For example, novel object recognition training induces a facilitation of CA3-CA1 field potentials, but there was no further potentiation following reconsolidation (Clarke et al., 2010). Interestingly, the field potentials were transiently depotentiated when measured immediately after reactivation. Similarly, the long-term facilitation of auditory-evoked field potentials in the LA was prevented when protein synthesis was inhibited (Doyère et al., 2007). Furthermore, reactivation of thalamic and cortical inputs to the LA does not cause further facilitation of field potentials (Li et al., 2013). While not indicative of a reconsolidation-dependent synaptic enhancement, these studies demonstrate that brief reactivation triggers a

transient depotentiation (that corresponds with memory destabilization) that is followed by a protein synthesis-dependent restabilization (reconsolidation). Therefore, the lack of reconsolidation effects in our studies (increased reinstatement, enhanced synaptic potentiation) does not imply a fundamental absence of reconsolidation. Our studies involving pharmacological manipulation of CaN and CaMKII, suggest that reconsolidation does occur. For example, activation of CaN following brief cue reactivation (3 CS re-exposure) leads to a decrease in T-LA synaptic strength and a decrease in cue-induced reinstatement that is not observed if the memory is not reactivated (0 CS re-exposure). Similar effects on reinstatement are observed following CaMKII inhibition, but again, only if the memory is reactivated. To fully rule out the possibility that brief reactivation fails to modify the drug-cue memory, future experiments should include a group of animals that is tested for reinstatement within the reconsolidation window (ie. 1 hour after reactivation). Evidence for this possibility stems from previous studies that have identified GluA2 endocytosis as a mechanism for reactivation-induced depotentiation, with reconsolidation then dependent on the insertion of GluA2-lacking Ca^{2+}-permeable AMPAR (Rao-Ruiz et al., 2011). Thus, we might predict that animals tested shortly after reactivation would exhibit a reduction in T-LA EPSC amplitude concurrent with a reduction in cue-induced reinstatement. It would also be useful for future studies to determine if drug-cue memory reconsolidation and extinction depend on AMPAR subunit specificity. During extinction of fear-associated memories there is a specific internalization of Ca^{2+}-permeable AMPAR (Clem and Huganir, 2010), but it is unclear if this extends to the extinction of drug-cue memories. Electrophysiological experiments could utilize subunit specific antagonists, such as Naspm, an antagonist for GluA2-lacking AMPAR (Ma et al., 2016). If EPSC amplitude was sensitive to Naspm, it would suggest that extinction does not internalize these specific receptors.

Recent experiments have taken advantage of the fact that memory reactivation initiates a transient depotentiation prior to reconsolidation in attempts to induce a persistent weakening of CS-US associations. Monfils and colleagues utilized a behavioral design in which they destabilized a fear memory via an isolated cue reactivation trial prior to a standard cue extinction session (Monfils et al., 2009). This retrieval-extinction paradigm creates a long-term disruption of fear memory that is resistant to reactivation, renewal, and spontaneous recovery. This and similar procedures have since been used to study the extinction of drug-associated memories. For example, CS-US retrieval-extinction in rats trained to self-administer heroin had long-lasting memory disruption effects (Xue et al., 2012). The same study also demonstrated clinical relevance, as this procedure also attenuated cue-induced craving of heroin for up to 180 days in abstinent heroin addicts. Retrieval-extinction has also been used to inhibit a cocaine-associated contextual memory (Sartor and Aston-Jones, 2014). Most recently, a slight modification to the procedure showed that brief US reactivation (drug re-exposure) prior to extinction of the CS can also inhibit drug-seeking (Luo et al., 2015). Overall, these procedures have promise as nonpharmacological methods to reduce drug-seeking behaviors; however, it should be noted that attempts by other labs to replicate the initial study by Monfils and colleagues have been unsuccessful (Goode et al., 2017; Ishii et al., 2015; Luyten and Beckers, 2017). Additionally, there is evidence that multiple reconsolidation events can make memories resistant to extinction (Dębiec et al., 2011), so the time course of reactivation and extinction is an important consideration if retrieval-extinction strategies are to be implemented as a therapeutic strategy.

Finally, future optogenetics experiments could continue to build towards identifying the specific synaptic mechanisms underlying reconsolidation and extinction. We have not yet examined whether cue reactivation/reconsolidation results in an increase in the amplitude of

optically-evoked MGN-LA EPSCs. A related experiment could test whether *in vivo* high frequency optical stimulation of MGN-LA terminals is sufficient to induce an LTP-like state (increased EPSC amplitude, AMPAR current) that enhances cue-induced reinstatement. These experiments would help validate LTP as a mechanism for drug-cue memory reconsolidation, a result that has potential therapeutic implications. Additional experiments should also consider the role of NMDAR in optically-induced LTP/LTD. In **Chapter 2** we demonstrate that *ex vivo* optical LTD is occluded in slices from animals that have already undergone cue extinction. MGN-LA synapses in these animals are already depotentiated, as indicated by decreased EPSC amplitude and AMPAR current relative to non-extinguished controls. If the extinction-induced depotentiation is dependent on NDMAR activation, then administration of an NMDAR antagonist such as APV or MK801 prior to cue extinction would block the extinction effects and allow for the generation of *ex vivo* LTD. Additionally, we show that brief cue reactivation induces significant increases in NMDAR current, suggesting enhanced expression and/or function at T-LA synapses. Future studies should continue to elucidate the potential role of NMDAR in drug-cue memory reconsolidation.

4.2 ROLE OF NEURAL ENSEMBLES IN DRUG-CUE MEMORY

In this dissertation we demonstrate that the formation of a cocaine-cue association results in potentiation of T-LA synapses and that extinction of the cocaine-cue association depotentiates T-LA synapses. Larger EPSC amplitudes were observed in slices from cocaine-trained animals relative to saline-trained controls, and this increase was reversed by cue extinction. On average,

for cocaine-trained non-extinguished animals, EPSC amplitude was correlated to the number of CS-US pairings received during SA. While there is some degree of variability both between individual neurons and between animals, most neurons tested exhibited relatively large EPSC amplitudes compared to both the saline and extinction groups. This is interesting, and perhaps somewhat surprising, given the concepts of Hebbian learning that were described above. Based on this theory, it has been proposed that sparsely distributed patterns of neurons called neuronal ensembles, may be responsible for the encoding of learned associations, such as those that underlie cue-dependent drug-seeking (Buzsáki and Moser, 2013; Pennartz et al., 2004). Reports suggest that only 1-5% of neurons within a brain region make up an ensemble, but there may be several ensembles within a given region, and individual neurons can contribute to more than one ensemble (Bossert et al., 2011a; Chawla et al., 2005; Schwindel & McNaughton, 2011). It is generally believed that the subset of neurons that receive the strongest, most persistent afferent input will be the ones recruited into any given ensemble. In our studies we fail to fully demonstrate the existence of distinct neuronal subsets that do not participate in the given memory processes. This may be due to a few reasons, including inherent limitations of our experimental approach. Unfortunately, with the use of whole-cell slice electrophysiology our data set is limited by the number of neurons that can be recorded from during a small timeframe, while the slices remain healthy. It is difficult to gauge whether such a small sample size is a true representation of all neurons in a brain region. We are left with the possibility that there are indeed neurons or at least individual synapses that are not affected by the memory process. This limitation can be overcome by correlating electrophysiology with Fos imaging from activated neurons (Cruz et al., 2015). Strong, correlated activity generates Ca^{2+} influx, which influences

plasticity at dendritic spines, but also activates the ERK/MAPK pathway, which stimulates c-fos promoter activity in the nucleus (Brami-Cherrier et al., 2005; Lüscher et al., 2000).

A novel approach known as the Daun02 inactivation procedure has been utilized to demonstrate the involvement of specific neuronal ensembles in mediating drug-seeking behaviors (Bossert et al., 2011; Cruz et al., 2014; Koya et al., 2009). In these experiments, *c-fos-lacZ* transgenic rats are used, in which translation of β-galactosidase occurs in strongly activated, *c-fos*-expressing neurons. Daun02 is then injected and catalyzed to daunorubicin by β-galactosidase, which is believed to then induce apoptotic cell death of only the previously activated neurons (Farquhar et al., 2002). Thus, the neural ensemble activated by a particular behavior would be eliminated. Unlike studies where entire brain regions are inactivated, this approach targets a small number of neurons and allows the investigation of how neuronal ensembles regulate drug-associated behaviors. For example, the elimination of a neuronal ensemble within the dorsolateral striatum can prevent context-induced seeking of methamphetamine, while inactivation of an ensemble within the dorsomedial striatum prevents incubation of methamphetamine craving (Caprioli et al., 2017; Rubio et al., 2015). This technique could be useful to confirm if ensembles of amygdala neurons are indeed involved in drug-cue memory formation, reconsolidation, and extinction. Future experiments could also utilize Fos-GFP transgenic rats so that electrophysiological recordings can be limited to Fos-activated neurons, which would be useful in determining if reconsolidation and extinction triggered LTP/LTD in an ensemble-specific manner. It is entirely likely that different sets of neuronal ensembles are activated during reconsolidation versus extinction and therefore the same molecular events in the same brain region may occur during reconsolidation and extinction, but in different populations of neurons (Gore et al., 2015; Suto et al., 2016; Warren et al., 2016). The

possibility exists that activating or inhibiting the same molecular processes in distinct neuronal ensembles could lead to opposing outcomes. This is one potential explanation for how a common biochemical manipulation (i.e., activating CaN; inhibiting CaMKII) in the same brain region can simultaneously interfere with reconsolidation and promote extinction.

4.3 CONTRIBUTION OF ADDITIONAL AMYGDALA AFFERENT PROJECTIONS

In addition to sensory thalamic and cortical glutamatergic projections, the amygdala receives input from a diverse collection of brain regions. Those that are most important for controlling memory-related behaviors include glutamatergic input from the mPFC and hippocampus, dopaminergic input from the VTA, serotoninergic input from the dorsal raphe, and adrenergic input from the locus coeruleus, among others (Giustino and Maren, 2015; Loh and Roberts, 1990; Pelloux et al., 2012; Quirk and Mueller, 2008; Wise, 2004). Local inhibitory projections from GABAergic interneurons and intercalated cells also have important effects (Asede et al., 2015b; Lucas et al., 2016; Pinard et al., 2012). While most of these are outside the scope of this dissertation, it is worthwhile to contemplate the impact that this input may have, thus providing a deeper insight into the role of the amygdala in regulating drug-cue memories.

As described, acute drug exposure increases dopaminergic transmission throughout the mesocorticolimbic system, including in the amygdala. When discrete cues are predictive of drug use, subsequent presentations of the cues alone cause DA neurons in the VTA to fire, increasing DA release (Phillips et al., 2003; Weiss et al., 2000). Although the precise impact of DA on reconsolidation and extinction of drug-cue memories has not been elucidated, one could

hypothesize that T-LA or C-LA synaptic activity would be modulated in a D1- or D2-specific manner. VTA DA has been shown to directly modulate the amygdala neuronal activity during Pavlovian conditioning. For instance, enhancements in activity that are observed by pairing a footshock with a particular odor can be blocked by administration of the nonspecific DA receptor antagonist, haloperidol (Rosenkranz and Grace, 2002). Furthermore, DA receptor activation increases kinase activity and downstream phosphorylation. For example, D1-receptor activation upregulates PKA, which can phosphorylate DARPP-32 (DA and cAMP-regulated phosphoprotein, 32kDa). DARPP-32 subsequently regulates excitatory transmission through effects at L-type voltage-gated calcium channels, AMPAR, and NMDAR (Bibb et al., 1999; Flores-Hernandez, 2002; Nishi et al., 1997). Conversely, D2-like receptor activation inhibits phosphorylation of DARPP-32, and a recent study showed that antagonism of D2 receptors in the amygdala impaired the extinction of a fear-associated memory (Nishi et al., 1997; Shi et al., 2017). Interestingly, antagonism of D1 receptors was found to attenuate methylphenidate-induced enhancements of cue-reward learning, while D2 antagonism increased task-irrelevant behavior, suggesting that D2 receptor activation suppresses this activity (Tye et al., 2010). Furthermore, the activation of presynaptically-located D2 receptors activates G-protein-coupled inwardly rectifying potassium (GIRK) channels, which can inhibit subsequent DA release, serving as an important feedback mechanism (Martel et al., 2011; Michaeli and Yaka, 2010). Future studies should investigate the role of D1- and D2-specificity during the reconsolidation and extinction of drug-associated memories. Finally, it is possible that DA could be modulating amygdala activity through indirect projections to the thalamus or mPFC. Studies have shown that the MGN has very low expression of DAT, suggesting that this subregion does not receive much dopaminergic input (García-Cabezas et al., 2009). However, other regions of the thalamus,

including mediodorsal and paraventricular nuclei (PVN), which also project to the amygdala, do express DAT, and could therefore play a role in drug-associated memory (Vertes et al., 2015). The PVN has already been associated with aspects of addiction; PVN projections to the NAc were shown to mediate the expression of physical symptoms and memory deficits during opiate withdrawal (Zhu et al., 2016). Midline thalamic subregions are spatially distinct from the MGN, removing the possibility that we accidentally targeted medial thalamus with our optogenetics experiments. Still, midline thalamic subregions may be important sites to investigate in future studies of drug-related memory.

Another circuit of interest involves reciprocal connections between the mPFC and the amygdala. Hypofunction of the mPFC can result in the loss of inhibitory control over drug seeking (Goldstein and Volkow, 2011; Jentsch and Taylor, 1999). Initially, evidence suggested that amygdala projections from the IL and PL had opposing roles in the control of fear-related memories, likely through differential recruitment of amygdala subregions (Arruda-Carvalho and Clem, 2015; Courtin et al., 2013). IL activity promotes fear extinction, possibly through projections to intercalated cell masses (Pinard et al., 2012; Quirk et al., 2000) while PL activation is associated with cue-elicted freezing, perhaps through direct projections to the basal nucleus or indirect projections to the PVN (Arruda-Carvalho and Clem, 2014; Do-Monte et al., 2015; Milad et al., 2004). The development of optogenetics techniques have allowed the further characterization of this circuitry and should be considered in future studies of drug-associated learning and memory. Notably, in this dissertation, the presence of a $GABA_A$ antagonist during electrophysiological recordings removes a likely important contribution from GABAergic projections. Because of evidence suggesting that PFC projects heavily to GABAergic neurons, future studies should assess the impact of inhibitory signaling.

4.4 IMPLICATIONS FOR ADDICTION THERAPY

In **Chapter 2** and **Appendix A** of this dissertation, we present two targets (CaN and CaMKII) for a pharmacology-based treatment that can simultaneously interfere with drug-cue memory reconsolidation and promote drug-cue memory extinction. Manipulation of these signaling pathways presents the possibility for eventual development of a medication that can consistently reduce craving and drug-seeking behaviors by targeting molecules that are important for T-LA synaptic regulation. This is supported by our electrophysiological experiments from **Chapters 1 and 2**, in which we demonstrate specific synaptic mechanisms that are at least partly responsible for the encoding of drug-related memories and show that evoking depotentiation of specific synapses can lead to a suppression of the conditioned drug-seeking response. Importantly, we also uncovered different synaptic modifications that were associated with one of two specific types of drug-related memories. Depotentiation of thalamo-amygdala synapses was driven by extinction of drug-cue specific memories while depotentiation at cortico-amygdala synapses was associated with extinction of the drug-seeking action. This is importantly clinically, as cue exposure therapy involves extinction of memories that are evoked by drug-associated stimuli, so pharmacological agents that induce depotentiation of thalamo-amygdala synapses may indeed prolong the therapeutic efficacy of cue exposure therapy. If a treatment were also to have nonspecific effects (e.g., at cortico-amygdala synapses) then other drug-related memories associated with drug taking may also be disrupted, which could have added benefit.

