Investigating Dopaminergic and Opioidergic Mechanisms of Cocaine Conditioned Reinforcement Using the New Response Acquisition Procedure

by

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Dedication

To my mom, dad, and brother, for teaching me how to work hard (and play hard)

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Abstract

Substance use disorders are chronically relapsing conditions, such that risk of relapse can persist for years despite maintained drug abstinence. Exposure to cues associated with drug-taking can elicit feelings of intense drug-craving in humans and drug-seeking behaviors in animal models. When neutral cues in the environment are paired repeatedly with drug-taking behavior, cues and drug form associations and acquire conditioned reinforcing properties. Elucidating the neurobiological mechanisms underlying conditioned reinforcing properties may help identify novel treatments for relapse. The dopaminergic and the opioidergic systems, have been implicated as mediators of cue-induced behaviors.

The experiments described in this thesis sought to delineate the role of dopaminergic and opioidergic systems in a stringent assay measuring the conditioned reinforcing properties of cocaine-paired cues. The New Response Acquisition procedure tests the ability of cocaine-paired cues to support new learning. New Response Acquisition begins with a Pavlovian Conditioning phase, in which rats receive noncontingent infusions of cocaine either with simultaneous (Paired) or separate (Unpaired) presentations of an arbitrary stimulus. Only in Paired conditions should the cue form an association with cocaine and develop conditioned reinforcing effects. In the second phase, called Acquisition, all subjects are allowed to make a novel, operant response to produce presentations of the stimulus alone. It is expected that Paired cues

induce more responses than Unpaired cues, indicative that conditioned reinforcement has occurred.

Following the establishment and optimization of a cocaine New Response Acquisition procedure, we assessed the role of dopamine in contributing to the behavior to earn cues. Dopamine levels in the nucleus accumbens shell or core were not different between groups of rats that underwent Paired or Unpaired Pavlovian Conditioning. Further, increasing dopamine levels by the administration of local or systemic indirect dopamine agonists did not potentiate responding for cues. Together, these data suggest dopamine does not mediate the reinforcing effects of cocaine-paired cues in this procedure.

To characterize the role of the opioidergic system in cocaine conditioned reinforcement, we examined the extent to which activation of opioid receptors alters responding for cues. Endogenous opioid peptides, specifically enkephalins, robustly increased responding for cocaine-paired cues, and this effect was antagonized by pretreatment of a delta opioid receptor selective antagonist. Further, activation of delta opioid receptors via direct agonists potentiated responding for cues, suggesting this system may mediate the conditioned reinforcing properties of cocaine-paired cues in New Response Acquisition.

Overall, I found that behavior maintained by cocaine-paired cues in the New Response Acquisition procedure may be modulated by the opioidergic system and is not dependent on dopamine. The work presented in this dissertation provides novel evidence for the mechanisms of cocaine conditioned reinforcement and potential new targets for reducing the ability of cues to elicit behavior and treating relapse.

Chapter 1
Introduction

Substance use disorders (SUDs; also known as drug addiction) are characterized by an inability to control drug use, continuing drug use despite adverse consequences, and relapse even after long periods of abstinence. Multiple risk factors contribute to vulnerability for developing of a SUD, such as genetic and environmental factors (for review, see: Volkow and Li, 2005). Due to its chronic relapsing nature, long-term abstinence and/or recovery is difficult.

People with SUDs often do not receive any treatment, but even with treatment the likelihood of relapse is high, between 40-60% (McLellan *et al.*, 2000). Many factors can influence relapse, such as exposure to stress, drug, or environmental stimuli that have been associated with drug-taking behaviors (Volkow and Li, 2005). Environmental stimuli include people, places, and things that were present while drug-taking or signal drug availability, such as a neon sign for a local bar. Exposure to these cues can elicit feelings of drug craving in humans (Bonson *et al.*, 2002; Volkow and Li, 2005; Volkow *et al.*, 2006). Drastic increases in intense cravings, or drug "wanting," increase vulnerability to relapse, despite conscious effort to abstain from drug use (Berridge and Robinson, 2016). Cue-induced drug craving remains a risk factor for significant periods of time, potentially contributing to relapse even after years of drug abstinence (Berridge and Robinson, 2016). Therefore, it would be useful to have treatments or medications that decrease drug-craving during exposure to cues. In order to develop effective

treatments, understanding of the underlying causes are likely necessary. After the late 1990's, the scientific community formed the general consensus that, "addiction is a brain disease, and it matters" (Leshner, 1997). From these perspectives, it was accepted that identifying neurobiological substrates for substance use disorders, and drug craving, would lead to new therapeutics for treating the disorders and relapse (Venniro *et al.*, 2020).

There are few FDA-approved pharmacological treatments for SUDs that target drug wanting, or craving, such as methadone and buprenorphine for opioid craving or nicotine replacement or varenicline for nicotine craving (Jordan and Xi, 2018). These treatments are agonist-based therapies, which have the same mechanism of action as the drugs of abuse but with optimized pharmacology to reduce symptoms of withdrawal that contribute to relapse, such as somatic symptoms and drug craving. In addition, cognitive behavioral therapy is used in conjunction with these therapies but is the only treatment option for treatment for some SUDs, such as psychostimulant use disorder (An *et al.*, 2017; Schwartz *et al.*, 2022). Alternative approaches to attenuating drug craving are in development, such as administration of psychedelics in clinical settings (DiVito and Leger, 2020; Calleja-Conde *et al.*, 2022), contingency management (Bigelow and Silverman, 1999; Caprioli *et al.*, 2015), community-reinforcement approach (Meyers *et al.*, 2011; Venniro *et al.*, 2018), or participation in telemedicine (Lin *et al.*, 2023). Because there are few FDA-approved medications to target drug craving, these newer approaches often incorporate non-pharmacological treatments for managing SUDs. While the development of therapies for SUDs has been an area of intense research for decades, future studies are required to better target neurobiological systems

responsible for craving and account for heterogeneity of patient populations (Schwartz *et al.*, 2022).

1.1 Preclinical assays to study substance use disorders

In order to develop pharmacological therapies to target drug craving, researchers utilize various preclinical assays of SUDs and relapse. Measures have been developed to investigate different behavioral features within each phase of the cycle of SUDs, including escalation of drug intake, periods of abstinence, withdrawal, and re-initiation of drug-taking or relapse (Venniro *et al.*, 2020). Because this is a complex phenomenon, researchers often divide this cycle into components that can be studied in an experimental setting. For example, drugs of abuse have primary reinforcing effects such that they maintain self-administration in both animal models and humans (Di Chiara *et al.*, 2004). Self-administration procedures are the standard for evaluating the primary reinforcing effects of drugs of abuse, yet other models are utilized as well, such as drug discrimination, conditioned place preference, and choice procedures. Positive reinforcing effects are characteristic of all drugs of abuse and is a component of initiation of drug use in humans (Venniro *et al.*, 2020).

For the purposes of this dissertation, we discuss in the introduction models of relapse in which researchers use distinct paradigms that are thought to measure “drug-seeking” behaviors. Researchers often do not investigate re-initiation of drug-taking, but rather focus on components that promote drug-seeking behavior or stimulate behaviors that would normally produce drug delivery but do not produce drug or occur after the behavior has been extinguished.

1.1.1 Reward-paired cues

"Drug-seeking" behaviors are meant to model aspects of behavior that influence relapse prior to reinitiating drug-taking. This term was first described in Davis and Smith, 1976, when reintroduction of a drug-paired cue induced responding on a lever that had previously lead to the delivery of drug (Davis and Smith, 1976). Therefore, reinstated behavior or reinstatement, such as lever pressing that previously produced drug infusions, was considered to be analogous to individuals initiating drug-seeking following history of drug exposure then abstinence, although this has been debated (Epstein *et al.*, 2006).