It should be noted that all of our experiments were performed on adult rats. However, there is evidence in both humans and rodents that adolescence represents a period of vulnerability to drugs of abuse. Epidemiological studies show that experimentation with drugs of

abuse is highest during adolescence or young adulthood, while reward-related brain circuits are still developing (Chambers et al., 2003; Dayan et al., 2010; Stanis and Andersen, 2014). In general, risk-taking behavior is elevated during this period, which can contribute to a relatively early onset of substance use disorders. Indeed, preclinical models have shown that greater risk taking during adolescence is predictive of greater cocaine use during adulthood as well as changes in dopaminergic signaling in the striatum (Mitchell et al., 2014). Furthermore, studies from our lab show that adolescent rodents will willingly self-administer a synthetic cannabinoid agonist (Kirschmann et al., 2017). However, specific components of drug-related memories have not been well elucidated in adolescent rats. It is possible that key differences in the neural circuitry might exist, so it would be beneficial for future studies to examine drug-cue memory processes (extinction and reconsolidation) during adolescence.

Although pharmacotherapy is one potential method to induce relapse-blocking synaptic changes, an alternative approach, which is supported by our optogenetics experiment from **Chapter 1**, involves stimulating affected brain regions to modulate or normalize neuronal firing. Clinically, two separate methods of brain stimulation have been utilized: deep brain stimulation (DBS) and repetitive transcranial magnetic stimulation (rTMS). DBS involves the surgical implantation of bipolar electrodes within specific subcortical brain regions in which a pulse generator delivers high-frequency stimulation that depolarizes or hyperpolarizes local neurons (McIntyre, et al., 2004). The mechanism of action remains unclear, but studies indicate that normal brain function can be reestablished by a synchronization or desynchronization of brain-wide circuits (Murrow, 2014). While DBS has been successful at improving outcomes for several neurological and psychiatric disorders, including Parkinson's disease and depression (Riva-Posse et al., 2014; Wagenbreth et al., 2015), relatively few studies have directly assessed

the effects of DBS on addictive behaviors. In animal studies, DBS has transiently suppressed cocaine-induced locomotor sensitization, reduced ethanol consumption, and attenuated cocaine-primed reinstatement (Creed et al., 2015; Knapp et al., 2009; Vassoler et al., 2013). Most addiction-related DBS findings in humans come from case reports or poorly controlled studies, but the results have yielded optimism. Studies have shown the effectiveness of DBS for alcohol, nicotine, and heroin. In a small clinical study of 5 alcohol-dependent individuals, bilateral DBS in the NAc resulted in the remission of craving in all individuals, and long-term abstinence in 2 of the 5 (Müller et al., 2016b). Similar results were achieved in a study of smokers, where rates of smoking cessation 30 months post-DBS were higher than rates of unaided smoking cessation in the general population (30% vs 8.7%) (Kuhn et al., 2009). Finally, two case reports of heroin-abusing individuals also yielded complete remission of drug use following DBS in the NAc (Valencia-Alfonso et al., 2012; Zhou, Xu, & Jiang, 2011). Together, these studies demonstrate the potential relapse-preventing potential of DBS and evidence from this dissertation suggests the amygdala as a novel target for stimulation-based therapy.

rTMS is a noninvasive form of brain stimulation in which magnetic pulses are delivered on top of the skull to indirectly generate electrical currents (Gorelick et al., 2014; Rossini et al., 2015). The frequency of stimulation can be varied, whereby low frequency stimulation (< 1 Hz) reduces neuronal activity and high frequency stimulation (between 5 and 20 Hz) enhances activity (Salling and Martinez, 2016; Speer et al., 2000). As with DBS, the mechanisms of action are not completely understood, but evidence suggests that TMS acts to locally alter blood flow, neuronal excitability and firing frequency, and neurotransmitter release (Grall-Bronnec and Sauvaget, 2014). Glutamatergic signaling appears to be involved, as NMDAR antagonists block the long-term effects of high frequency TMS (Huang et al., 2008a). Most clinical rTMS studies

of addiction have utilized high-frequency stimulation in the dorsolateral PFC (dlPFC), which is involved in cognitive processing, working memory, and impulse control (Barbey et al., 2013; Steinbeis et al., 2012). Although the effects of rTMS are generally limited to cortical tissue, there are reports of possible downstream effects in deeper brain structures (Fox et al., 1997). Studies of rTMS have produced mixed results in drug-dependent individuals; some have demonstrated acute reductions in craving and drug use (Eichhammer et al., 2003; Johann et al., 2003; Mishra et al., 2010; Politi et al., 2008), whereas others have shown no effect on craving (Herremans et al., 2012). Recently, Bonci and colleagues have suggested that rTMS rescues drug-induced hypoactivity of the PFC (Chen et al., 2013; Terraneo et al., 2016). In a rodent model, long-term cocaine self-administration decreased excitability of PL pyramidal neurons, while *in vivo* optogenetic PL stimulation prevented compulsive drug seeking (Chen et al., 2013). In a clinical study of cocaine abusers, rTMS-driven stimulation of the DLPFC resulted in a significantly higher number of cocaine-negative drug tests as well as significantly lower levels of craving compared to a control group (Terraneo et al., 2016).

Despite the mixed results of brain stimulation for the treatment of addiction, these techniques do offer promise for targeting neuroplasticity in the long-term prevention of craving and relapse. It is important to continue to study the mechanisms responsible for the normalization of drug-induced brain deficits. Circuit-specific approaches, like the ones presented in this dissertation may prove helpful at determining appropriate stimulation parameters and specific brain regions to target.

4.5 SUMMARY AND CONCLUSIONS

In summary, this dissertation describes a previously unstudied thalamo-amygdala circuit in the regulation of a memory associated with self-administered cocaine. Collectively, we have shown that MGN specific thalamic projections to the amygdala are recruited during cocaine self-administration leading to the formation of a cocaine-cue association. Subsequent re-exposure to the cocaine-associated cues can either initiate reconsolidation or extinction, depending on the amount of unreinforced cue re-exposure. Reconsolidation maintains, while extinction, which is associated with a reversal in thalamo-amygdala synaptic potentiation, inhibits the original cocaine-cue association. Furthermore, optically-induced depotentiation of the MGN-LA projection mimics cue extinction and suppresses cue-evoked reinstatement of drug-seeking. Finally, pharmacological agents that promote depotentiation of this circuit (CaMKII inhibitors, CaN activators) present with high therapeutic potential. We propose a model for the bidirectional regulation of cocaine-cue memories (**Figure 24**). Brief cue re-exposure initiates a relatively large increase in Ca^{2+} that activate kinases such as CaMKII, leading to AMPAR phosphorylation and expression at the synapse that strengthens or maintains the cocaine-cue association (reconsolidation). Longer cue re-exposure is accompanied by lower Ca^{2+} influx, preferential activation of phosphatases such as CaN, AMPAR dephosphorylation and internalization, and a weakening of the cocaine-cue association. Manipulation of these events, either by pharmacology or circuit-specific stimulation can affect memory outcome and have potentially beneficial effects that prevent relapse.

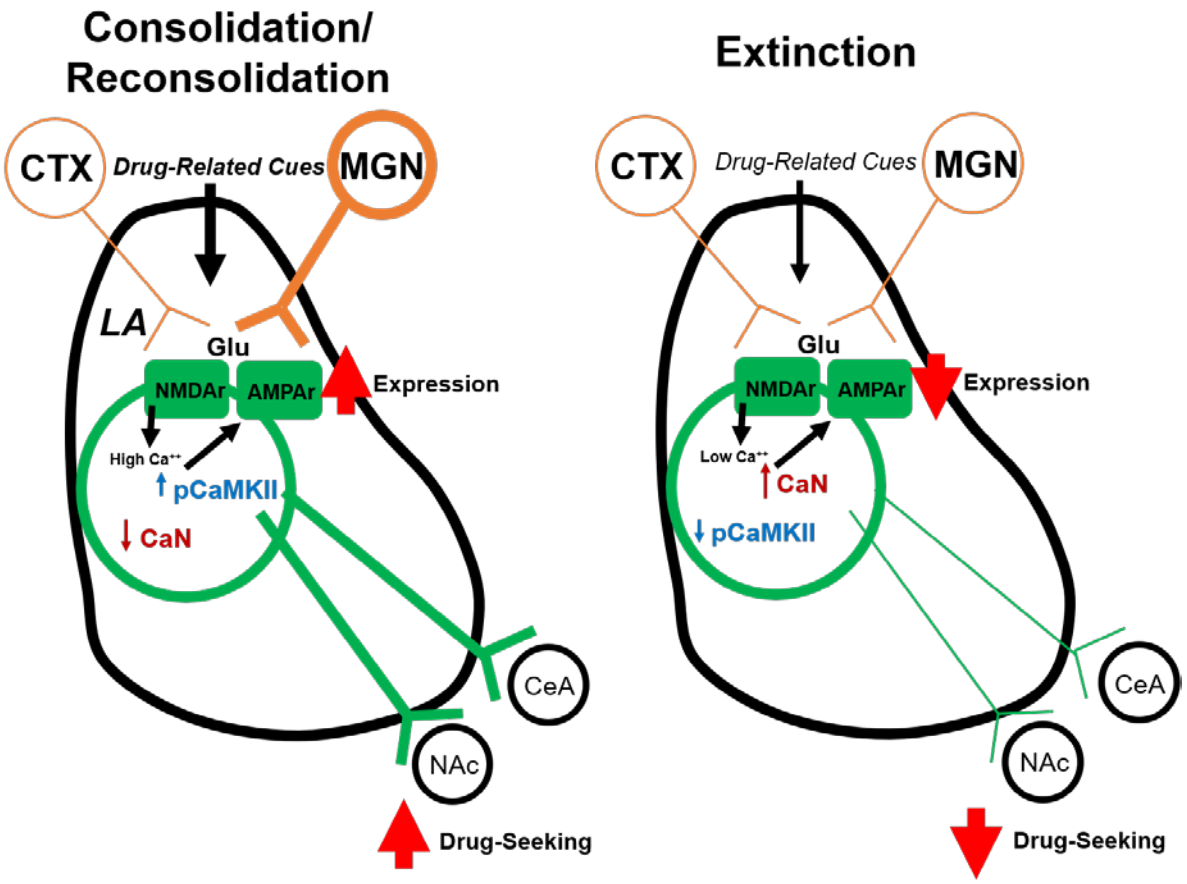

Figure 24. Model summarizing major conclusions.

Simplified schematic showing the cellular mechanisms involved in the formation, reconsolidation, and extinction of a cocaine-cue memory within the LA. Initial formation of a cocaine-cue association involves enhanced thalamo-amygdala synaptic activity, including MGN-specific activity, likely through increased postsynaptic AMPAR. Brief re-exposure to drug-related cues triggers increased CaMKII activity/decreased CaN activity, likely because of a large increase in Ca^{2+}. CaMKII phosphorylates synaptic targets that further increases/stabilizes AMPAR at the postsynaptic membrane and strengthens/maintains the original drug-cue association and leads to increased drug seeking, possibly by enhanced NAc/CeA activity. Extended cue re-exposure results in a smaller influx of Ca^{2+} that activates CaN and inhibits CaMKII. CaN dephosphorylates synaptic targets, resulting in internalization of AMPAR. This weakens the original drug-cue association and decreases subsequent cue-evoked drug-seeking, possibly by decreased downstream activity at the NAc/CeA.

APPENDIX A

PHOSPHOPROTEOMIC ANALYSIS REVEALS A NOVEL MECHANISM OF CAMKII ALPHA REGULATION INVERSELY INDUCED BY COCAINE MEMORY EXTINCTION VERSUS RECONSOLIDATION

Matthew T. Rich, Thomas B. Abbott, Lisa Chung, Erol E. Gulcicek, Kathryn L. Stone, Christopher M. Colangelo, TuKiet T. Lam, Angus C. Nairn, Jane R. Taylor, Mary M Torregrossa
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Successful addiction treatment depends on maintaining long-term abstinence, making relapse prevention an essential therapeutic goal. However, exposure to environmental cues associated with drug use often thwarts abstinence efforts by triggering drug using memories that drive craving and relapse. We sought to develop a dual approach for weakening cocaine memories through phosphoproteomic identification of targets regulated in opposite directions by memory extinction compared with reconsolidation in male Sprague-Dawley rats that had been trained to self-administer cocaine paired with an audiovisual cue. We discovered a novel, inversely-regulated, memory-dependent phosphorylation event on calcium-calmodulin dependent kinase II alpha (CaMKIIα) at serine (S)331. Correspondingly, extinction-associated S331 phosphorylation

inhibited CaMKIIα activity. Intra-BLA inhibition of CaMKII promoted memory extinction and disrupted reconsolidation, leading to a reduction in subsequent cue-induced reinstatement. CaMKII inhibition had no effect if the memory was neither retrieved nor extinguished. Therefore, inhibition of CaMKII represents a novel mechanism for memory-based addiction treatment that leverages both extinction enhancement and reconsolidation disruption to reduce relapse-like behavior.

A.1 INTRODUCTION

The successful treatment of addictive disorders requires maintaining long-term abstinence from drug use (Kalivas and Volkow, 2005; Milton and Everitt, 2010). However, individuals frequently encounter environmental cues previously associated with drug use that can increase craving and the likelihood of relapse (Fuchs et al., 2009; Kalivas, 2009). The ability of drug-associated memories to induce relapse is perhaps the greatest obstacle to overcome for the successful treatment of addictive disorders (Bossert et al., 2013; Torregrossa et al., 2011). Thus, reducing the strength of drug-associated memories has therapeutic potential for individuals struggling with addiction.

Behavioral and pharmacological interventions can disrupt or weaken drug-associated memories through two primary mechanisms. The first is to pharmacologically prevent reconsolidation of the memory into long-term storage after the memory is retrieved or reactivated (Taylor et al., 2009; Torregrossa and Taylor, 2013). It is possible to disrupt reconsolidation because when a memory is retrieved it can enter a labile state that requires

protein synthesis-dependent restabilization (reconsolidation) for the memory to be maintained in long-term storage. During the period of lability, memories are susceptible to interference, and can be weakened or strengthened with specific pharmacological manipulations (Tronson and Taylor, 2007). The second mechanism for weakening drug-associated memories is through the process of extinction. Extinction involves repeatedly presenting drug-associated cues in the absence of the drug. In this situation, the individual learns that the cues are no longer predictive of drug availability, and thus, subsequent encounters with the cue produce less craving and relapse. Extinction memories undergo their own consolidation and reconsolidation processes that can also be enhanced or inhibited with pharmacological manipulations (Holmes and Quirk, 2010; Nic Dhonnchadha et al., 2010; Vurbic and Bouton, 2011). Disrupting reconsolidation and enhancing extinction have long been proposed as potential strategies to disrupt aversive memories that form the basis of anxiety disorders (Agren et al., 2012; Gamache et al., 2012; Monfils et al., 2009), and these strategies have been extended to the addiction field (Arguello et al., 2014; Torregrossa et al., 2010; Tronson et al., 2012b). However, clinical application of extinction- or reconsolidation-based treatments has met with limited success, likely because both processes involve overlapping molecular mechanisms, making selective targeting of one or the other challenging. Indeed, previous attempts to disrupt drug-associated memories using pharmacological agents combined with extinction training may have been unsuccessful due to unintentional memory strengthening (Hofmann et al., 2012; Price et al., 2013).