Extinction and reinstatement of learned behaviors is discussed in early classical conditioning and operant work by Pavlov and BF Skinner. Pavlov reported that following extinction of a classically conditioned response, re-exposure to the unconditioned stimulus restored the response in dogs (Pavlov, 1927). Skinner demonstrated that following a period of operant extinction, noncontingent delivery of food or water reinstated lever pressing (Skinner, 1938). This work was eventually expanded to operant behavior maintained by drugs.

Previous work demonstrated that priming injections of drug or exposure to drug-paired cues could reinstate responding following extinction of the drug-reinforced behavior (de Wit and Stewart, 1981; Shaham *et al.*, 2003). Reinstatement of responding appeared to have face validity to human relapse, such that factors that influenced relapse in humans also reinstated responding in animal models (Shaham *et al.*, 2003; Bossert *et al.*, 2013), including drug (de Wit and Stewart, 1981), stress (Brown *et al.*,

1995; Tabbara *et al.*, 2020), and drug-paired cues (Figure 1.1) (Ludwig *et al.*, 1974; Davis and Smith, 1976; de Wit and Stewart, 1981).

Drug-paired cues elicit behavior because these cues acquire value in their own right via Pavlovian Conditioning (Williams, 1994) through associative properties with the primary reinforcer (Fantino, 2022). Arbitrary cues gain reinforcing properties following pairing of the cue with a primary reinforcer, described as conditioned reinforcement. Behaviors elicited and maintained by conditioned reinforcers are a measure of the reinforcing properties of cues. Cue-induced reinstatement of operant behaviors is one way to measure the conditioned reinforcing properties of cues.

In addition to cue-induced reinstatement (Shaham *et al.*, 2003; Epstein *et al.*, 2006; Bossert *et al.*, 2013), commonly used assays to measure the conditioned reinforcing effects of drug-paired cues are: Pavlovian Instrumental Transfer (PIT) (Heinz *et al.*, 2019), sign-tracking (Meyer *et al.*, 2014), second-order schedules of reinforcement (Everitt and Robbins, 2000; Ito *et al.*, 2000), cue reactivity (Childress *et al.*, 1993), reinstatement of conditioned place preference (CPP) (Shaham *et al.*, 2003; Hillhouse *et al.*, 2021), and New Response Acquisition (Taylor and Robbins, 1984; Bertz and Woods, 2013). While these procedures attempt to mimic some aspects of relapse in the human condition, they do not always have predictive validity (Epstein *et al.*, 2006; Bossert *et al.*, 2013) because often drugs that work to reduce reinstatement or drug-seeking are not effective in reducing drug-craving the clinic (Namba *et al.*, 2018; Venniro *et al.*, 2020). Therefore, newer assays are being developed to better incorporate aspects of effective human paradigms using a post-translatable approach, with the goal of increasing translatability (Venniro *et al.*, 2020). However, the most common way to

evaluate the conditioned reinforcing properties of drug-paired cues remains cue-induced reinstatement (Nasser *et al.*, 2017).

1.1.1.1 Cue-induced reinstatement

Cue-induced reinstatement procedures (Figure 1.1) typically begin with operant drug self-administration, in which subjects learn to make responses to earn contingent drug (or non-drug reward) delivery which is paired with a cue (often a light or tone). Cues can be discriminative (Weiss *et al.*, 2000), in which they are presented during the session signaling when drug is available to be self-administered, contextual (Crombag and Shaham, 2002), in which they are part of the context/environment in which drug is delivered, or discrete (Meil and See, 1996), in which cues are delivered transiently coinciding with drug delivery. In humans, discrete and contextual drug cues include places were drug use has occurred, people with whom drugs were taken with, and items used to administer drugs, among other complex environmental cues (Volkow *et al.*, 2006; Namba *et al.*, 2018).

After drug self-administration has been acquired, responding is often extinguished by removing drug from the environment, such that responding has no scheduled consequences or is replaced with saline. Forced abstinence may take the place of operant extinction in certain procedures, in which subjects remain in their home cage for a period of time before continuing through the procedure (Gál and Gyertyán, 2006). Then, cues can be reintroduced into the session and operant responding is often reinstated, interpreted as increased drug-seeking. During reinstatement, researchers can initiate the session with a noncontingent cue presentation, often done in discriminative cue-induced reinstatement (Weiss *et al.*, 2000) or allow subjects to make

operant responses that result in contingent cue deliveries, common with discrete drug-paired cues (Parsegian and See, 2014). Interestingly, drug-priming and exposure to drug-paired cues may differentially induce reinstatement depending on the type of primary reinforcer (LeSage *et al.*, 2004). Drug-seeking may be further interpreted as drug-wanting, which would likely recruit motivational processes (Robinson and Berridge, 2001). Because the operant response to earn cues had been associated with prior drug delivery during self-administration, researchers cannot definitively conclude that responding is controlled by cues alone (Mackintosh, 1974; Williams, 1994), as there could be motivating factors for potential drug delivery (Weiss *et al.*, 2001; Berridge, 2007) that contribute to reinstatement.

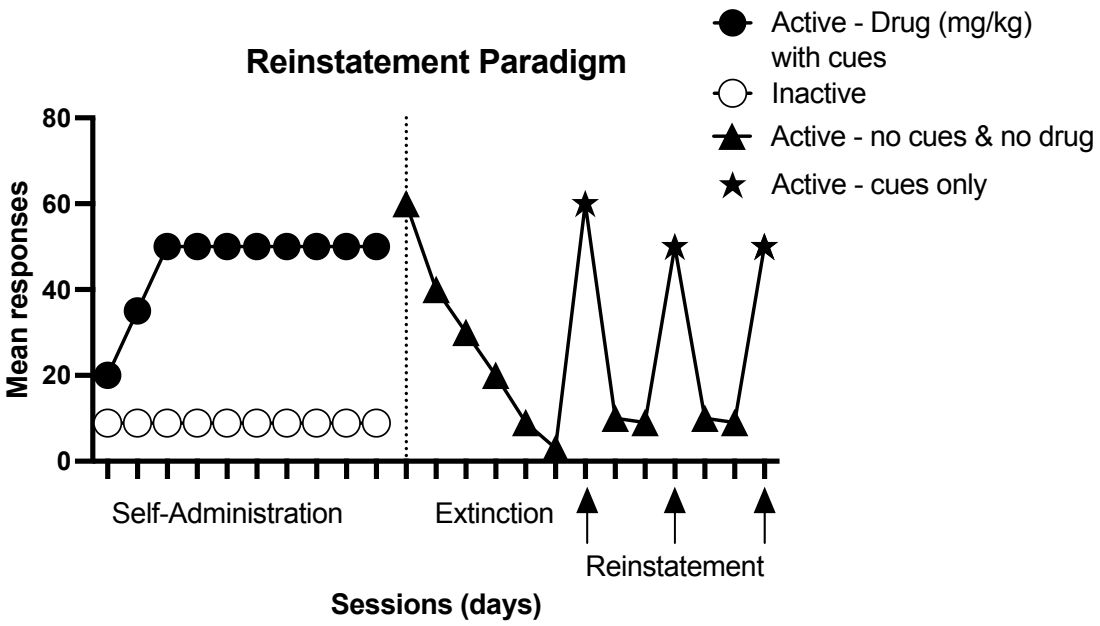

Figure 1.1 Schematic of a representative cue-induced reinstatement experimental procedure.