Therefore, the goal of the present study was to identify cellular signaling events that are regulated in opposition by extinction relative to reconsolidation of a memory associated with self-administered cocaine. Such molecules, once identified, could represent targets for the development of a single medication that can enhance extinction while inhibiting reconsolidation

to ensure memory weakening and a reduction in relapse. We used a discovery-based phosphoproteomics strategy to identify proteins that exhibit a divergent pattern of phosphorylation following reconsolidation relative to extinction, as initial memory consolidation events involve the activation of kinase and phosphatase cascades (Merlo et al., 2014; Sanchez et al., 2010). The basolateral amygdala (BLA) was analyzed as it is the locus for associative learning and mediates the encoding of drug-associated memories (Fuchs et al., 2006; Nic Dhonnchadha et al., 2013; Sanchez et al., 2010). The proteomics analysis found a small number of opposing signaling events, including identification of a novel phosphorylation event on calcium-calmodulin dependent kinase II alpha (CaMKIIα) on serine (S) 331. Although CaMKIIα phosphorylation events on the highly characterized autophosphorylation site at threonine (T) 286 have been previously linked to drug-related learning (Easton et al., 2013, 2014; Salling et al., 2016), the role of S331 phosphorylation has not been investigated. Here, we report a functional role for S331 phosphorylation in vitro and describe the behavioral effects of CaMKII inhibition on both the reconsolidation and extinction of a cocaine-associated memory in vivo. Our findings suggest that molecular mechanisms exist that could allow for the combined enhancement of extinction and disruption of reconsolidation.

A.2 MATERIALS AND METHODS

A.2.1 Subjects

Naïve, adult male Sprague-Dawley rats, weighing 275-325 g on arrival, were used in all studies. All rats were housed in a temperature- and humidity-controlled room. Animals were housed in pairs, given ad libitum access to food and water, and maintained on a 12 h light-dark cycle. Rats were given at least 5 days to acclimate to the facility before undergoing surgical procedures. Following surgery, rats were individually housed and given at least 1 week to recover before the start of behavioral training. Rats were food-deprived 24 h prior to the start of behavioral experiments and maintained at ~90% of their free-feeding body weight (~20 g of chow per day) for the duration of testing. All behavioral experiments were run during the light-cycle. The experiments used to generate samples for proteomic analysis were conducted at Yale University in the Connecticut Mental Health Center, while all subsequent experiments were conducted at the University of Pittsburgh. Sprague-Dawley rats were obtained from Charles River (Kingston, NY) at Yale and from Harlan (Frederick, MD) at the University of Pittsburgh. We used different vendors to minimize animal shipping time to both facilities. At Yale rats were housed in shoebox cages with water bottles on standard racks, while at the University of Pittsburgh, rats were housed in auto-ventilated racks with an automated watering system. All other housing and procedural parameters were the same between the two universities, unless otherwise noted. In addition, all procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by each institution's Institutional Animal Care and Use Committee.

A.2.2 Self-administration test chambers

Rats were trained to self-administer cocaine in standard operant conditioning chambers (MedAssociates, St. Albans, VT). Experiments were counterbalanced across one of two chamber designs, providing two distinct contexts. The first type of chamber consisted of bar floors and 2 nosepoke apertures. The second type of chamber consisted of grid floors and 5 nosepoke apertures. All chambers contained two retractable levers on one wall of the chamber, a tone-generator, stimulus-light above each lever, house light, and infusion pump. Operant boxes were kept in sound-attenuating chambers equipped with a fan for background noise.

A.2.3 Drugs

Cocaine hydrochloride (generously provided by the National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in sterile 0.9% saline (2 mg/ml) and filter-sterilized for self-administration. KN-62 (Tocris, Bristol, UK) was dissolved in 0.9% saline + 62% DMSO. KN-93 (Tocris) was dissolved in 1X PBS + 10% DMSO.

A.2.4 Surgical procedures

Rats were fully anesthetized with ketamine hydrochloride (87.5 mg/kg; i.m.) and xylazine hydrochloride (5 mg/kg; i.m.) and then received an analgesic (Rimadyl, 5 mg/kg; s.c.) and 5 ml of Lactated Ringer's (s.c.) prior to surgery. Betadine and 70% ethanol were applied to all incision sites. All rats were implanted with a chronic indwelling intravenous (i.v.) catheter

(CamCaths, Cambridge, UK) into the right jugular vein, as described previously (Torregrossa and Kalivas, 2008; Torregrossa et al., 2010). Catheters were fed subcutaneously to the midscapular region, where they exited through a round incision. For experiments involving intracranial infusions, rats were immediately placed into a stereotaxic instrument (Stoelting, Kiel, WI), and implanted with bilateral stainless steel guide cannulae (22 gauge; Plastics One) targeting the area just dorsal to the BLA (AP –3.0 mm, ML ±5.3 mm, DV –7.9 mm, relative to bregma; Paxinos and Watson, 1998). Guide cannulae were secured to the skull with 3 miniature screws and dental acrylic resin. Dummy cannulae were inserted the length of the guide cannulae to maintain patency. Rimadyl (5 mg/kg; s.c.) was administered for the first two days after surgery. Catheters were kept patent by daily infusions of 0.1 ml of an antibiotic solution of cefazolin (10.0 mg/ml) dissolved in heparinized saline (30 USP heparin/ml).

A.2.5 Cocaine self-administration procedures

Rats administered cocaine during daily sessions for 1 h, on a fixed ratio 1 (FR1) schedule of reinforcement with a 10 s timeout. The designated active lever (counterbalanced across left and right levers) produced a cocaine infusion paired with a 10 s tone-light conditioned stimulus (CS). Pump durations were adjusted daily according to body weight in order to deliver the correct dose of drug (1.0 mg/kg of body weight per infusion). Responses on the other, inactive, lever were recorded, but had no programmed consequences. Rats underwent training for at least 10 d and until they administered at least 8 infusions per day over 3 consecutive days. Rats that did not meet acquisition criteria by 14 d were excluded from the study. The program was controlled by and data were collected using MedPC (MedAssociates).

A.2.6 Instrumental lever extinction

After successful acquisition of self-administration, rats underwent instrumental lever extinction for at least 7 d. During these daily 1 h sessions, responses on both the active and inactive levers were recorded, but had no programmed consequences. Lever extinction continued until extinction criteria had been met (an average of < 25 lever presses on the last two days of extinction). Throughout lever extinction, rats received no cocaine or cocaine-associated cue reinforcement. Lever extinction was conducted to reduce responding to a stable, low rate to later assess cue-induced reinstatement. In addition, lever extinction reduces the motivational value of other cues in the self-administration context, such as the levers, so that subsequent testing specifically isolates the memory for the discrete cue associated with cocaine infusion.

A.2.7 Identification of cellular signaling events regulated by cocaine-associated memory extinction and reconsolidation

Memory manipulations. Following cocaine self-administration and instrumental lever extinction, rats were assigned to a memory manipulation group (extinction, memory reactivation, or no manipulation controls) based on a matching procedure that ensured that each group had no statistical differences in their cocaine infusions acquired over days, or differences in lever extinction behavior. All groups were placed in the opposite context from which they received self-administration training (different in flooring texture, shape, and smell), for a 30 min session on 2 consecutive days. During these sessions, rats had no opportunity for instrumental responding, i.e., the levers were retracted.

Extinction: For cue extinction, the cocaine-associated CS was presented for 10 s, 60 times, with each presentation separated by 30 s, on each of the two days. Thus, the rats were exposed to a total of 120 CS presentations, which we have previously shown to significantly reduce cue-induced reinstatement on a subsequent test day (Torregrossa et al., 2010).

Memory Reactivation: For cue reactivation, the CS was presented 3 times at the end of the last session on the second day, with each CS presentation separated by 1 min. Previous work from our lab has shown that 3 CS presentations is sufficient to induce memory reactivation and reconsolidation, but is not sufficient to produce extinction (Sanchez et al., 2010; Wan et al., 2014).

No Memory Manipulation Control: The control group was placed in the operant chambers for the same amount of time as rats in the cue extinction and reactivation groups, but with no CS presentations. Thus, the time spent in the operant boxes and the type of operant box was equivalent between groups prior to sacrifice. Fifteen minutes following memory manipulations, rats were lightly anesthetized with isofluorane to minimize stress prior to euthanasia by focused microwave irradiation. Focused microwave irradiation was used to preserve the phosphorylation state of proteins. Importantly, because no group had the opportunity to make instrumental responses prior to sacrifice, differences in behavioral activity should not substantially affect levels of protein phosphorylation. The brains were immediately dissected and individual brain regions, including the basolateral amygdala complex were obtained and stored at -80 C until processing.

Label-free quantitative proteomics: sample preparation. Brain regions of interest, including the amygdala reported here, were homogenized by sonication in a buffer containing urea (8 M), ammonium bicarbonate (0.4 M), and phosphatase inhibitor cocktails (Sigma). Samples from 2

rats in each experimental group were randomly pooled to create a total of 4-5 sample pools per group. The extinction group included one sample "pool" that consisted of just one rat due to an odd number of rats meeting acquisition criteria. Pooled samples were then analyzed by the Yale/NIDA Neuroproteomics Center at Yale. 20 µL of 45 mM DTT was added to each sample and incubated at 37 ºC for 20 min to reduce Cys residues. Samples were cooled and 20 µL of 100 mM iodoacetamide (IAM) was added to each sample and incubated at RT in the dark for 20 min for alkylation of the reactive free sulfhydro of the reduced Cys. Dual enzymatic digestion was carried out by adding 600 µL of dH2O and 30 µL of 1 mg/mL Lys C followed by incubation at 37 ºC for 4 hrs, with subsequent digestion by incubation with 30 µL of 1 mg/mL trypsin overnight at 37 ºC. Samples were macrospin desalted and dried by speedvac. Pellets were dissolved in 50 µL of a solution containing 0.5% TFA and 50% acetonitrile. Samples were then subjected to titanium dioxide (TiO2) phosphopeptide enrichment using TopTips (Glygen, Columiba, MD). The TopTip was washed 3 times at 2000 rpm for 1 min with 40 µL 100% acetonitrile, then 0.2 M sodium phosphate (pH 7.0), 0.5% TFA, and 50% acetonitrile. The acidified digest supernatants were loaded into the TopTip, spun at 1,000 rpm for 1 min, and then at 3,000 rpm for 2 min. The flow through from these washes (less phosphorylated fraction) was saved for analysis by LC-MS/MS as described below. Phosphopeptides were eluted from each TopTip by 3 washes with 30 µL of 28% ammonium hydroxide. Both the flow through and eluted fractions were dried by speedvac. Enriched fractions were dissolved in 10 µL of 70% formic acid and 30 µL of 50 mM sodium phosphate. Peptide concentrations were determined by Nanodrop to load 0.3 µg/5µL of each sample.

LC/MS-MS. 5 µL of each sample was injected onto a LTQ Orbitrap LC-MS/MS system. Peptide separation was performed on the nanoACQUITY™ ultra-high pressure liquid chromatography

(UPLC™) system (Waters, Milford, MA), using a Waters Symmetry® C18 180 µm x 20 mm trap column and a 1.7 µm, 75 µm x 250 mm nanoACQUITY™ UPLC™ column (35 ºC). Trapping was done at 15 µL/min, with 99% Buffer A (0.1% formic acid in water) for 1 min. Peptide separation was performed over 120 min at a flow rate of 300 nL/min beginning with 95% Buffer A and 5% Buffer B (0.075% formic acid in acetonitrile) to 40% B from 1–9 min, to 85% B from 9-91 min, held at 85% B from 91-95 min, then returned to 5% B from 95-96 min. Two washes were made between each sample run to ensure no carry over (1. 100% acetonitrile, 2. Buffer A). The LC was in-line with an LTQ-Orbitrap mass spectrometer. MS was acquired in the Orbitrap using 1 microscan, and a maximum inject time of 900 ms followed by 3-6 data dependent MS/MS acquisitions in the ion trap (with precursor ion threshold of >3000). The total cycle time for both MS and MS/MS fragmentation by collision induced dissociation (CID) were first isolated with a 2 Da window followed by normalized collision energy of 35%. Dynamic exclusion was activated where former target ions were excluded for 30 sec. Three technical replicates were injected for each sample and all samples and replicates were randomized across an entire run time.

Data analyses. Feature extraction, chromatographic/spectral alignment, data filtering, and statistical analysis used Nonlinear Dynamics Progenesis LC-MS software (www.nonlinear.com). Raw data files were imported into the program and detected mass spectral features were aligned based on retention time of the detected m/z peaks based on a randomly selected reference run. All other runs were automatically aligned to the reference run to minimize retention time (RT) variability between runs. No adjustments were necessary in the m/z dimension due to high mass accuracy of the spectrometer (typically < 3 ppm). All runs were selected for detection with an automatic detection limit. Features within retention time ranges of 0-5 min were filtered out, as

were features with charge state greater than +6 or singly charged peptides (as no MS/MS fragmentations were taken for these charge states during data collection) for reduction of false positive peptide assignments. A normalization factor was then calculated for each run to account for differences in sample load between injections. The experimental design grouped multiple injections from each condition. The algorithm then calculated and tabulated raw and normalized abundances, max fold change, and Anova p values for each feature in the data set. Stringent conditions were set in MASCOT to filter out low scoring identified peptides by imposing a confidence probability score (p) of < 0.05. Additionally a positively identified protein that was quantified contained at least two unique identified tryptic peptides. The filtered MS/MS spectral features along with their precursor spectra were exported in the form of an .mgf file (Mascot generic file) for database searching using the Mascot algorithm (Hirosawa et al., 1993). The data was searched against the Uniprot (Rattus norvegicus) database. The confidence level was set to 95% within the MASCOT search engine for peptides assigned hits based on randomness. MS/MS analysis was based on the use of trypsin and the following variable modifications: carboamidomethyl (Cys), Oxidation (Met), Phospho (Ser, Thr, Tyr). Other search parameters included peptide mass tolerance of ± 15 ppm, fragment mass tolerance of ± 0.5 Da, and maximum missed cleavages of 3. A decoy search (based on the reverse sequence search) was performed to estimate False Discovery Rate (FDR), with setting of acceptable protein ID having FDR of 2%. Using the Mascot database search algorithm, a protein is considered identified when Mascot lists it as significant (bold red) and more than 2 unique peptides match the same protein. The Mascot significance score match is based on a MOWSE score and relies on multiple matches to more than one peptide from the same protein. The Mascot search results were exported to an .xml file using a significance cutoff of < 0.05, and ion score cutoff of 28, and a

requirement of at least one bold (first time any match to the spectrum has appeared in the report) and red (top scoring peptide match for this spectrum) peptide. The .xml file was then imported into the Progenesis LCMS software, where search hits were assigned to corresponding detected features, identified as described above.