The timing between drug self-administration, extinction, and reinstatement also plays a role in the ability of cues to elicit and maintain behavior. In humans, it was reported that cue-induced drug craving increased over time, throughout extended periods of abstinence (Gawin and Kleber, 1986). This was replicated in animal models,

termed incubation of drug craving, such that rodents demonstrated time-dependent increases in cue-induced drug seeking following periods of abstinence (Lu *et al.*, 2004; Pickens *et al.*, 2011). This time-dependent effect on reinstatement may be selective for discrete cue-induced reinstatement, and not context- or drug-induced reinstatement (Adhikary *et al.*, 2017). The time between drug self-administration and the reinstatement test, either via forced abstinence or operant extinction, influences the level of reinstated responding during the test.

Cue-induced reinstatement is often designed as a within-subjects experiment, yet researchers can utilize additional control groups for a between-subjects design. Cues develop reinforcing properties after forming an association with a primary reinforcer (during self-administration); therefore, one control condition used is to deliver cues with saline administration in a separate group of animals thereby eliminating conditioned reinforcement. Because animals do not self-administer saline (it does not have primary reinforcing properties), this is often done in a yoked-saline procedure. Control animals receive noncontingent saline infusions and cue presentations whenever a matched drug self-administering subject receives contingent primary rewards (and cues) (Parsegian and See, 2014). Operant manipulanda are present in the chamber of yoked-saline subjects, but do not have scheduled consequences. Yoked-saline groups control for the association between a cue and a primary reinforcer, such that during reinstatement tests, the cue should not have reinforcing properties and should not maintain high levels of responding. An alternative control procedure uses yoked-drug controls, in which noncontingent drug and cues are delivered at the same time and patterns of matched contingent drug infusions. The drug exposure is similar between self-administering and

yoked-control groups; however, the contingency between operant responding and drug and cue delivery is removed (Di Ciano *et al.*, 1998). Cues can still develop conditioned reinforcing properties in yoked-drug control groups, as drug is delivered simultaneously with cue.

Overall, cue-induced reinstatement is a useful behavioral paradigm to model different aspects of relapse in humans. However, these procedures lack predictive validity and have limitations and caveats when interpreting results. Some aspects of self-administration and reinstatement that are not analogous to the human condition are that drug access is restricted by the experimenter and these paradigms often do not include choice between reinforcer or an alternative. Also, forced abstinence may model abstinence that is not chosen, but does not model human abstinence that is chosen and has motivational aspects (Epstein *et al.*, 2006; Venniro *et al.*, 2020). Cue-induced reinstatement has some face validity to relapse, but it is important to note that these procedures are controlled models of complex conditions that influence drug-craving and relapse; therefore, other factors are likely involved in the human condition that cannot be fully captured in experimental settings.

1.1.1.2 New Response Acquisition

Other behavioral paradigms seek to isolate the conditioned reinforcing properties of drug-paired cues by measuring the ability of drug-paired cues to elicit behavior in animals and support new learning (Mackintosh, 1974; Hyde, 1976; Williams, 1994). Importantly, these procedures do not seek to model relapse. New Response Acquisition typically begins with a classical Pavlovian Conditioning phase. A primary reinforcer (drug or non-drug) is delivered noncontingently to animals while presentation of an

arbitrary stimuli, often a light or tone or both in combination, is given simultaneously. This is done in an operant chamber with no manipulanda present. The arbitrary stimuli should form an association with the primary reinforcer and acquire conditioned reinforcing properties after repeated pairings. Groups of animals in which both the reinforcer and cue are paired together are the experimental group, often called the Paired group.

After a period of Pavlovian Conditioning, subjects continue to the next phase of New Response Acquisition, called Acquisition. In this second phase, novel operant manipulanda, either nosepokes or levers, are introduced into the operant chamber. Animals then learn to make operant responses to earn contingent presentations of the conditioned reinforcer from Pavlovian Conditioning. Typically, there are two operant manipulanda, one active and one inactive. To establish that acquisition is dependent on instrumental contingency between a particular response and the cue, only responses on the active manipulanda have scheduled consequences of contingent cue delivery (Bertz and Woods, 2013). Importantly, there is no delivery or administration of primary reinforcers during Acquisition sessions. Cues that have developed conditioned reinforcing properties likely support new learning to a greater extent than novel cues (Bastle *et al.*, 2012) and subjects will acquire a new response to earn them (Mackintosh, 1974; Williams, 1994). Conditioned reinforcers can maintain high levels of responding in these sessions (Bertz and Woods, 2013; Robertson and Jutkiewicz, 2020) and for a significant periods of time, upwards of 40 (Chapter 2) to 60 days (Di Ciano and Everitt, 2004*a*). The reinforcing properties of the cues, therefore, last long past final primary

reinforcer delivery, and the association between cue and primary reinforcer extinguishes slowly.

During Acquisition, the cue functions as a conditioned reinforcer by increasing behavior during Acquisition through its association with primary reinforcer (Williams, 1994). It is unlikely that the cue has discriminative properties, as it did not signal imminent availability of primary reinforcers (Williams, 1994; Fantino, 2022). The cue may be predictive of drug effects in Paired groups, as cues are delivered simultaneously with primary reinforcer delivery (Savastano and Miller, 1998; Schultz, 2006). The behavior to earn contingent cues during Acquisition is not thought of as drug-seeking behavior, as the operant response has never been associated with prior primary reinforcer delivery (either contingent or noncontingent) (Mackintosh, 1974).

To demonstrate that conditioned cues maintain behavior, between-subjects control groups are used. During Pavlovian Conditioning, separate groups of animals can receive saline simultaneously with cues; therefore, conditioning should not occur. Saline conditioned cues maintain low levels of responding, indicating that the novelty of contingent cues may contribute to the behavior (preliminary data by Dr. Stephen Robertson). Other control groups receive primary reinforcer delivery and cue presentations that do not occur simultaneously. While the latter controls receive the same overall exposure to primary reinforcers and cue presentations as Paired groups, the cues should not form an association with primary reinforcers and should not develop conditioning reinforcing properties (Rescorla, 1967). There are generally two methods to deliver primary reinforcers and cues separately, either randomly or in an explicitly unpaired paradigm. With random conditioning, primary reinforcers and cues are often

delivered separately (Rescorla, 1967), but due to the random nature of presentations there are some incidental pairings in which the two events occur together (Robertson and Jutkiewicz, 2020). In explicitly unpaired conditioning, primary reinforcer delivery and cue presentations are scheduled such that they will never occur simultaneously (Chapter 2). These two groups can be referred to as Random and Unpaired groups, respectively. In both groups, the cue should not form an association with primary reinforcer and should not develop conditioned reinforcing properties (Rescorla, 1967; Mackintosh, 1974); however, depending on the half-life of the drug it is entirely possible that the cue is presented during near-peak drug effects. After conditioning sessions, subjects continue to the next phase where the ability of the cue to support acquisition of a novel response is tested. Overall, contingent cue presentations do not maintain high levels of responding in control groups since cues were not contiguously paired with drugs/reinforcers.

New Response Acquisition procedures are not a model of relapse but attempt to isolate conditioned reinforcement from other properties of cues (i.e., discriminative). Responding for conditioned reinforcers is not considered drug-seeking behavior, as operant responses for cues have never been associated with prior contingent drug-delivery, as in reinstatement paradigms. Reinstatement and New Response Acquisition procedures both measure conditioned reinforcement, but the utility of these preclinical models is distinct for modelling conditions that influence relapse.

Cues can develop conditioned reinforcing properties when paired with a variety of primary reinforcers. It has been shown that water- (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Wolterink *et al.*, 1993), sucrose- (Phillips *et al.*, 1994, Di Ciano and

Everitt, 2004*a*), heroin- (Di Ciano and Everitt, 2004*a*), remifentanil- (Bertz and Woods, 2013; Robertson and Jutkiewicz, 2020), and cocaine- (Di Ciano, 2008) associated stimuli can maintain robust responding during acquisition of a novel response for cues.