SRM proteomics. Identified peptides and their modifications were mined to a list of approximately 80 unique peptides for quantitative analysis between groups by selective reaction monitoring (SRM). The list was created based on ionization signal quality and to reduce the number of high abundance, highly modified phosphoproteins (e.g., neurofilament proteins) and proteins with unclear function. Analyses were carried out on a 5500 Q-TRAP instrument coupled online to a Waters nanoACQUITY™ UPLC™ system. Four µL of each sample was loaded onto a 5 µm, 180 µm x 20 mm Symmetry C18 nanoAcquity trapping column with 100% water/0.1% formic acid at a flow rate of 15 µL/min for 1 min. Peptides were then separated on a 1.7 µm, 75 µm x 100 mm BEH130 C18 nanoAcquity column with a 30 min, 2-40% acetonitrile/0.1% formic acid linear gradient at a flow rate of 0.75 µL/min. SRM scanning was conducted using 211 transitions and a cycle time of 2.4 s with a 5 ms dwell time per transition (5 transitions/peptide) in positive polarity. Data were processed using MRMPilot 2.0, Analyst 1.5 with MIDAS, and Multiquant 2.0 software. Peptide identification was further confirmed using MASCOT. Data were analyzed using nested linear models with transition nested within peptide. Interaction effects were included in the model and significant group x transition effects within a peptide were used to identify outlying transitions for exclusion from further analysis. Statistics were calculated using SRMstats with restricted scope (freely available in R (Chang et al., 2012). We report phosphopeptides with significantly different abundances in either the extinction or reactivation group relative to control with $P < 0.05$ considered significant after correcting for

multiple comparisons. The statistical methods used here, as described by Chang et al., are robust in detecting statistical differences from SRM experiments, particularly when there is little underlying biological variability in the protein abundance. This statistical method was chosen for hypothesis generation, as the primary aim of the present study was to identify phosphorylation events that are bi-directionally regulated by the extinction vs. reconsolidation of cocaine-associated memories. The analysis was therefore biased towards potentially identifying false positives rather than creating false negatives. We also include analytical results using a more conservative linear mixed effects model for comparison in Table 1. Within the table, the first column lists the Uniprot defined protein abbreviations (not gene names), the second column shows the peptide sequence identified with lower case “p”s indicating sites of phosphorylation and “ox” indicating oxidation of Methionine. The next set of 6 columns (orange) gives data for the extinction group relative to control, and the next 6 columns (purple) represent data from the reactivation group relative to controls. Within in each group, the green columns are the results from the SRM stats analysis, while the blue columns are the linear mixed effects model analysis. Each analysis includes the unadjusted p-value, the estimated fold change from controls, and the p-value after correcting for multiple comparisons.

A.2.8 Assessment of effect of CaMKIIα phosphorylation at S331 on catalytic activity

Site-directed mutagenesis. CaMKIIα phospho-deficient (serine 331 to alanine; S331A) and phospho-mimetic (serine 331 to glutamate; S331E) mutants were created using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The PCR mixture

contained 50 ng template plasmid DNA (pCMV6-CaMKIIα-Myc-DDK plasmid; Origene, RR201121), 125 ng of each oligonucleotide

(For: GGCGTGAAGGAATCCGCTGAGAGCACCAACACC)

(Rev: GGTGTTGGTGCTCTCAGCGGATTCCTTCACGCC),

1 μL dNTP mix, 2.5 U PfuUltra DNA polymerase, 5 μL 10X Reaction buffer, and 3 μL Quiksolution reagent in a final volume of 50 μL. PCR was performed under the following conditions; denaturation at 95°C for 50 sec, annealing at 60°C for 50 sec and extension at 68°C for 1 min/kb. The PCR product was digested with 10 U of DpnI for 1 h at 37°C and then transformed into 45 μL of XL10-Gold Ultracompetent E. coli cells. Presence of the mutation was confirmed by DNA sequencing of the construct.

Cell culture and plasmid transfection. HEK293T cells were maintained in culture in a humidified 5% CO2 atmosphere at 37°C in medium consisting of DMEM/F12 supplemented with 10% fetal bovine serum, and penicillin-streptomycin. Transfections were carried out using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA). HEK293T cells were seeded in 6-well plates at 3 X 105 cells per well, grown for 24h and then incubated overnight with 2 mL of serum-free medium containing 5 μg wildtype (WT) or mutant pCMV6-CaMKIIα expression vectors, and 7.5 μg Lipofectamine. Control cells were incubated with a plasmid-free mixture of medium and Lipofectamine. After overnight incubation, the medium was replaced by fresh medium supplemented with 500 μg/μL geneticin (Invitrogen) to select for transfected-cells. To determine basal levels of CaMKIIα expression in HEK293T cultures, a subset of the control cells did not receive geneticin treatment.

Immunoblot and kinase activity assay. HEK293T whole-cell lysates were prepared by washing cultures with ice-cold 1X PBS followed by treatment with ice-cold lysis buffer containing

protease inhibitors (1:100). Cells were collected and incubated on ice for 30 min, then centrifuged at 16,000 g for 20 min at 4°C. Supernatants from two 6-well plates were collected and pooled together, and protein content was quantified using the Pierce BCA assay kit. Supernatants with equal amounts of protein (10 μg) were resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dairy milk in PBST (phosphate-buffered saline + 0.1% Tween 20). Membranes were then probed with the following primary antibodies: Rabbit Anti-CaMKII (Millipore07-1496, 1:500; Millipore, Billerica, MA) and Mouse Anti-GAPDH (Millipore MAB374, 1:1000). Goat Anti-Rabbit IgG (Li-Cor 926-32211, 1:5000; Li-Cor, Licoln, NE) and Goat Anti-Mouse IgG (Li-Cor 926-68070, 1:5000) secondary antibodies were used. Odyssey Infrared Imager (Li-Cor) was used for the detection of protein bands. Kinase activity was quantified following the instructions of the ADP-GloTM Kinase Assay (Promega, Madison, WI) in the presence of CaCl2, MgCl2, calmodulin, and the CaMKII selective substrate, Autocamtide-2. For each reaction, relative amounts of protein supernatant (~2.5 μg) were loaded based on total protein levels in the lysate, corrected for loading of GAPDH. Reactions were initiated by the addition of 250 μM ATP then incubated for 15 min at 30°C. Reactions were terminated and the unconsumed ATP depleted by the addition of ADP-Glo Reagent, then incubated for 40 min at RT. Kinase Detection Reagent was then added to each reaction to convert ADP back to ATP while also introducing luciferase and luciferin to detect ATP. This was followed by another 40 min incubation at RT. Luminescence was measured using the FLx800 Multi-Detection Microplate Reader (BioTek, Winooski, VT) Using a linear standard curve, the amount of depleted ADP was determined, and these values were converted to specific kinase activity.

A.2.9 CaMKII inhibition and reinstatement of cocaine seeking

Memory manipulations. Memory manipulations took place as described in the proteomics experiment above with the following differences. Pavlovian cue extinction was conducted in the same context in which rats received self-administration training, and took place for either a single 30 min session (moderate extinction) or a 30 min session on 2 consecutive days (extended extinction). For cue reactivation, rats were placed in operant chambers, in the opposite context from which they were trained, for a single memory reactivation session. During this session, the cocaine-associated cue was presented for 10 sec, 3 times, with each presentation separated by 1 min. Control rats were again placed in the opposite context form which they were trained, but were never presented with the cocaine-associated cues.

Intracranial infusions. Drugs were administered in a volume of 0.5 µl/hemisphere. KN-62 was given at one of two doses: 340 or 680 ng/side. KN-93 was given at either 5 or 10 µg/side. These doses were based on effective doses in prior publications using intracranial infusions (Rodrigues, 2004; Sakurai et al., 2007). Infusions were given immediately following the memory manipulations, by removing dummy cannuale and inserting injection cannulae (28 gauge; Plastics One; Roanoke, VA) that extended 1 mm beyond the guide cannulae. The injectors were connected to Hamilton syringes (Hamilton Robotics, Reno, NV) controlled by a syringe pump via polyethylene tubing. Infusions were given over the course of 2 min and injectors were left in the cannulae for an additional 1 min to allow for drug diffusion.

Cue-induced reinstatement. 24 h after the final Pavlovian cue extinction session or memory reactivation session, cue-induced reinstatement was assessed during a 1 h session that took place in the original self-administration context. A lever press on the active lever produced a 10 sec

presentation of the cocaine-associated cue on an FR1 schedule, but no drug reinforcement. Lever presses on the inactive lever were recorded but had no programmed consequences.

Histological analysis. After the completion of experiments, rats with intracranial cannulae were sacrificed via decapitation. Brains were dissected and placed in 10% formalin for at least 3 d then transferred to 30% sucrose for at least 3 d. Brains were then frozen and sectioned coronally through the BLA on a cryostat. Sections were taken at 50 µm and placed on slides for visualization of infusion placements. The investigator was blind to treatment group when analyzing histology, and animals with infusions outside of the BLA were removed from the main analysis. Schematic of acceptable infusion locations is seen in **Figure 25**.

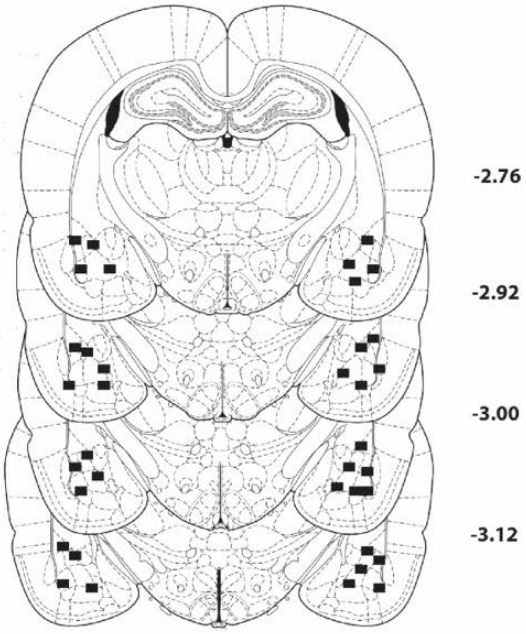

Figure 25. Histological representation of infusion locations in the BLA.

The black squares represent the outermost range in the medial-lateral and dorsal-ventral planes that were considered a hit in the BLA, all other placements were located within these bounds. Numbers by each section represent millimeters from bregma.

A.2.10 Code availability

All behavioral training and testing was conducted using custom MedPC programs. The computer code used is available to researchers upon request.

A.2.11 Statistical analyses

Behavioral and kinase activity data were analyzed using GraphPadPrism for Windows. For proteomic data, the normalized average intensity across transitions for each phosphopeptide was computed for each sample and these values were averaged within groups. Group averages were compared statistically using a SRMstats restricted analysis and p-values corrected for multiple comparisons. For immunoblot data, intensities of protein bands were quantified using Odyssey Imaging Software ver 3.0. Total CaMKII amount was normalized based on the signal intensity of GAPDH and expressed as %WT. For kinase activity data, samples were run in duplicate. Luminescence was normalized to a 'no-substrate control' and converted to kinase specific activity based on a standard conversion curve. Data was analyzed using a one-way ANOVA. Reinstatement tests were analyzed by two-way ANOVA with repeated measures, with the between-subjects factor being responding on the last day of lever extinction versus reinstatement responding and the within-subjects factor being the dose of drug. For the no reactivation control group, vehicle groups were statistically similar, and therefore collapsed across treatment groups. For the anatomical control experiment, data were analyzed using an independent samples t-test. For all analyses, significant effects were further analyzed by Bonferroni's post hoc tests, with significance set at $P < 0.05$. All data were determined to be normally distributed using the

Shapiro-Wilk test, and Bartlett's test was used to determine that there were no significant differences in the estimated variance between groups.

A.3 RESULTS

A.3.1 Identification of novel signaling events that are regulated by cocaine-cue memory extinction and reconsolidation

To identify candidate signaling events that are regulated in opposition by extinction vs. reconsolidation of a memory associated with self-administered cocaine, we employed a high resolution tandem mass spectrometry-based phosphoproteomics approach, where we examined differential protein phosphorylation events after memory manipulations as an index of increased or decreased protein activity. Rats were trained to self-administer cocaine paired with an audiovisual cue (conditioned stimulus (CS)) for 10 days, followed by 5-7 days of extinction of instrumental lever responding (**Figure 26A**). Each rat was assigned to a memory manipulation group in a random manner that ensured no training differences between groups. There were no significant differences or interactions with day of training for infusions earned (**Figure 26B**) or active lever presses during extinction (**Figure 26C**; two way repeated-measures ANOVA: $P > 0.05$, n = 7-10 rats per group). No statistical differences were observed for active lever presses during acquisition or inactive lever presses at any stage (data not shown). Following lever extinction, rats were exposed to one of three memory conditions: reactivation, extinction, or