Multiple variables within the New Response Acquisition paradigm influence the ability of the cue to develop conditioned reinforcing properties and subsequent behavioral responding maintained by cues. First, the primary reinforcer used during Pavlovian Conditioning plays a role in responding for cues, such that drug-paired cues maintain different levels of behavior depending on type of primary reinforcer. For example, remifentanil-associated stimuli maintain on average 25-30 active responses (Bertz and Woods, 2013; Robertson and Jutkiewicz, 2020) while cocaine-associated cues maintain on average 60 responses (Chapters 2, 3, and 4) in Paired groups during a single, 60-min Acquisition session. Second, conditioned reinforcing effects of cues are related to dose of primary reinforcer (drug) (Davis and Smith, 1976; Bertz *et al.*, 2016; Robertson and Jutkiewicz, 2020). Third, the timing interval between pairings likely influences subsequent responding for the cue. For example, water and sucrose solution conditioning paradigms have used 30 seconds random interval schedules between trials (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Wolterink *et al.*, 1993; Phillips *et al.*, 1994). Remifentanil, a short acting mu opioid receptor agonist, was delivered noncontingently via a variable time 3 min schedule (Bertz and Woods, 2013; Bertz *et al.*, 2015, 2016; Robertson and Jutkiewicz, 2020, 2021), selected by doubling the half-life of remifentanil (Crespo *et al.*, 2005). There is the possibility that cues delivered during varying physiological effects of the primary reinforcer develop differential reinforcing properties. For example, in one study (Goddard and Leri, 2006), conditioning with

cocaine on a variable time 4 min schedule did not induce conditioned reinforcement during Acquisition, as levels of operant responses for the conditioned reinforcer were similar to that of a novel cue. Other factors, such as food restriction during conditioning, likely alter the conditioned reinforcing properties of cues (Robertson and Jutkiewicz, 2021) and all of these factors may influence conditioned reinforcement differentially depending on sex (Bertz *et al.*, 2015).

An important determinant for the formation of the association between cue and primary reinforcer is the total number of pairings during Pavlovian Conditioning. Total pairings are, generally, positively correlated with stronger conditioned reinforcing properties of cues (Di Ciano and Everitt, 2004*a*; Bertz and Woods, 2013). In Bertz and Woods (2013), 100 total pairings of remifentanil and cues during Pavlovian Conditioning elicited more robust behavior controlled by cues than 20 pairings. It is likely that more pairings allow for stronger learning of the association between cue and primary reinforcer and stronger conditioned reinforcing properties of the cues (Schultz, 2006). Sufficient learning is necessary to elicit differentiated behavior for conditioned reinforcers over novel cues (Bastle *et al.*, 2012). The number of pairings during conditioning is related to the duration of the conditioning sessions as well as the total number of sessions. Therefore, it is also possible that time plays a role in the formation of the association between cue and primary reinforcer, such that more time allows for the underlying neuroadaptations of learning to occur.

Lastly, other paradigms utilize a version of New Response Acquisition in which the primary reinforcer is delivered contingently (during self-administration) then subjects learn a different operant response to produce conditioned reinforcers alone

(Acquisition). Contingency of primary reinforcer delivery likely influences the conditioned reinforcing properties of the cues (also delivered contingently with drug infusion) and the acquisition of a novel response to earn cues (Namba *et al.*, 2018). Drug-paired cues develop conditioned reinforcing properties in these paradigms and support the learning of a novel operant response (Di Ciano and Everitt, 2004*a*; Di Ciano *et al.*, 2007, 2008; Di Ciano, 2008). It is important to note that acquisition of a different operant response may be augmented in subjects with a history of instrumental conditioning via self-administration due to better facilitation of learning the contingency between response and conditioned reinforcer.

In summary, New Response Acquisition procedures seek to isolate the conditioned reinforcing properties of drug- (or non-drug reward) paired cues. This is achieved because the operant response to produce presentations of conditioned reinforcers has never been associated with prior drug delivery (Mackintosh, 1974; Williams, 1994). Therefore, conditioned reinforcers support new learning (Mackintosh, 1974) and maintain high levels of operant responding in subjects that underwent Paired Pavlovian Conditioning. Some limitations of New Response Acquisition are that within the control groups (Random and Unpaired Pavlovian Conditioning), cue presentations are not always separated from the physiological effects of primary reinforcers. Therefore, cues likely overlap with these effects to some extent, and develop weak conditioned reinforcing properties (Robertson and Jutkiewicz, 2020). Control groups for New Response Acquisition are meant to control for the association between cue and primary reinforcer, while keeping total primary reinforcer and cue exposure the same as experimental groups; however, there are limitations to these controls.

1.2 Underlying neurobiology of reward-paired cues

Preclinical paradigms used to evaluate drug-paired cues can be useful for examining the neurobiology underlying behaviors mediated by conditioned reinforcers. Understanding the neuroadaptations that occur following association of a cue with a drug may provide targets for potential treatments of relapse in people. To that end, there are many techniques for probing neurobiological mechanisms of behavior, such as *in vivo* measurement of neurotransmitters or peptides, pharmacological manipulations of different neurotransmitter systems, or circuit manipulation via optogenetics or chemogenetics.

The underlying neurobiological mechanisms of reinstatement, or drug-seeking behaviors, have been studied for decades. Many different neurotransmitter or peptide systems have been implicated in contributing to the behavior elicited and maintained by drug-paired cues (for reviews, see: Meil and See, 1996; Everitt and Robbins, 2000; Shalev *et al.*, 2002; Shaham *et al.*, 2003; See, 2005; Torregrossa and Kalivas, 2008; Bossert *et al.*, 2013; Lüscher, 2016; Namba *et al.*, 2018). These include, but are not limited to, glutamatergic (Kalivas and McFarland, 2003; Wise, 2009; Scofield *et al.*, 2016), cholinergic (See, 2005; Zhou *et al.*, 2007; Collins *et al.*, 2016), serotonergic (Burmeister *et al.*, 2003; Bossert *et al.*, 2013), and dopaminergic systems (Di Chiara and Bassareo, 2007) (described below). Further, neuropeptides have been shown to modulate “classic” neurotransmitter systems in cue-controlled behaviors, such as endocannabinoids (Namba *et al.*, 2018) or opioids (Pellissier *et al.*, 2018; Rysztak and Jutkiewicz, 2022). Brain regions mediating these effects lie in the mesolimbic dopamine

pathway, but also include connected regions such as the ventral pallidum or hippocampus (Namba *et al.*, 2018)

While the dopaminergic and glutamatergic system (Kalivas and McFarland, 2003) likely play a significant role in drug-seeking and reinstatement behaviors, these systems are hard to target for therapeutics due to their essential functions in many physiological processes. Therefore, modulatory systems, such as the opioidergic system or cholinergic system, may provide better targets for altering neurotransmission that contribute to drug-craving and relapse. Future work is required to further evaluate the role of modulatory systems in conditioned reinforcement. This introduction is not meant to be an exhaustive review of the different neurotransmitter systems in conditioned reinforcement, but rather to highlight findings relevant to the dissertation. Dopaminergic and opioidergic mechanisms of conditioned reinforcement, as evaluated in Pavlovian Conditioning, cue-induced reinstatement, or New Response Acquisition procedures, are briefly summarized below.

1.2.1 Neurobiology of Pavlovian Conditioning

Arbitrary cues acquire conditioned reinforcing properties through the association with a primary reinforcer (Williams, 1994), through either classical Pavlovian Conditioning or instrumental conditioning (Schultz, 2006). In classical Pavlovian Conditioning, the primary reinforcer is delivered noncontingently following a stimulus. After sufficient pairings, presentations of a conditioned stimulus alone is able to induce increases in dopamine concentrations in the nucleus accumbens (NAc) (Datla *et al.*, 2002; Bassareo *et al.*, 2007). In order to determine how dopamine dynamics are changing across conditioning of cue with reinforcer, techniques with high temporal

resolution can time-lock dopamine signals with pairings between cues and reinforcers and were used to evaluate changes in dopamine between the first pairings to later in conditioning. During initial pairings, dopamine concentrations are transiently increased in the NAc following delivery of the primary reinforcer (Day *et al.*, 2007; Sunsay and Rebec, 2008). Following repeated pairings of the primary reinforcer with the cue, dopamine is transiently increased upon presentation of the cue, rather than delivery of the primary reinforcer (Flagel *et al.*, 2011). Indeed, the magnitude of the phasic increases in dopamine to stimuli are positively correlated with the number of pairings (Day *et al.*, 2007), particularly in the NAc core (Sunsay and Rebec, 2008; Aragona *et al.*, 2009). These data suggest dopamine encodes the strength of the associations between cue and primary reinforcer and reflects anticipation of reward upon perception of the cue (Mirenowicz and Schultz, 1996).

Consistent with these findings, manipulating the dopaminergic system influences conditioned behaviors. Amphetamine administration is sufficient to increase conditioned approach behaviors (Wan *et al.*, 2007; Wan and Peoples, 2008) and selectively activating mesolimbic dopamine neurons with optogenetics during cue presentation, even without delivery of primary reinforcers, can promote approach to cues (Saunders *et al.*, 2018). Conversely, blocking dopamine signaling with D1 antagonists decreased a conditioned approach behavior (Dalley *et al.*, 2005). These findings indicate that modulation of the dopamine system can alter conditioned behaviors. Together, this demonstrates that dopamine is a likely mediator of conditioned responses.

During instrumental conditioning, cues are associated with contingent drug-delivery through self-administration procedures, and dopamine is also increased upon

noncontingent presentation of cues alone, to a greater extent in the NAc core than shell (Phillips *et al.*, 2003). Therefore, dopamine likely plays a role in encoding the associations between cues and primary reinforcers regardless of contingency.

The exact function of dopamine in mediating the formation of associations between cues and primary reinforcers is complex, with multiple theories in the field. Commonly, dopamine is thought to encode reward prediction errors (RPEs), such that it is increased with an unexpected reward and promotes learning between cues and the unpredicted outcome. Increases in dopamine will then transfer to the cue that predicted the reward and will bias behavior towards that outcome in the future (Schultz, 2006; Steinberg *et al.*, 2013; Nasser *et al.*, 2017). This idea was further developed to include dopamine encoding unexpected aversive stimuli in addition to reinforcing stimuli (Nasser *et al.*, 2017). Other theories are that dopamine plays a role in motivation and driving behavior towards an outcome (Mohebi *et al.*, 2019), stimulus-change (Winterbauer and Balleine, 2007) or attentional processes to changing stimuli (Nasser *et al.*, 2017), retrospective learning of cue outcomes (Jeong *et al.*, 2022), or encoding value of outcomes, including predicted omissions (Kutlu *et al.*, 2022). Overall, the neurobiological processes by which cues form associations with primary reinforcers (or rewarding outcomes) involve dopamine, although its exact role is complex.

Less work has been done examining other brain regions or neurotransmitter systems in Pavlovian Conditioning, yet they are likely involved. The glutamatergic system is directly involved with learning, and indeed antagonizing the glutamatergic system via an NMDA receptor antagonist immediately following conditioning blocks conditioned approach behaviors (Dalley *et al.*, 2005). Brain regions such as the

amygdala, which has been shown to be important for reinstatement behaviors, is also involved in the expression of a conditioned response (See, 2005). Dopamine (Berglind *et al.*, 2006) as well as acetylcholine (Squire and Davis, 1981; See *et al.*, 2003; See, 2005) in the amygdala contribute to the formation of cue- or context-drug associations and have been discussed in the context of drug-cue memories (Kelley *et al.*, 2007; Wan *et al.*, 2014).

1.2.2 Dopamine and reward-paired cues

"Classic" drugs of abuse, such as psychostimulants, opioids, and nicotine, that maintain self-administration behavior in both animal models and humans, induce a characteristic elevation in dopamine in the NAc after administration (for review, see: Di Chiara *et al.*, 2004). This can occur via stimulation (or disinhibition) of dopamine neurons in the ventral tegmental area (VTA) projecting to the NAc and/or by inhibiting the reuptake of dopamine in the NAc. Dopamine receptors are differentially expressed on cell types within the NAc. Excitatory D1 receptors are expressed on D1-medium spiny neurons (MSNs) which project to the VTA and co-express GABA and dynorphin while inhibitory D2 receptors are located on D2-MSNs projecting to the ventral pallidum and co-expressing GABA and enkephalins (Zahm *et al.*, 1985; Yager *et al.*, 2015).

1.2.2.1 Dopamine and cue-induced reinstatement

Drug-paired cues, one factor that can contribute to relapse, can also increase dopamine in the NAc, which supports other frameworks explaining the role of dopamine in various aspects and stages of SUDs, such as the opponent process and incentive salience theories (for reviews, see: Berridge, 2007; Trigo *et al.*, 2010). Dopamine levels in the dorsal striatum are correlated with increased subjective craving in people

abstinent from cocaine use following the presentation of a cocaine-associated cue (Volkow *et al.*, 2006). Further, in cue-induced reinstatement paradigms, researchers have measured whether dopamine is altered during drug-seeking behaviors. Dopamine concentrations measured via microdialysis are increased in the NAc, amygdala, or medial prefrontal cortex in the presence of a discriminative stimulus (Weiss *et al.*, 2000) and while responding to earn discrete cues previously associated with psychostimulants during reinstatement (Parsegian and See, 2014). These findings are consistent with the dopaminergic neurobiology of Pavlovian Conditioning. They further suggest that dopamine may have a role in mediating behavior to earn conditioned reinforcers during reinstatement procedures.

Researchers often implicate a role of the dopamine system using pharmacological manipulations to alter cue-induced reinforcement behaviors. Briefly, administration of indirect dopamine agonists systemically or into the NAc or medial prefrontal cortex potentiated responding for cocaine-paired cues (Park *et al.*, 2002). Blockade of dopamine signaling by administration of D1, D2, or D3 receptor antagonists has been shown to reduce cue-induced drug-seeking behaviors (Ciccocioppo *et al.*, 2001; Alleweireldt *et al.*, 2002; Gilbert *et al.*, 2005; Sun and Rebec, 2005; Gál and Gyertyán, 2006; Liu *et al.*, 2010). Interestingly, D1 or D2 agonists have also been observed to reduce cue-induced reinstatement (Self *et al.*, 1996; Alleweireldt *et al.*, 2002) (for review, see: Namba *et al.*, 2018). The discrepancies between dopamine receptor activation or blockade on attenuating cue-induced reinstatement behaviors may be due to behaviorally disrupting effects at certain doses. The ability of dopamine to mediate reinforcement may be dependent on stimulus availability, as D2 agonists

maintained operant responding when responses resulted in a conditioned stimulus but not when there were no scheduled consequences (Collins and Woods, 2009; Collins *et al.*, 2012).

Further, indirect dopamine agonists into the NAc augmented reinstatement behaviors induced by noncontingent presentations of drug-paired discriminative stimulus (Saunders *et al.*, 2013), which were blocked by nonselective or D2/D3 receptor antagonists (Cervo *et al.*, 2003; Saunders *et al.*, 2013). Therefore, dopamine is likely involved in the discriminative effects of drug-paired cues.