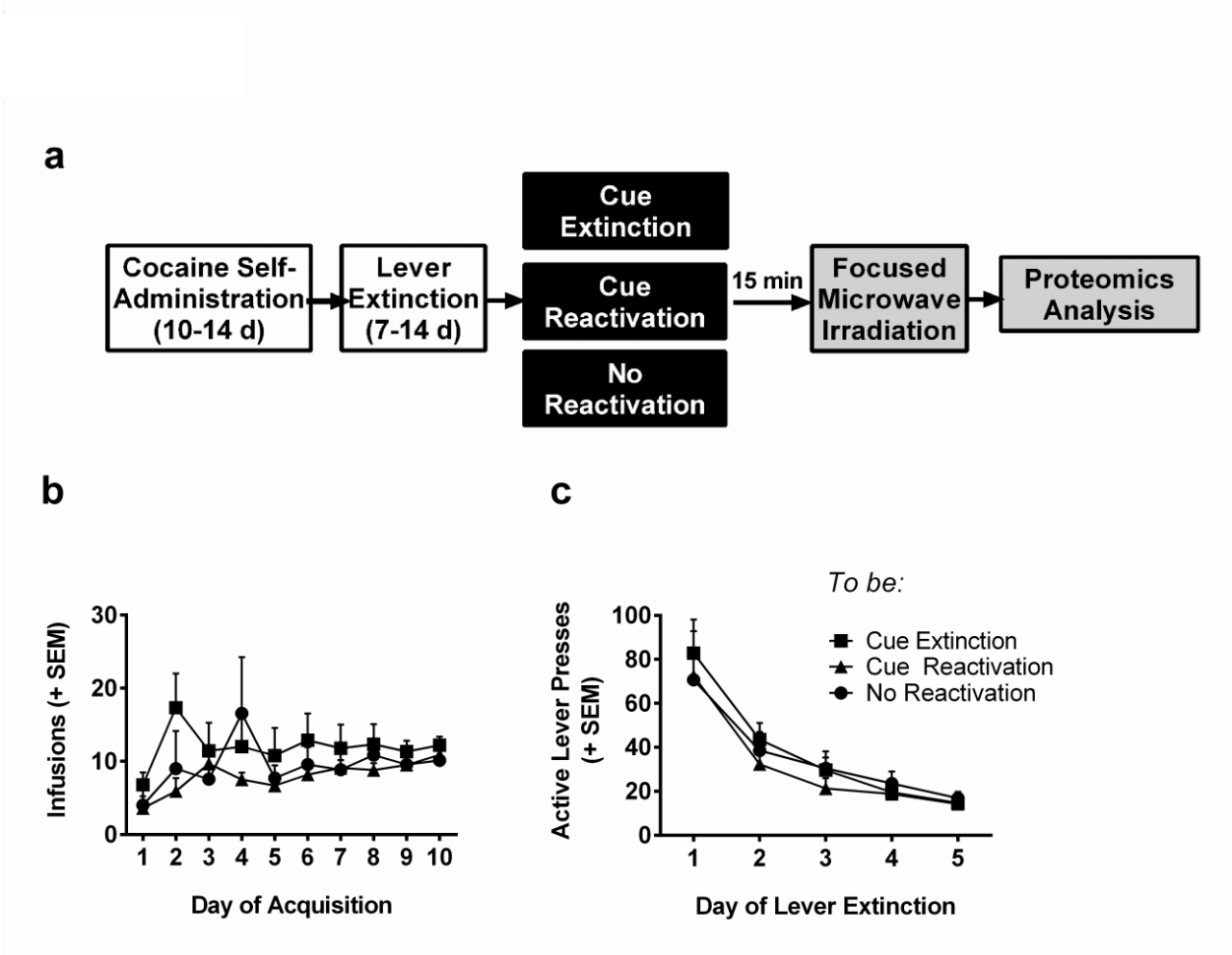

Figure 26. Self-administration and extinction training data from rats used for phosphoproteomic analysis. (**A**) Timeline for proteomics experiments. (**B**) Mean number of cocaine infusions per day during acquisition and (**C**) mean number of active lever presses per day during instrumental extinction in rats prior to memory manipulations. There were no differences in acquisition or instrumental extinction between manipulation groups (both $p > 0.05$, n = 7, 9, and 10 rats, respectively, for control, extinction, and reconsolidation). Data are expressed as mean + s.e.m.

control context exposure. Fifteen min following the memory manipulation, rats were euthanized by focused microwave irradiation to maintain protein post-translational modifications, and the brains were dissected for proteomics analysis. Fifteen min was chosen as it is thought to represent the time when many of the intracellular signaling cascades regulating the extinction or reconsolidation of a memory would be active. Here we describe results from the analysis of the BLA, as it is the locus for associative learning and mediates the encoding of drug-associated memories (Schafe and LeDoux, 2000; See, 2005).

Total homogenates were enriched for phosphopeptides, and discovery-based, label free quantitative analysis was used to identify putative memory-regulated proteins. A large number of phosphopeptides were detected corresponding to 355 unique proteins. From this list of phosphopeptides, we pseudorandomly chose ~80 phosphopeptides for quantitative validation by selective reaction monitoring (SRM) mass spectrometry. We prioritized the phosphopeptides to be chosen by selecting those with robust ionization intensity that were likely to provide clear MS signals and to exclude highly abundant, heavily phosphorylated proteins, such as neurofilaments and microtubule associated proteins. Phosphorylation of these proteins are potentially relevant to memory processes, but because of their abundance and high degree of phosphorylation, they may be over-represented in the analysis. A total of 72 unique phosphopeptides had sufficient data quality from the SRM experiment for data analysis. **Table 1** contains the statistical analysis, as described in the methods, and estimated fold change in the level of phosphopeptides in the extinction and reactivation groups relative to the control. **Figure 27** summarizes the data from all phosphopeptides that showed a significant change from control when analyzed using SRMstats with a significance cut-off of $p < 0.05$ after correction for multiple comparisons. Each panel is organized to illustrate the phosphopeptides significantly regulated by both extinction and

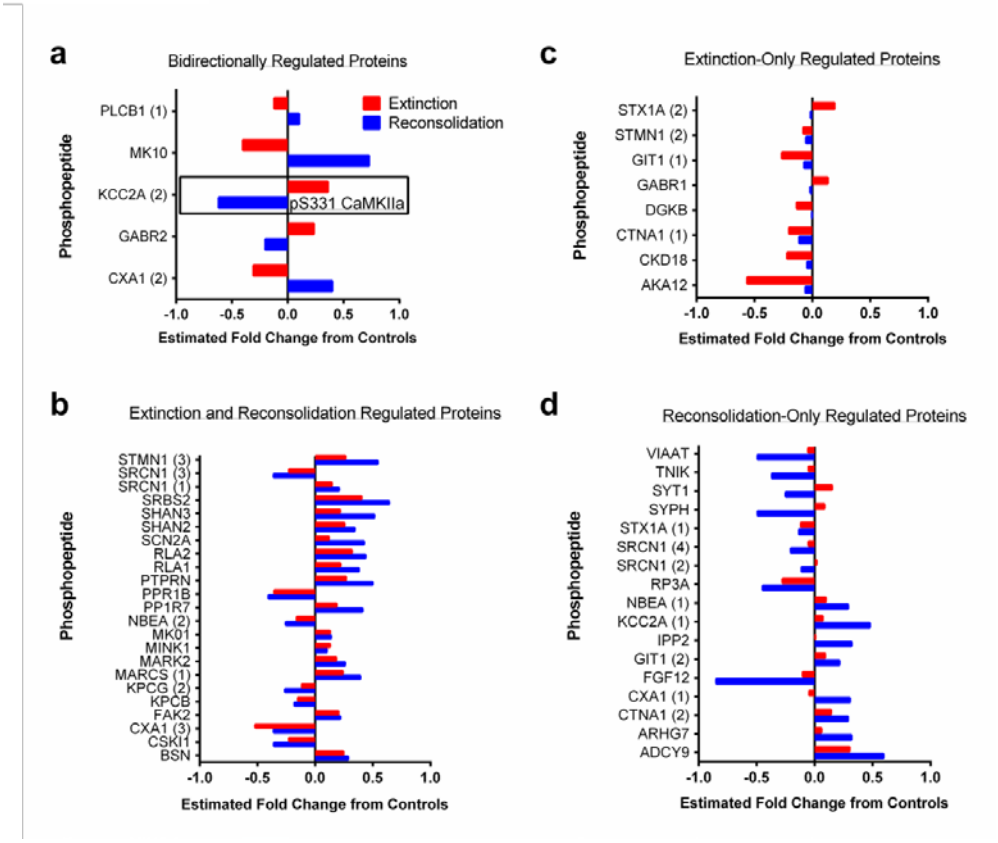

Figure 27. Identification of signaling pathways regulated by cocaine-associated memory extinction and reconsolidation.

(**A-D**) Phosphoproteomic analysis from tissue collected 15 min following memory manipulations reveals several phosphopeptides that were significantly regulated (**A**) in the opposite direction by extinction vs. reconsolidation, (**B**) in the same direction by both memory conditions, (**C**) by memory extinction only (only red bars significantly different from control), or (**D**) by memory reconsolidation only (only blue bars significantly different from control). The y-axes list the Uniprot database protein abbreviation. The number in parenthesis following an abbreviation is the ID we assigned when multiple unique phosphopeptides were identified from the same protein. Bars represent the estimated fold change in phosphopeptide abundance relative to non-memory manipulated controls. Positive values indicate significant increases in the abundance of the phosphopeptide, and negative values decreased abundance. All $p < 0.05$ relative to control after correction for multiple comparisons using SRMstats for statistical analysis of SRM data (Chang et al., 2012).

reconsolidation in the opposite direction (**Figure 27A**); those significantly regulated in the same direction by both conditions (**Figure 27B**); those only significantly regulated in the extinction condition (**Figure 27C**); and those only significantly regulated in the reactivation condition (**Figure 27D**). Of greatest interest for our study were the 5 phosphopeptides regulated in the opposite direction by extinction vs. reconsolidation, as these could represent targets that can both enhance extinction while inhibiting reconsolidation to ensure sufficient weakening of a drug-associated memory. These phosphopeptides included CaMKIIα (KCC2A, pS331), c-Jun N-terminal kinase 3 (JNK3 or MK10, pT221+Y223), the gap junction protein connexin 43 (CXA1, pS328+pS330), the GABAB receptor subunit 2 (GABR2, pS883), and phospholipase C beta 1 (PLCB1, pS988). Interestingly, CaMKIIα, phospholipase C, and GABAB receptors are important regulators of Ca2+ signaling (Chalifoux and Carter, 2011; Lyon and Tesmer, 2013), and JNK3 activation can be regulated by PLC (Buckley et al., 2004), indicating a convergence of opposing calcium-related cellular events dependent on whether a cocaine memory is reconsolidated or extinguished.

A.3.2 Phosphorylation of CaMKII at S331 decreases catalytic activity

CaMKIIα is known to be an important regulator of learning and memory (Coultrap and Bayer, 2012; Sanhueza and Lisman, 2013), and is an abundant protein, thus it is not surprising that CaMKIIα was identified in our analysis. Yet, somewhat surprisingly, the differentially regulated phosphorylation event on CaMKIIα was on residue S331. S331 phosphorylation was significantly increased after memory extinction, but was significantly decreased after memory reactivation (**Figure 28A**). To our knowledge, S331 phosphorylation has never been previously

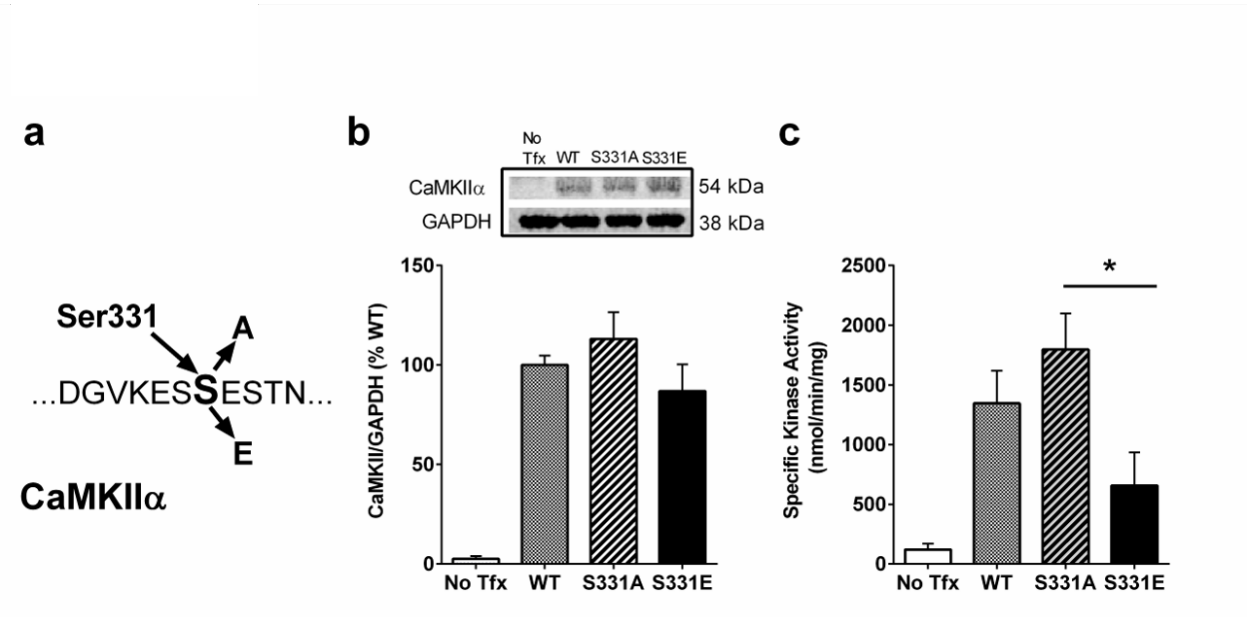

Figure 28. Phosphorylation of CaMKIIα at S331 inhibits enzyme activity.

(**A**) Schematic representation of site-directed mutagenesis of CaMKIIα at S331 to generate phospho-deficient (S331A) or phospho-mimetic (S331E) mutants. (**B**) Wild-type (WT), S331A, and S331E plasmids were expressed in HEK293T cells. Supernatants were collected and CaMKIIα expression was quantified via immunoblot, normalized to GAPDH, and expressed as %WT. Results indicate a lack of CaMKIIα in the non-transfected control (No Tfx) condition, but similar expression of all recombinant forms of CaMKIIα (n = 8 samples per group). Representative immunoblots are shown in the upper panel. (**C**) Quantification of CaMKII activity from whole cell lysates. Kinase activity was regulated by the phospho-mimetic mutation of S331 (n = 8 samples per group). Catalytic activity of the CaMKIIα S331E mutant was significantly lower than that of the S331A mutant . Data are expressed as mean + s.e.m. $*p < 0.05$.

reported in relation to learning and memory or any other biological process. However, one recent study has found that S331 is a potential autophosphorylation site (Baucum et al., 2015). Therefore, we proceeded to determine if phosphorylation of S331 regulates the enzymatic activity of CaMKIIα. Phospho-deficient (S331A) and phospho-mimetic (S331E) forms of CaMKIIα DNA were generated (**Figure 28A**) and, along with a wild-type (WT) form, expressed in HEK293T cells. The absence of endogenous CaMKIIα in HEK293T cells, as previously reported (Jenkins et al., 2010; Tsui et al., 2005), was confirmed by immunoblot analysis, while expression of all recombinant forms of CaMKIIα was comparable (**Figure 28B**; one-way ANOVA: $F_{(3,28)} = 25.84$, $P < 0.001$, n = 8 samples per group). CaMKIIα activity in whole-cell lysates was measured in the presence of Ca2+, calmodulin, ATP, and the CaMKIIα selective substrate, autocamtide-2. Kinase activity differed across plasmid types (**Figure 28C**; one-way ANOVA: $F_{(3,28)} = 8.714$, $P < 0.001$, n = 8 samples per group). The catalytic activity of phospho-mimetic CaMKIIα (S331E) was significantly lower than that of the S331A mutant enzymes ($P < 0.05$). The kinase activity of WT CaMKIIα was between the levels observed for S331A- and S331E-mutated lysates. Although the phosphorylation state of the WT CaMKIIα at S331 is unknown, it is likely that the total protein contains a mixture of phosphorylated and non-phosphorylated S331, and as an autophosphorylation site, had the potential to be phosphorylated in the presence of Ca2+, calmodulin, and ATP. However, our results suggest that in HEK293 cells, WT CaMKIIα is predominantly not phosphorylated, as kinase activity more closely mimicked that of the phospho-deficient mutant.