In addition to pharmacological studies, there has been much work to elucidate the circuitry involved in cue-induced reinstatement behaviors. The NAc is regarded as a central hub for mediating primary reinforcement as well as conditioned reinforcement by drug-paired cues (Willuhn *et al.*, 2010). Previous work has shown functional differences between the NAc core and shell subregions, such that the shell may be more responsible for cue-controlled reinstatement (Vassoler *et al.*, 2013; Guercio *et al.*, 2015; Augur *et al.*, 2016), although other studies have shown the opposite (Fuchs *et al.*, 2004). Multiple brain regions have shown functional interactions with the NAc to contribute to reinstatement behaviors. The mesolimbic dopamine circuit, particularly VTA to NAc projections, is particularly important for drug-primed reinstatement (McFarland and Kalivas, 2001; Kalivas and McFarland, 2003), but has also shown to be involved in cue-induced drug seeking through dopaminergic (Halbout *et al.*, 2019; Jing *et al.*, 2022) mechanisms in addition to other mechanisms, such as cholinergic transmission (Zhou *et al.*, 2007). The mesocortical dopamine circuits, such as medial prefrontal cortex or prelimbic cortex projections to the NAc, can drive behaviors maintained by drug-paired

cues ((Park *et al.*, 2002; Parsegian and See, 2014; McGlinchey *et al.*, 2016) for reviews, see: Bossert *et al.*, 2013; Namba *et al.*, 2018)). Functional differences between drug-seeking and conditioned behaviors between the mesolimbic and mesocortical dopamine systems have been observed (Halbout *et al.*, 2019). Further, NAc efferents to downstream brain regions, such as the ventral pallidum (Heinsbroek *et al.*, 2017, 2020), are important for modulating cue-induced reinstatement (O'Neal *et al.*, 2020).

In addition to the NAc, the amygdala has been extensively studied for its role in cue-induced drug seeking. Dopamine within the amygdala is associated with greater responding for drug-paired cues (Weiss *et al.*, 2000), such that potentiating dopamine receptor signaling via agonists further drives drug-seeking behaviors ((Di Ciano and Everitt, 2004*b*; Berglind *et al.*, 2006), for reviews, see: Schmidt *et al.*, 2005; See, 2005)).

Overall, there is extensive data indicating that dopamine is involved in the conditioned reinforcing properties of drug-paired cues such that it is often assumed that dopamine plays a role in mediating behavior maintained by cues. However, there are numerous studies that do not implicate dopamine in cue-induced craving or cue-controlled behavior. In people abstinent from cocaine use, dopamine levels in the dorsal striatum, but surprisingly not in the nucleus accumbens, were associated with subjective craving score following presentation of cocaine-associated visual cues (Volkow *et al.*, 2006). In preclinical models, contingent presentation of drug-paired cues did not always stimulate increases in dopamine in the NAc (Brown *et al.*, 1992; Bradberry *et al.*, 2000; Ito *et al.*, 2000). Similarly, during reinstatement procedures, increased dopamine concentrations were not associated with higher levels of responding for cues, measured either via microdialysis (Neisewander *et al.*, 1996; Katner and Weiss, 1999) or with

chronoamperometry (Di Ciano *et al.*, 2001). The latter study demonstrated that contingent cue delivery during cue-induced reinstatement did not lead to increases in dopamine transients in the NAc, measured with high temporal resolution (Di Ciano *et al.*, 2001). These studies highlight that the function of dopamine in mediating the ability of drug-paired cues to induce behavior is not straightforward. There are many different factors that could explain differential recruitment of the dopaminergic systems in these behaviors, such as contingency of cue delivery (Ito *et al.*, 2000; Namba *et al.*, 2018). Overall, these studies demonstrate important differences in the potential function of dopamine in cue-controlled reinstatement behaviors.

1.2.2.2 Dopamine and New Response Acquisition

Differences in the function of drug-paired cues, due to history of drug delivery, may involve similar but distinct neurobiological processes in reinstatement and New Response Acquisition procedures. Studies have investigated the dopamine system in mediating the conditioned reinforcing properties of drug-paired cues selectively using New Response Acquisition procedures, such that manipulating the dopaminergic system via indirect or direct agonists has altered behavior. Intra-NAc or systemic amphetamine dose-dependently enhanced novel responses to produce water-paired stimuli in groups of rats in which the cue was positively correlated with water (Paired group) (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Burton *et al.*, 2011), and was blocked by either intra-NAc D1 or D2 antagonists (Wolterink *et al.*, 1993). Further, D1 or D2/D3 agonists (quinpirole) into the NAc enhanced responding for the water conditioned reinforcers (Wolterink *et al.*, 1993). These effects were replicated for sucrose-associated cues (Phillips *et al.*, 1994). Dopamine receptor agonists may

differentially alter responding depending on time of administration, such that pramipexole (D2/D3 agonist) also potentiated responding for a remifentanil-associated stimulus, but only after 6 days of repeated administration (Bertz *et al.*, 2015). Systemic cocaine increased responding for cocaine-paired cues, albeit levels of responding for the conditioned reinforcers did not differ to that of novel cues prior to cocaine administration (Goddard and Leri, 2006). Administration of cocaine also potentiated conditioned reinforcement in mice that are bred to exhibit high psychomotor sensitization (Bailey *et al.*, 2023).

The NAc appears to be a central mediator of these drug effects, as activity in the dorsal striatum, thalamus, and medial orbital frontal cortex did not influence conditioned reinforcement (Taylor and Robbins, 1984; Jenni *et al.*, 2023). Other catecholamines have been investigated in conditioned reinforcement, and activation of serotonin receptors can potentiate responding for cues (Guy *et al.*, 2014; Fletcher *et al.*, 2017), while norepinephrine concentrations in the NAc did not influence responding (Cador *et al.*, 1991).

Different factors likely alter the ability of dopaminergic drugs to alter conditioned reinforcement, such as sex (Bertz *et al.*, 2016), food restriction or water deprivation (Ostlund *et al.*, 2011; Tabbara *et al.*, 2016; Robertson and Jutkiewicz, 2021), and contingency of cues. A version of New Response Acquisition utilizes a novel operant response following contingent drug self-administration. In these procedures, cocaine-paired cues alone maintain high levels of responding that persisted for months (Di Ciano and Everitt, 2004*a*) in a dopamine dependent manner (Di Ciano, 2008), potentially involving both the NAc and the medial prefrontal cortex (Di Ciano *et al.*, 2007). It is

important to note the caveat of prior experience with instrumental responding on the acquisition or learning of a novel operant response, such that there may be some overlap of instrumental responding with primary reinforcer delivery.

Together, these studies suggest dopamine, particularly in the NAc, modulates and contributes to responding for conditioned reinforcers. Therefore, dopamine may mediate the conditioned reinforcing effects of cues and influence the ability of these cues to elicit behavior in animals, consistent with the role of dopamine from reinstatement literature.

1.2.3 Opioids and reward-paired cues

In addition to dopamine, numerous neurotransmitter and receptor systems have been implicated in the adaptations caused by drugs of abuse and in the transition from recreational use to substance use disorders. The endogenous opioid system, comprised of multiple opioid receptor types and endogenous ligands, is highly expressed in reward circuitry (Figure 1.2) and has been proposed to be a crucial modulator of SUDs (for reviews, see: Trigo *et al.*, 2010; Rysztak and Jutkiewicz, 2022).

There are three primary opioid peptide gene families: proopiomelanocortin (*POMC*), proenkephalin (or preproenkephalin; *PENK*), or prodynorphin (*PDYN*). These genes are translated into prepropeptides (proopiomelanocortin, proenkephalin A, and prodynorphin, respectively) before being cleaved into the final functional peptides, beta-endorphin, enkephalin, and dynorphin. The primary peptides share a common amino acid N-terminal sequence Tyr-Gly-Gly-Phe-X (Met/Leu for enkephalin). A fourth family of opioid peptide, nociceptin, is derived from prepronociceptin.