A.3.3 Inhibition of CaMKII in the BLA enhances drug-cue memory extinction

Based on the apparent increase in an inhibitory phosphorylation event at S331 on CaMKIIα after extinction, and a decrease in phosphorylation after reactivation, we hypothesized that memory reconsolidation must require active CaMKIIα in the BLA, while extinction requires a deactivation of CaMKIIα. Therefore, we investigated if CaMKII inhibition could both inhibit drug-cue memory reconsolidation and enhance extinction to reduce relapse-like cue-induced reinstatement. For this set of experiments, rats again underwent standard cocaine self-administration and lever extinction training before placement into memory manipulation groups. Following memory manipulations rats received immediate infusion of one of two doses of two related CaMKII inhibitors (KN-93 or KN-62) or the vehicle directly into the BLA, and the propensity to reinstate cocaine-seeking in response to the cue was assessed 24 h later (**Figure 29A**). For all experiments involving CaMKII inhibition, there were no pre-existing differences between drug-treatment groups in number of reinforcers earned, active lever responding, or inactive lever responding during acquisition or lever extinction (KN-93: **Figure 29B**; KN-62: **Figure 29C**; all $P > 0.05$, n = 5-11 rats/group). This ensures that any memory manipulation or drug effect on cue-induced reinstatement was not due to pre-existing differences in propensity to self-administer drug or extinguish drug seeking.

Pavlovian cue extinction has previously been demonstrated as an effective method for reducing drug-cue motivated behavior (Torregrossa et al., 2010, 2013)). To determine if the effects of cue extinction on reinstatement could be amplified by inhibiting CaMKII, rats were given intra-BLA infusions of a CaMKII inhibitor immediately following two types of extinction training: 60 vs. 120 CS presentations. In this manner, we could determine if CaMKII inhibition

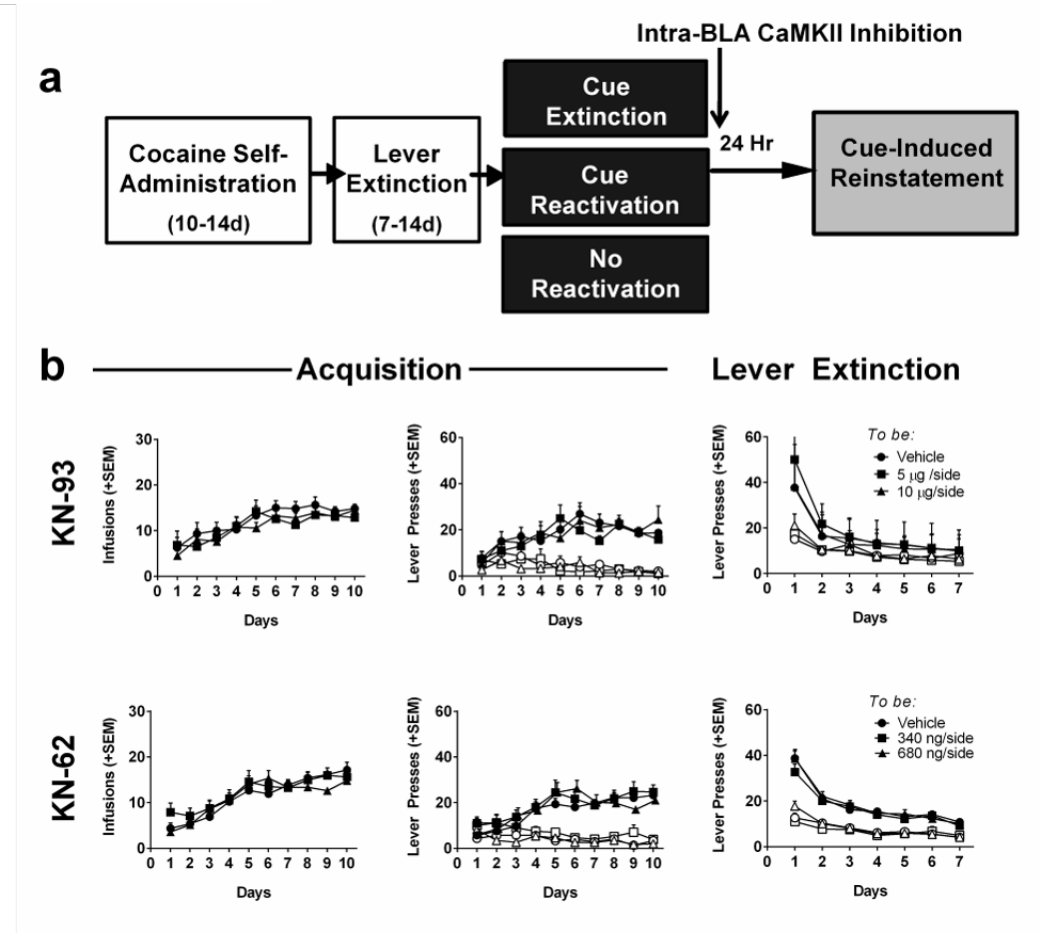

Figure 29. No differences in training data between groups prior to memory manipulations/BLA infusions.

(**A**) Experimental timeline. Rats received bilateral infusions of one of two CaMKII inhibitors (KN-93 or KN-62) or vehicle into the BLA immediately following memory manipulation sessions. 24 h later, rats were tested for cue-induced reinstatement under drug free conditions. Data are displayed separately for groups treated with the CaMKII inhibitor KN-93 (**B**) or KN-62 (**C**). *Left panels:* Mean number of cocaine infusions per day during acquisition. *Center panels:* Mean number of active (solid shapes) and inactive (open shapes) lever presses per day during acquisition. *Right panels:* Mean number of active and inactive lever presses per day during instrumental extinction. For simplicity, data is grouped via dose of CaMKII inhibitor, collapsed across memory manipulation. There are no differences, in any measures, during acquisition or instrumental extinction between treatment groups for any of the memory manipulations (all $p > 0.05$). Data are expressed as mean + s.e.m.

could facilitate sub-optimal (moderate), as well as extended, extinction training. All cue extinction and reinstatement testing occurred in the same context to specifically understand extinction consolidation processes, rather than contextual encoding or renewal. CaMKII inhibition with either KN-93 or KN-62 following moderate cue extinction reduced subsequent cue-induced reinstatement (**Figure 30A**; KN-93: $F_{(2,15)} = 6.671$, $P = 0.009$, n = 5-7 rats per group; KN-62: $F_{(2,17)} = 5.036$, $P = 0.019$, n = 6-7 rats per group; both two-way ANOVAs). These data suggest that CaMKII inhibition after extinction training can enhance the effects of moderate cue extinction training to reduce reinstatement. On the other hand, an extended cue extinction-duration protocol (120 cues) immediately followed by infusion of the CaMKII inhibitor KN-62, failed to reduce reinstatement relative to vehicle-treated controls. As a whole, all rats that experienced extended cue extinction in the self-administration context exhibited the expected extinction-induced reduction in reinstatement that could not be further potentiated by CaMKII inhibition (**Figure 30A**; two way ANOVA: $P > 0.05$, n = 6-8 rats per group), indicating that 120 CS presentations likely produces a floor effect. These results are similar to previously published work demonstrating that extended cue extinction in the self-administration context could not be potentiated by treatment with D-cycloserine (DCS), even though DCS can promote extinction learning under other conditions (Torregrossa et al., 2010).

A.3.4 Inhibition of CaMKII in the BLA interferes with drug-cue memory reconsolidation

We then tested if CaMKII inhibition in the BLA following reactivation of the cocaine cue memory (3 CS presentations) could disrupt reconsolidation. After cocaine self-administration and lever extinction training, the cocaine-associated cue memory was reactivated in a novel

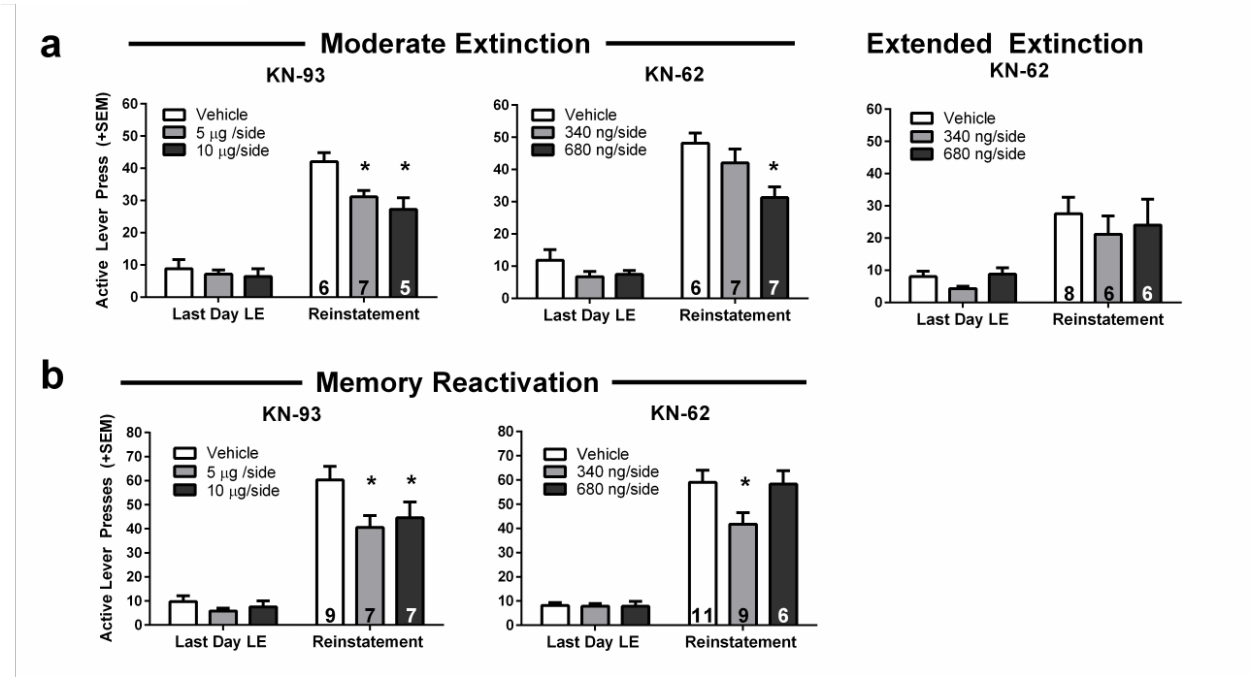

Figure 30. CaMKII inhibition enhances drug-cue memory extinction and disrupts memory reconsolidation to reduce cue-induced reinstatement.

(**A**) Mean number of active lever presses during cue-induced reinstatement, 24 h after extinction manipulations. CaMKII inhibition by KN-93 (left panel) and KN-62 (middle panel) enhanced the effects of a single (moderate) cue extinction session. Both doses of KN-93 and the 680 ng/side dose of KN-62 significantly decreased reinstatement in the moderate extinction condition. CaMKII inhibition by KN-62 (right panel) had no effect after extended extinction training ($p > 0.05$). (**B**) Mean number of active lever presses during cue-induced reinstatement, 24 h after memory reactivation manipulations. CaMKII inhibition by KN-93 (left panel) and KN-62 (right panel) disrupted drug-cue memory reconsolidation. Both doses of KN-93, but only the 340 ng/side dose of KN-62 reduced reinstatement. Data are expressed as mean + s.e.m.; number of rats per group indicated by number inside bars. *$P < 0.05$ versus vehicle.

context, followed by immediate infusion of CaMKII inhibitors into the BLA. Rats were then tested for cue-induced reinstatement of cocaine seeking 24 h later. KN-93 at both doses decreased active lever responding during reinstatement relative to VEH-treated controls, while only the low dose of KN-62 significantly reduced reinstatement (**Figure 30B**; KN-93: main effect: $F_{(2,20)} = 3.587$, $P = 0.047$, n = 7-9 rats per group; KN-62: significant interaction between treatments: $F_{(2,23)} = 4.413$, $P = 0.024$, n = 6-11 rats per group; both two-way ANOVA). Due to the consistency of effects with both doses of KN-93 and the low dose of KN-62, we suspect that the high dose of KN-62 produced non-specific effects that occluded any influence of CaMKII inhibition; however, we cannot rule out the possibility that there is a U-shaped dose effect function in the ability of CaMKII inhibition to interfere with memory reconsolidation. Nevertheless, together these data suggest that CaMKII may be important for the reconsolidation (restabilization) of the drug-cue memory following reactivation, and inhibition of CaMKII has the potential to interfere with this reconsolidation.

A.3.5 Intra-BLA CaMKII Inhibition does not affect reinstatement in the absence of memory manipulations

Finally, we wanted to confirm that the effects of CaMKII inhibition were selective to memory manipulations rather than non-specific effects on locomotion or motivation, and that inhibitor actions were mediated within the BLA. First, we determined whether the highest dose of either CaMKII inhibitor could affect cue induced reinstatement if it was given following placement of rats in a novel operant context without cue presentation or the opportunity to make an instrumental response (i.e., a no reactivation control experiment). Post-session treatment with the

high dose of either KN-93 or KN-62 had no significant effect on active responding during cue-induced reinstatement, indicating no effect on cocaine seeking in the absence of a memory manipulation (**Figure 31A**; Two-way ANOVA: $P > 0.05$, n = 5-9 rats per group). Therefore, effects of CaMKII inhibition on cue-induced reinstatement are likely due to specific actions on extinction and reconsolidation processes. Next, we determined if infusions of CaMKII inhibitors outside of the BLA had any effect on subsequent cue-induced reinstatement. We analyzed the data from animals whose vehicle or inhibitor infusion locations were found to lie outside of the BLA after histological analysis. We included rats whose infusion locations were dorsal, lateral, and medial to the BLA. We found no significant effect of CaMKII inhibitors infused after extinction or reactivation of the cocaine memory on cue-induced reinstatement if the infusions were outside of the BLA, suggesting that drug spread into other regions does not explain our behavioral results (**Figure 31B,C**; both unpaired t-test: $P > 0.05$, n = 5-8 rats per group).

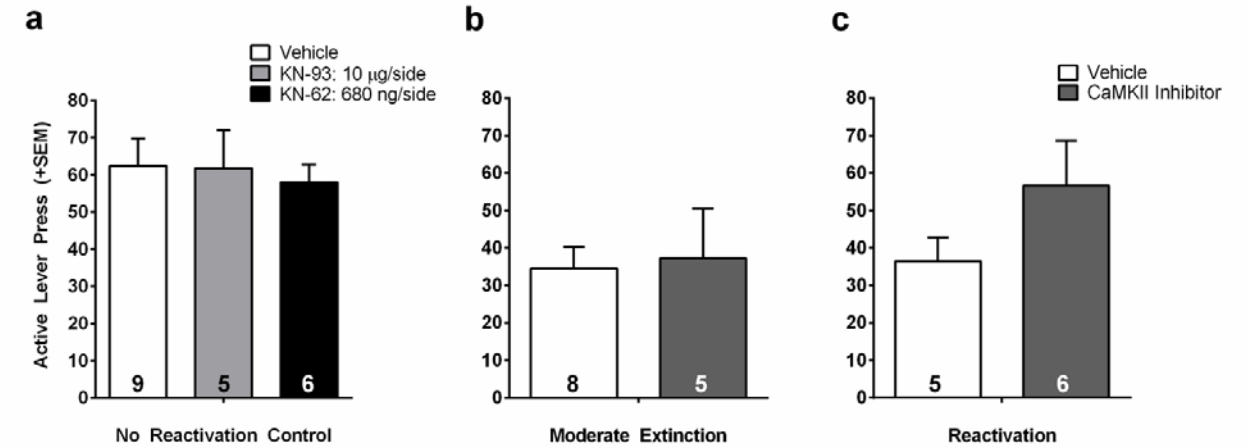

Figure 31. CaMKII inhibition has no effect on cue-induced reinstatement if the memory was not reactivated or infusions occurred outside of the BLA.