Opioid peptides bind to opioid receptors, which are G-protein coupled receptors (GPCRs). These receptors are coupled to the Gi/o proteins, leading to inhibition of cAMP, inhibition of Ca^{2+} channels, activation of inwardly rectifying K^{+} channels and MAP kinase pathway, which ultimately inhibits neuronal activation and neurotransmitter release (Law *et al.*, 2000). Each receptor is encoded by separate genes, MOR: *Oprm1,* DOR*: Oprd1,* KOR*: Oprk1,* and ORL1*: Oprl1.* Canonically, it is believed that beta-endorphin, met-/leu-enkephalin, and dynorphin preferentially bind to the mu opioid receptor (MOR), delta opioid receptor (DOR) and kappa opioid receptor (KOR), respectively. Nociceptin/orphanin FQ binds to the nociceptin opioid peptide receptor (NOPR; or opioid receptor-like 1 [ORL1]). Enkephalins bind with high affinity to DOR and MOR (with slightly greater affinity (10-fold) for DOR than MOR; measured under nonphysiological conditions) (Raynor *et al.*, 1994), but more recently, all opioid peptides have been shown to bind to each of the opioid receptors to some extent (Gomes *et al.*, 2020). For example, beta-endorphin, met-enkephalin and dynorphin have been shown to be full agonists at MOR and partial agonists at DOR. Shorter forms of beta-endorphin, generally thought to have limited activity at opioid receptors, may also be agonists at MOR (Gomes *et al.*, 2020). Therefore, focusing on enkephalin-DOR or enkephalin-MOR interactions in studies investigating substance use disorders may be overlooking other important interactions of other endogenous opioid peptides and receptor types. Overall, while many studies implicate enkephalin in multiple aspects of SUDs, there is likely distinct and overlapping roles of other endogenous opioid peptides as well.

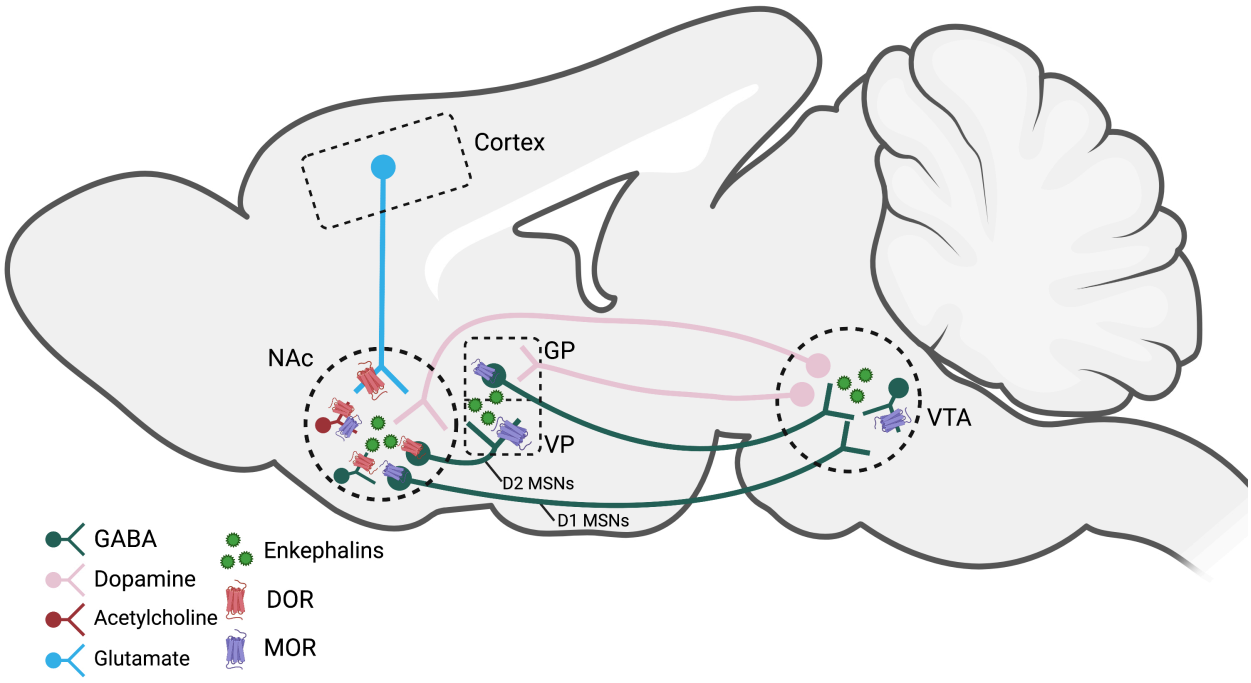

Figure 1.2 Adapted from Rysztak and Jutkiewicz, 2022. Brain regions and pathways implicated in opioid-mediated reward-related behaviors. Dopamine neurons in the VTA that project to the NAc are modified by MORs on GABAergic interneurons. Activation of MORs and DORs, likely by enkephalins, within the NAc modulate dopamine, GABA, glutamate, and acetylcholine release. D2 MSNs express enkephalin and project to the VP and are believed to be a crucial circuit for reinstatement behaviors. Figure created using Biorender.com. NAc = nucleus accumbens; GP = globus pallidus; VP = ventral pallidum; VTA = ventral tegmental area; MOR = mu opioid receptor; DOR = delta opioid receptor; MSNs = medium spiny neurons

The opioidergic system modulates multiple neurotransmitters systems within the mesolimbic dopamine pathway, such as dopaminergic, glutamatergic, and cholinergic systems, to influence reward-related behaviors (Figure 1.2) (Mongi-Bragato *et al.*, 2018; Rysztak and Jutkiewicz, 2022). There is evidence that neuroadaptations in the endogenous opioid system occur following exposure to drugs of abuse to promote reinforcement (Rysztak and Jutkiewicz, 2022). For example, repeated cocaine may increase expression of endogenous enkephalin peptides which contribute to the primary

reinforcing effects of cocaine (Sun *et al.*, 2020), behavioral sensitization (Mongi-Bragato *et al.*, 2016, 2021) and likely conditioned reinforcement.

1.2.3.1 Opioids and cue-induced reinstatement

Drug-seeking behaviors measured via cue-induced reinstatement procedures have been shown to be influenced by opioidergic signaling (Burattini *et al.*, 2008). Naltrexone, a nonselective opioid receptor antagonist, dose-dependently decreases reinstatement of responding for cocaine-paired cues following a period of extinction (Burattini *et al.*, 2008). Individual opioid receptors have also been evaluated to determine whether they contribute to responding maintained by drug-paired cues. MOR and DOR knockout mice show reductions in cue-induced reinstatement (Gutiérrez-Cuesta *et al.*, 2014) and consistently, exogenous administration of beta-endorphin or enkephalins promotes reinstatement (Simmons and Self, 2009). However, a MOR agonist has also been shown to reduce cue-induced food-seeking (Guy *et al.*, 2011). Activating or antagonizing the KOR system with either spiradoline or JDTIC, respectively, both reduced cue-induced reinstatement of responding, suggesting there may be differential involvement of dynorphins or KORs due to primary reinforcers type or contingency of drug-paired cues (Morani *et al.*, 2009; Schank *et al.*, 2012). It is possible that the different opioid peptide or receptor types have overlapping functions within reinforcement behaviors, such that both the MOR and DOR systems may be critical for the formation of drug-cue associations, which are necessary for conditioned reinforcement (Skoubis *et al.*, 2005; Le Merrer *et al.*, 2011; Bertran-Gonzalez *et al.*, 2013; Gutiérrez-Cuesta *et al.*, 2014) while the MOR system alone contributes to the

primary reinforcing effects of opioids and cocaine (Ward *et al.*, 2003; Schroeder *et al.*, 2007; Charbogne *et al.*, 2014).