(**A**) Cue-induced reinstatement in animals that did not receive drug-cue memory extinction or reactivation. For this control, rats were placed back in self-administration chambers, but received 0 CS presentations. There were no effects of CaMKII inhibition on active lever presses during the cue-induced reinstatement test. (left bar: vehicle, middle bar: KN-93, right bar: KN-62). (**B-C**) Cue-induced reinstatement in animals with infusions outside of the BLA. There were no effects of CaMKII inhibition on active lever presses during the cue-induced reinstatement test in rats that received infusions outside of the boundaries of the BLA after (**B**) a single (moderate) cue extinction session or (**C**) reactivation. Reinstatement data is from rats infused with either the effective dose of KN-93 or KN-62 (5 μg/side and 680 ng/side, respectively). Rats were pooled due to the low numbers of animals in each individual inhibitor condition, and because there was no difference in reinstatement behavior between the compounds. Data are expressed as mean + s.e.m.; number of rats per group indicated by number inside bars.

A.4 DISCUSSION

The present study identified brain-region specific protein phosphorylation events that occur in response to the reactivation or extinction of cocaine-cue-specific memories. Our proteomic analysis indicated that several cellular signaling cascades were activated in the same manner by both extinction and reconsolidation, including increased phosphorylation of extracellular regulated kinase 2 (ERK2) and 60S ribosomal subunits, which regulate protein synthesis. These results are consistent with prior findings in the literature indicating that ERK activity and protein synthesis are required for both the reconsolidation and extinction of memory (Cestari et al., 2006; Duvarci et al., 2005; Tronson and Taylor, 2007). The proteomic analysis also revealed several phosphorylation events that were selective to one memory condition or the other, and importantly, identified 5 proteins that exhibited opposing directions of phosphorylation under extinction vs. reactivation conditions. Thus, we report the identification of several signaling pathways that may be viable targets for developing medications to be used in conjunction with memory-based behavioral interventions. In particular, phospholipase C and c-jun N-terminal kinase 3 are two proteins that have been associated with memory regulation in other paradigms, and deserve further study. It should be noted that it is possible that some of the protein phosphorylation changes observed are not related to memory, but rather could be associated with a change in motivational state. Nonetheless, use of this discovery-based approach represents a novel method for determining signaling events involved in specific memory processes.

Specifically, we found a novel residue on CaMKIIα, S331, where phosphorylation was increased after extinction and decreased after reactivation. Mutagenesis experiments indicated that phosphorylation of S331 reduced enzymatic activity of CaMKIIα. Additionally, we found

that pharmacological inhibition of CaMKII within the BLA can lead to a reduction in drug-cue motivated behavior if the inhibition occurs immediately following either memory reactivation or memory extinction. This is consistent with both a partial impairment in the reconsolidation of the original drug-cue memory and an enhancement in the learning/consolidation of the new cue-extinction memory, effectively weakening the drug-associated memory trace.

A.4.1 Targeting memory processes as a therapeutic strategy

Manipulations that inhibit drug-cue memory reconsolidation and promote extinction have high therapeutic potential for relapse prevention in addiction (Sorg, 2012; Torregrossa et al., 2011). Despite a remarkable effort to develop pharmacotherapies for drug addiction, there has been little success in helping addicted individuals maintain long-term abstinence (Koob and Volkow, 2010). Currently, clinical efforts that combine treatment medications with behavioral therapies have demonstrated the highest success rates (Carroll and Onken, 2005). A common behavioral approach involves exposing individuals to multimodal cues in an extinction-based therapy to decrease subsequent craving and relapse when drug-associated cues are re-encountered (Price et al., 2013). Previous research has demonstrated some effectiveness of this approach to inhibit fear-associated memories in rodent models and in humans (Hofmann et al., 2012; Monfils et al., 2009; Ressler et al., 2004). In the present study, we show that a single cue extinction session is mildly effective in reducing responding for cocaine during a cue-induced reinstatement session, but that this reduction is amplified by the addition of a second cue extinction session, consistent with the fact that the number of unreinforced cue presentations is a critical determinant of extinction efficacy (Price et al., 2013; Unrod et al., 2014).

Although cue extinction therapy can have moderate success (Kantak and Nic Dhonnchadha, 2011; Price et al., 2010), developing medications that can be given in conjunction with exposure therapy to augment extinction learning will likely improve treatment outcomes. In particular, intra-BLA CaMKII inhibition immediately following a single cue-extinction session (moderate extinction) potentiated the reduction in cue-induced reinstatement observed 24 h later. CaMKII inhibition also disrupted reconsolidation, indicating that insufficient extinction training would not prevent efficacy, as may be the case for glutamatergic agonists like DCS (Lee et al., 2009; Price et al., 2013). Therefore, inhibiting CaMKII signaling pathways in conjunction with exposure therapy may be a viable treatment strategy. However, CaMKII inhibition had no additional benefit after extended cue-extinction indicating a potential floor effect. Extended extinction alone was sufficient to drastically reduce levels of responding during reinstatement, even in VEH-treated animals, suggesting that sufficient exposure therapy could be beneficial, though the addition of CaMKII inhibition may have been able to inhibit renewal of cocaine seeking after a change of context or reduce spontaneous recovery, which could be tested in future experiments.

A.4.2 Mechanisms of CaMKII action in memory processes

Identifying CaMKIIα as an important component in the maintenance of drug-associated memories is not altogether surprising. It is well established that CaMKII activity is necessary for NMDAR-dependent forms of long-term potentiation (LTP) and synaptic modifications (Coultrap and Bayer, 2012; Lisman et al., 2012; Sanhueza et al., 2011). However, the identification of a phosphorylation event that is bidirectionally regulated by reconsolidation relative to extinction of

a memory is novel. CaMKII is activated by Ca2+ influx through NMDAR or voltage-gated Ca2+ channels, resulting in its autophosphorylation at T286. CaMKII then translocates to synapses, initiating a biochemical cascade, including phosphorylation of glutamatergic receptors that potentiates synaptic transmission (Barria, 1997; Bayer, 2006; Coultrap et al., 2014; El Gaamouch et al., 2012; Lemieux et al., 2012; Lu et al., 2010; Raveendran et al., 2009; Sanhueza and Lisman, 2013). Moreover, CaMKIIα knockout mice have deficits in LTP(Silva et al., 1992a), and in learning a hippocampus-dependent spatial task (Silva et al., 1992b), further demonstrating that CaMKIIα is necessary for memory formation. On the other hand, CaMKII activity, including phosphorylation of T286, is also needed for some forms of long-term depression (LTD) (Pi et al., 2010). Whereas CaMKII mediates LTP via phosphorylation of GluA1 at S831, LTD is likely evoked by CaMKII-mediated phosphorylation of GluA1 at S567 (Coultrap et al., 2014). Thus, in our study, the effect of CaMKII inhibitors on reconsolidation and extinction might be explained by differential effects on LTP vs. LTD, respectively.

Consistent with this possibility, it has been shown that increased activation of CaMKIIα can impair an NMDAR-dependent form of synaptic depotentiation in mice lacking cytoplasmic polyadenylation element binding protein 3 (CPEB3) (Huang et al., 2014). Coupled with the finding that CPEB3 KO mice exhibit deficits in the extinction of contextual fear memory (Chao et al., 2013), this further supports the notion that CaMKII inhibition could enhance extinction learning, and highlights a potential involvement of an LTD-like synaptic mechanism in this process. Additionally, there are a few reports that CaMKII also regulates memory reconsolidation. For example, reconsolidation of an amphetamine-conditioned place preference was disrupted by infusion of KN-93 into the hippocampus prior to re-exposure to the conditioned context (Sakurai et al., 2007). Likewise, CaMKII activity in the hippocampus is important for the

maintenance of a spatial memory after retrieval (Da Silva et al., 2013). On the other hand, the reconsolidation of a cocaine-associated contextual memory was not affected by intra-BLA CaMKII inhibition (Arguello et al., 2014). Although inconsistent with our results, the procedural differences between the two studies, including manipulating contextual rather than discrete cue memories, likely explains the differential findings.

A.4.3 Role of CaMKII signaling in regulating drug-associated behaviors

CaMKIIα phosphorylation events have recently been linked to drug-related learning (Easton et al., 2013, 2014; Salling et al., 2016). For example, mice without the capacity for CaMKIIα autophosphorylation (T286A mice) exhibited a delay in the establishment of a preference for cocaine, (Easton et al., 2014), and increased phosphorylation of CaMKIIα at T286 was observed in the amygdala after rats lever press for alcohol (Salling et al., 2016). In addition, reduction of CaMKIIα function in the NAc shell prevented amphetamine-associated plasticity and reduced self-administration (Loweth et al., 2013). Together these studies are consistent with our conclusions that CaMKIIα activity is a critical component of drug-related memory processes. Our proteomics results further suggest a very precise and novel, memory-specific regulation of CaMKIIα at S331. Phosphorylation of S331 has not been previously reported to change in a biological context, but a recent study has found S331 to be a putative autophosphorylation site that exists in vivo (Baucum et al., 2015). Interestingly, S331 is located within the linker region of CaMKIIα, and the linker region has the potential to regulate the function of CaMKII via multiple mechanisms in addition to kinase activity (Chao et al., 2011; Stratton et al., 2013). The present study is the first to report that S331 phosphorylation may inhibit kinase activity, suggesting that

CaMKIIα may have the ability to autoinhibit, potentially providing a negative feedback mechanism and/or a mechanism for mediating LTD. Future studies will further explore how and when this novel phosphorylation site on CaMKIIα is regulated, if memory manipulation-specific phosphorylation events occur in distinct sub-populations of BLA neurons, and how CaMKIIα S331 phosphorylation affects synaptic plasticity.

A.4.4 Summary

Taken together, the present study identified several signaling cascades regulated by the extinction and reconsolidation of a memory associated with self-administered cocaine. In particular, we identified a novel mechanism by which CaMKIIα can regulate both memory processes. Furthermore, our results suggest that inhibiting CaMKII or related signaling cascades could be a novel approach for combined behavioral and pharmacological therapy in the treatment of addictive disorders.

Table 1. Results from SRM Proteomics Analysis

		Extinction vs. Control					
		SRM Stats Restricted Scope			Linear Mixed Effects Model		
Protein	**Peptide Sequence + Modification(s)**	**non adjusted pval**	**Fold Change-estimate**	**adjusted pval**	**non adjusted pval**	**Fold Change-estimate**	**adjusted pval**
ADCY9	GQGTASpPGSpVSDLAQTVK	0.2413	0.3017	0.3475	0.5004	0.3017	0.7506
AKA12	ALGSpLGGSpPSLPDQDK	0.0172	-0.5640	0.0403	0.3275	-0.5640	0.7284
ARHG7	MSpGFIYQGK	0.4373	0.0582	0.5524	0.6516	0.0582	0.8530
BAIP2	SSSpMAAGLER	0.7626	0.0362	0.8133	0.8628	0.0362	0.9557
BSN	SPQVLYSpPVSpPLSPHR	0.0118	0.2460	0.0340	0.1478	0.2460	0.7284
CKD18	RASpLSDIGFGK	0.0000	-0.2172	0.0000	0.4012	-0.2172	0.7284
CSKI1	KVPLPGPGSpPEVK	0.0173	-0.2256	0.0403	0.1374	-0.2256	0.7284
CTNA1	SRTpSpVQTpEDDQLIAGQSAR	0.0144	-0.2016	0.0370	0.1565	-0.2016	0.7284
CTNA1	SRTSpVQTpEDDQLIAGQSAR	0.0345	0.1416	0.0653	0.3944	0.1416	0.7284
CTNB1	RTSMGGTpQQQFVEGVR	0.2586	-0.0518	0.3581	0.8423	-0.0518	0.9557
CTND2	ALQSpPEHHIDPIYEDR	0.9798	-0.0032	0.9798	0.9794	-0.0032	0.9925
CXA1	M(ox)GQAGSTISpNSpHAQPFDFPDDNQNAK	0.3219	-0.0462	0.4292	0.8552	-0.0462	0.9557
CXA1	MGQAGSTISpNSpHAQPFDFPDDNQNAK	0.0152	-0.3070	0.0377	0.3096	-0.3070	0.7284
CXA1	VAAGHELQPLAIVDQRPSSpRASpSpR	0.0000	-0.5198	0.0000	0.0540	-0.5198	0.7284
DGKB	GAITpPPRSSpPANTCSPEVIHLK	0.0032	-0.1344	0.0117	0.5449	-0.1344	0.7846
EF1D	ATAPQTQHVSpPMR	0.7508	-0.1949	0.8133	0.7508	-0.1949	0.8862
FAK2	RNSpLPQIPTLNLESR	0.0000	0.2027	0.0000	0.2270	0.2027	0.7284
FGF12	EPSpLHEIGEK	0.2060	-0.1028	0.3027	0.4973	-0.1028	0.7506
GABR1	HPPTpPPDPSGGLPR	0.0002	0.1330	0.0011	0.4323	0.1330	0.7284
GABR1	RHPPTpPPDPSGGLPR	0.0556	0.1371	0.1002	0.3379	0.1371	0.7284
GABR2	DPIEDINSpPEHIQR	0.0035	0.2341	0.0119	0.2114	0.2341	0.7284
GIT1	HGSpGAESDYENTQSGEPLLGLEGK	0.0000	-0.2619	0.0002	0.0726	-0.2619	0.7284
GIT1	NQSDLDDQHDYDSpVASpDEDTDQEPLPSAGATR	0.1288	0.0910	0.2065	0.3636	0.0910	0.7284
IF3M	LLYLTpSpAK	0.5401	0.2342	0.6418	0.5401	0.2342	0.7846
IPP2	EQESpSpGEEDNDLSPEER	0.9515	0.0051	0.9787	0.9830	0.0051	0.9925
KCC2A	ESSESTpNTpTpIEDEDTK	0.5437	0.0698	0.6418	0.8973	0.0698	0.9762
KCC2A	ESSpESTNTTIEDEDTK	0.0000	0.3596	0.0004	0.0737	0.3596	0.7284

Table 1. Results from SRM Proteomics Analysis

KCNQ2	HGTSpPVGDHGSLVR	0.1291	-0.2891	0.2065	0.1291	-0.2891	0.7284
KPCB	HPPVLTpPPDQEVIR	0.0000	-0.1477	0.0002	0.5564	-0.1477	0.7855
KPCG	AAPALTpPPDR	0.5667	-0.2324	0.6535	0.5902	-0.2324	0.8058
KPCG	TpFCGTPDYIAPEIIAYQPYGK	0.0008	-0.1151	0.0034	0.4350	-0.1151	0.7284
MARCS	EAAEAEPAEPGSpPSAETEGASASSTSSPK	0.0000	0.2408	0.0000	0.1891	0.2408	0.7284
MARCS	GEAAAERPGEAAVASpSPSK	0.3813	0.2147	0.4992	0.3813	0.2147	0.7284
MARK2	DQQNLPFGVTPASpPSGHSQGR	0.0020	0.1821	0.0075	0.0738	0.1821	0.7284
MINK1	SDSVLPASHGHLPQAGSpLER	0.0001	0.1289	0.0005	0.2101	0.1289	0.7284
MK01	VADPDHDHTGFLTpEYpVATR	0.0002	0.1278	0.0014	0.3636	0.1278	0.7284
MK10	TAGTSFMMTpPYpVVTR	0.0236	-0.4020	0.0499	0.4244	-0.4020	0.7284
NBEA	EIEDLSQSQSpPESpETDYPVSTDTR	0.2533	0.0973	0.3576	0.4144	0.0973	0.7284
NBEA	TPLENVPGNLSpPIKDPDR	0.0180	-0.1595	0.0406	0.3981	-0.1595	0.7284
NCAM1	DESpKEPIVEVR	0.9721	0.0015	0.9798	0.9925	0.0015	0.9925
NCAM2	ITNHEDGSpPVNEPNETTPLTEPEK	0.0423	0.1622	0.0781	0.2935	0.1622	0.7284
NCAM2	ITNHEDGSpPVNEPNETTpPLTEPEK	0.3886	0.4858	0.4996	0.3886	0.4858	0.7284
NMDE2	HSQLSDLYpGK	0.9273	0.0241	0.9676	0.9608	0.0241	0.9925
PAK1	TVSETPAVPPVSpEDEDDDDDATPPPVIAPRPEHTK	0.7681	0.0103	0.8133	0.9220	0.0103	0.9762
PEA15	QPSpEEEIIK	0.7666	-0.0792	0.8133	0.8489	-0.0792	0.9557
PLCB1	SEPSSPDHGSSpAIEQDLAALDAEMTQK	0.0129	-0.1209	0.0344	0.4324	-0.1209	0.7284
PLCB1	VNLKSpPSpSEEVQGENAGR	0.1122	0.1155	0.1878	0.3816	0.1155	0.7284
PP1R7	HGGGIVADLSpQQSpLK	0.0211	0.1865	0.0460	0.3519	0.1865	0.7284
PPR1B	IAESHLQTISNLSENQASpEEEDELGELR	0.0000	-0.3538	0.0001	0.5932	-0.3538	0.8058
PTPRN	LPEEGGSSpRAEDSSpEGHEEEVLGGHGEK	0.0263	0.2670	0.0513	0.4188	0.2670	0.7284
RLA1	KEESpEESpEDDM(ox)GFGLFD	0.0002	0.2178	0.0013	0.1795	0.2178	0.7284
RLA2	KEESpEESpDDDM(ox)GFGLFD	0.0003	0.3184	0.0016	0.1069	0.3184	0.7284
RP3A	WHQLQNENHVSSpD	0.4637	-0.2760	0.5757	0.4625	-0.2760	0.7506
SCN2A	RFSSpPHQSpLLSIR	0.0005	0.1191	0.0021	0.6348	0.1191	0.8464
SHAN2	RAPSpPVVSpPTELSK	0.0002	0.2541	0.0014	0.3608	0.2541	0.7284
SHAN3	SRSpPSpPSpPLPSPSPGSGPSAGPR	0.0263	0.2147	0.0513	0.3748	0.2147	0.7284
SRBS2	SESpMGSpLLCDEGSK	0.0068	0.4027	0.0204	0.1111	0.4027	0.7284

Table 1. Results from SRM Proteomics Analysis

SRCN1	DSGSSSVFAESpPGGK	0.0051	0.1440	0.0161	0.2492	0.1440	0.7284
SRCN1	KAESpEELEIQKPQVK	0.6499	0.0178	0.7312	0.9149	0.0178	0.9762
SRCN1	RFSpNVGLVHTSER	0.0005	-0.2261	0.0021	0.0394	-0.2261	0.7284
SRCN1	RGSpDELTVPR	0.1518	-0.0512	0.2377	0.6823	-0.0512	0.8773
SRCN1	SSpGATpPVSGPPPPAVSSTPAGQPTAVSR	0.3208	0.1193	0.4292	0.4850	0.1193	0.7506
STMN1	DLSpLEEIQK	0.2030	0.0679	0.3027	0.7102	0.0679	0.8862
STMN1	ESVPEFPLSpPPK	0.0251	-0.0784	0.0513	0.7438	-0.0784	0.8862
STMN1	RASpGQAFELILSpPR	0.0123	0.2622	0.0340	0.3793	0.2622	0.7284
STX1A	HSAILASpPNPDEK	0.0676	-0.1172	0.1187	0.3204	-0.1172	0.7284
STX1A	TAKDSpDDDDDVTVTVDR	0.0004	0.1918	0.0020	0.1052	0.1918	0.7284
SYPH	LHQVYpFDAPSCVK	0.0847	0.0850	0.1452	0.7301	0.0850	0.8862
SYT1	DQALKDDDAETGLTpDGEEK	0.4814	0.1493	0.5875	0.4814	0.1493	0.7506
TNIK	SEGSpPVLPHEPSK	0.1999	-0.0503	0.3027	0.3956	-0.0503	0.7284
VIAAT	GGAPLPPSGSpK	0.5718	-0.0553	0.6535	0.7363	-0.0553	0.8862
		Reactivation vs. Control					
		SRM Stats Restricted Scope			**Linear Mixed Effects Model**		
Protein	**Peptide Sequence + Modification(s)**	**non adjusted pval**	**Fold Change-estimate**	**adjusted pval**	**non adjusted pval**	**Fold Change-estimate**	**adjusted pval**
ADCY9	GQGTASpPGSpVSDLAQTVK	0.0041	0.5934	0.0084	0.1647	0.5934	0.4146
AKA12	ALGSpLGGSpPSLPDQDK	0.7666	-0.0604	0.8119	0.9193	-0.0604	0.9734
ARHG7	MSpGFIYQGK	0.0030	0.3177	0.0065	0.1157	0.3177	0.3783
BAIP2	SSSpMAAGLER	0.7281	0.0759	0.8119	0.8649	0.0759	0.9730
BSN	SPQVLYSpPVSpPLSPHR	0.0063	0.2866	0.0120	0.0908	0.2866	0.3783
CKD18	RASpLSDIGFGK	0.1156	-0.0447	0.1734	0.8288	-0.0447	0.9730
CSKI1	KVPLPGPGSpPEVK	0.0305	-0.3568	0.0500	0.0260	-0.3568	0.2341
CTNA1	SRTpSpVQTpEDDQLIAGQSAR	0.0604	-0.1133	0.0945	0.5393	-0.1133	0.7949
CTNA1	SRTSpVQTpEDDQLIAGQSAR	0.0000	0.2878	0.0000	0.0124	0.2878	0.1491
CTNB1	RTSMGGTpQQQFVEGVR	0.6712	0.0640	0.7922	0.8436	0.0640	0.9730
CTND2	ALQSpPEHHIDPIYEDR	0.7668	0.0369	0.8119	0.7588	0.0369	0.9419
CXA1	M(ox)GQAGSTISpNSpHAQPFDFPDDNQNAK	0.0000	0.3038	0.0000	0.1234	0.3038	0.3783
CXA1	MGQAGSTISpNSpHAQPFDFPDDNQNAK	0.0000	0.3992	0.0001	0.1918	0.3992	0.4359
CXA1	VAAGHELQPLAIVDQRPSSpRASpSpR	0.0000	-0.3599	0.0000	0.0198	-0.3599	0.2037
DGKB	GAITpPPRSSpPANTCSPEV	0.9499	-0.0044	0.9738	0.9901	-0.0044	0.9901

Table 1. Results from SRM Proteomics Analysis

	IHLK						
EF1D	ATAPQTQHVSpPMR	0.7183	-0.2315	0.8119	0.7183	-0.2315	0.9403
FAK2	RNSpLPQIPTLNLESR	0.0000	0.2167	0.0001	0.1670	0.2167	0.4146
FGF12	EPSpLHEIGEK	0.0000	-0.8485	0.0000	0.2585	-0.8485	0.5444
GABR1	HPPTpPPDPSGGLPR	0.5901	-0.0188	0.7201	0.9144	-0.0188	0.9734
GABR1	RHPPTpPPDPSGGLPR	0.4862	-0.0397	0.6251	0.8359	-0.0397	0.9730
GABR2	DPIEDINSpPEHIQR	0.0054	-0.2011	0.0105	0.3330	-0.2011	0.5994
GIT1	HGSpGAESDYENTQSGEP LLGLEGK	0.3029	-0.0707	0.4194	0.5410	-0.0707	0.7949
GIT1	NQSDLDDQHDYDSpVASp DEDTDQEPLPSAGATR	0.0002	0.2143	0.0005	0.0370	0.2143	0.2661
IF3M	LLYLTpSpAK	0.7412	0.1111	0.8119	0.7412	0.1111	0.9419
IPP2	EQESpSpGEEDNDLSPEER	0.0052	0.3180	0.0104	0.2948	0.3180	0.5736
KCC2A	ESSESTpNTpTpIEDEDTK	0.0003	0.4759	0.0008	0.0322	0.4759	0.2578
KCC2A	ESSpESTNTTIEDEDTK	0.0000	-0.6186	0.0001	0.4973	-0.6186	0.7949
KCNQ2	HGTSpPVGDHGSLVR	0.1937	-1.4748	0.2847	0.1937	-1.4748	0.4359
KPCB	HPPVLTpPPDQEVIR	0.0000	-0.1782	0.0000	0.6023	-0.1782	0.8339
KPCG	AAPALTpPPDR	0.3605	-0.3204	0.4806	0.3961	-0.3204	0.6633
KPCG	TpFCGTPDYIAPEIIAYQPY GK	0.0000	-0.2607	0.0000	0.1253	-0.2607	0.3783
MARCS	EAAEAEPAEPGSpPSAETE GASASSTSSPK	0.0000	0.3923	0.0000	0.0730	0.3923	0.3726
MARCS	GEAAAERPGEAAVASpSP SK	0.2646	-1.9668	0.3736	0.2646	-1.9668	0.5444
MARK2	DQQNLPFGVTPASpPSGH SQGR	0.0000	0.2598	0.0001	0.0023	0.2598	0.0559
MINK1	SDSVLPASHGHLPQAGSp LER	0.0085	0.1011	0.0153	0.3707	0.1011	0.6509
MK01	VADPDHDHTGFLTpEYpV ATR	0.0004	0.1374	0.0008	0.3844	0.1374	0.6590
MK10	TAGTSFMMTpPYpVVTR	0.0002	0.7296	0.0004	0.1492	0.7296	0.4132
NBEA	EIEDLSQSQSpPESpETDYP VSTDTR	0.0088	0.2889	0.0155	0.0659	0.2889	0.3648
NBEA	TPLENVPGNLSpPIKDPDR	0.0003	-0.2541	0.0006	0.1069	-0.2541	0.3783
NCAM1	DESpKEPIVEVR	0.4820	-0.0317	0.6251	0.8555	-0.0317	0.9730
NCAM2	ITNHEDGSpPVNEPNETTP LTEPEK	0.9769	-0.0021	0.9769	0.9894	-0.0021	0.9901
NCAM2	ITNHEDGSpPVNEPNETTp PLTEPEK	0.5354	-0.3821	0.6763	0.5354	-0.3821	0.7949
NMDE2	HSQLSDLYpGK	0.9603	0.0113	0.9738	0.9690	0.0113	0.9901
PAK1	TVSETPAVPPVSpEDEDD DDDATPPPVIAPRPEHTK	0.2036	-0.0521	0.2932	0.6999	-0.0521	0.9383
PEA15	QPSpEEEIIK	0.7288	0.0539	0.8119	0.8991	0.0539	0.9734

Table 1. Results from SRM Proteomics Analysis

PLCB1	SEPSSPDHGSSpAIEQDLAALDAEMTQK	0.0348	0.1013	0.0558	0.7037	0.1013	0.9383
PLCB1	VNLKSpPSpSEEVQGENAGR	0.5520	-0.1562	0.6853	0.5302	-0.1562	0.7949
PP1R7	HGGGIVADLSpQQSpLK	0.0001	0.4104	0.0002	0.0546	0.4104	0.3275
PPR1B	IAESHLQTISNLSENQASpEEEDELGELR	0.0000	-0.4040	0.0000	0.5816	-0.4040	0.8211
PTPRN	LPEEGGSSpRAEDSSpEGHEEEVLGGHGEK	0.0001	0.4983	0.0003	0.1004	0.4983	0.3783
RLA1	KEESpEESpEDDM(ox)GFGLFD	0.0000	0.3790	0.0000	0.0048	0.3790	0.0868
RLA2	KEESpEESpDDDM(ox)GFGLFD	0.0001	0.4373	0.0003	0.0009	0.4373	0.0559
RP3A	WHQLQNENHVSSpD	0.0067	-0.4470	0.0124	0.5687	-0.4470	0.8189
SCN2A	RFSSpPHQSpLLSIR	0.0000	0.4239	0.0000	0.0446	0.4239	0.2922
SHAN2	RAPSpPVVSpPTELSK	0.0000	0.3424	0.0000	0.2148	0.3424	0.4686
SHAN3	SRSpPSpPSpPLPSPSPGSGPSAGPR	0.0009	0.5152	0.0019	0.1261	0.5152	0.3783
SRBS2	SESpMGSpLLCDEGSK	0.0002	0.6422	0.0005	0.0801	0.6422	0.3726
SRCN1	DSGSSSVFAESpPGGK	0.0000	0.2076	0.0001	0.1560	0.2076	0.4146
SRCN1	KAESpEELEIQKPQVK	0.0146	-0.1133	0.0250	0.4970	-0.1133	0.7949
SRCN1	RFSpNVGLVHTSER	0.0000	-0.3600	0.0001	0.0083	-0.3600	0.1191
SRCN1	RGSpDELTVPR	0.0001	-0.2053	0.0004	0.1774	-0.2053	0.4259
SRCN1	SSpGATpPVSGPPPPAVSSTPAGQPTAVSR	0.9596	0.0141	0.9738	0.9630	0.0141	0.9901
STMN1	DLSpLEEIQK	0.6268	0.0313	0.7522	0.8983	0.0313	0.9734
STMN1	ESVPEFPLSpPPK	0.3329	-0.0552	0.4523	0.7488	-0.0552	0.9419
STMN1	RASpGQAFELILSpPR	0.0000	0.5433	0.0001	0.1049	0.5433	0.3783
STX1A	HSAILASpPNPDEK	0.0193	-0.1344	0.0323	0.3284	-0.1344	0.5994
STX1A	TAKDSpDDDDDVTVTVDR	0.7533	-0.0147	0.8119	0.8233	-0.0147	0.9730
SYPH	LHQVYpFDAPSCVK	0.0037	-0.4914	0.0079	0.2792	-0.4914	0.5583
SYT1	DQALKDDDAETGLTpDGEEK	0.0828	-0.2498	0.1269	0.0828	-0.2498	0.3726
TNIK	SEGSpPVLPHEPSK	0.0000	-0.3677	0.0001	0.0019	-0.3677	0.0559
VIAAT	GGAPLPPSGSpK	0.0002	-0.4927	0.0005	0.3042	-0.4927	0.5763

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