1.2.3.2 Opioids and New Response Acquisition

Few studies have investigated how the opioid system might alter responding maintained by drug-paired cues in stringent tests of conditioned reinforcement, or New Response Acquisition. Opioid-paired cues can facilitate new learning, such that morphine-paired (Davis and Smith, 1976) or remifentanil-paired cues (Bertz and Woods, 2013; Robertson and Jutkiewicz, 2020) elicit robust responding. Further, manipulating the opioidergic system via intra-NAc, but not intra-VTA, infusions of either a MOR agonist, a DOR agonist, or MOR/DOR agonist increased responding for sucrose-associated cues (Phillips *et al.*, 1994). KOR agonists did not alter conditioned reinforcing effects of either ethanol- or sucrose-paired cues (Tabbara *et al.*, 2020). On the other hand, naltrexone reduced responding for a sucrose-associated conditioned reinforcer (Burton *et al.*, 2011). It is possible the opioidergic system mediates the ability of other drugs to alter conditioned reinforcement, demonstrated by the finding that naltrexone slightly reduced the ability of nicotine to potentiate responding for water-associated cues (Guy *et al.*, 2014). Together, these data suggest that the endogenous opioid system modulates behavior maintained by both drug- or non-drug-paired cues, potentially interacting with the dopaminergic system in mesolimbic circuitry. Overall, the opioidergic system remains relatively understudied in assays that attempt to isolate conditioned reinforcement.

Overall, cue-induced drug-seeking behaviors have complex underlying neurobiological mechanisms and recruit multiple different neurotransmitter and

neuropeptides systems across multiple brain regions. The function of the drug-paired cue; however, is complicated by the fact that operant responding to earn cues had previously earned contingent drug-delivery. Therefore, drug-paired cues likely develop motivational, discriminative, and/or conditioning reinforcing properties (Mackintosh, 1974; Williams, 1994). The function of the cue in stringent tests of conditioned reinforcement likely recruits similar neurobiological processes, such that dopamine and opioids have been shown to modulate cue-controlled behaviors. However, it is important to note that these two behavioral procedures measure different properties of drug-paired cues and may have distinct underlying neurobiology.

1.3 Gaps in Knowledge

Understanding the underlying neurobiology by which drug-paired cues elicit feelings of craving and promote relapse may provide potential targets for pharmacological therapies to intervene and prevent drug relapse. While researchers use different preclinical approaches to investigate neurobiological mechanisms of drug-seeking behavior, these models are not perfect and are comprised of many complex behaviors. Dopamine has been heavily implicated in the formation of cue+primary reinforcer associations (Schultz, 2006), and has been associated with greater responding for drug-paired cues (studies described above). However, the dopaminergic system is not solely responsible for cue-controlled behaviors. Further, antagonizing the dopaminergic system has had some clinical efficacy in reducing cue reactivity (Weber *et al.*, 2016), but adverse effects such as motor deficits limit the utilization of these drugs (Meisenzahl *et al.*, 2008). Many studies report that dopamine is correlated with or mediates behaviors controlled by cues formerly paired with drugs; however, there are

some studies that found that dopamine is not involved. Whether dopamine is mediating behavior may depend on the assays used to evaluate neurochemical changes. Additionally, a history of contingent drug self-administration may influence the role of dopamine in the effects of drug-paired cues.

Behavioral measures of conditioned reinforcement require more development. Early works utilized cues paired with water or sucrose, yet fewer studies have been modified for drug-paired cues. A short acting opioid, remifentanil, has been shown to induce conditioned reinforcing effects in paired cues (Bertz and Woods, 2013; Robertson and Jutkiewicz, 2020), while tests of psychostimulant conditioned reinforcement are mostly lacking. Assays for cocaine conditioned reinforcement have utilized contingent cocaine self-administration prior to tests of conditioned reinforcement (Di Ciano, 2008), potentially overlapping the conditioned reinforcing properties of cues with more motivational aspects from prior drug delivery. One study, to our knowledge, has used cocaine as a conditioning drug; however, this paradigm was not sufficient for the cue to acquire conditioned reinforcing effects, as responding for the cocaine-paired cue was not higher than that for a novel cue (Goddard and Leri, 2006). The ability of drug-paired cues to elicit and maintain behavior in New Response Acquisition procedures is influenced by many factors, such as dose of primary reinforcer, number of pairings, and interval between pairings. Therefore, these procedures require optimization for each primary reinforcer tested in order to best model conditioned reinforcement selectively.

Behavioral measures of conditioned reinforcement, such as New Response Acquisition procedures, provide valuable models to measure the conditioned reinforcing

properties of drug-paired cues, but the underlying neurobiology has not been as well characterized as for reinstatement paradigms. The neurobiology of reinstatement has been extensively probed via pharmacological, optogenetic, and chemogenetic studies which are lacking for New Response Acquisition. Additionally, individual differences in dopamine-dependent cue-controlled behaviors have been observed (Homberg *et al.*, 2004; Flagel *et al.*, 2011; Shaw *et al.*, 2021), but these have not been investigated in tests of conditioned reinforcement.

Furthermore, the potential involvement of the opioidergic system in behaviors elicited by the conditioned reinforcing properties of drug-paired cues has not been extensively studied. Pharmacological manipulations of the opioid system is often the use of non-selective opioid receptor antagonists, which only indirectly implicate endogenous opioid peptides in certain reward-related behaviors (Rysztak and Jutkiewicz, 2022). Therefore, activating opioid receptors with endogenous peptides to alter behavior is a useful tool for implicating the opioidergic system. Overall, evaluating the opioidergic system in conditioned reinforcement will provide better insight into the underlying neurobiology of drug-paired cues to elicit behavior in preclinical assays, and potentially add further characterization to dopamine-opioid interactions in cue-maintained behaviors. These studies will provide important insight into the reinforcing properties of drug-paired cues and may highlight novel targets for selectively reducing cue-induced drug craving in people with substance use disorders.

1.4 Goals of Dissertation

The objective of this dissertation was to investigate the underlying neurobiology of the conditioned reinforcing properties of drug-paired cues. To that end, we focused

on involvement of both dopaminergic and opioidergic mechanisms. In an attempt to selectively measure the conditioned reinforcing effects of cues, we utilized the New Response Acquisition procedure. In Aim 1, we optimized the New Response Acquisition procedure to evaluate cocaine conditioned reinforcement. In Aim 2, we characterized dopaminergic involvement in responding for cocaine-paired cues via neurochemical measurements of dopamine in the NAc and local and systemic pharmacological manipulations. In Aim 3, we investigated the role of the opioidergic system during acquisition of a novel response for cocaine-paired cues using pharmacological manipulations.

1.4.1 Aim 1: Optimize a stringent test of conditioned reinforcement for cocaine-paired cues

We sought to establish a procedure in which arbitrary cues develop conditioned reinforcing properties due to an association with cocaine and support learning of a novel operant response maintained by cues alone. We manipulated various aspects of the Pavlovian Conditioning phase to alter conditioned reinforcement and elicit high levels of responding in Paired groups for subsequent studies probing the underlying neurobiological mechanisms of the behavior (Chapters 3 and 4). We hypothesized that cues would acquire conditioned reinforcing effects in a dose-dependent manner and that more pairings between cue+cocaine would lead to stronger effects. Indeed, higher doses of cocaine paired with cues during Pavlovian Conditioning promoted conditioned reinforcement and induced more responding for cues. The behavior to earn cocaine-paired cues persisted for upwards of 40 days. Interestingly, more pairings (100 total pairings vs. 50 total) did not reliably induce greater reinforcing properties of the cocaine-

paired cue, but more days of conditioning did. The explicitly Unpaired paradigm induced greater behavioral differences between control and experimental groups. Further, we characterized how schedule of reinforcement for contingent cue presentations influenced acquisition of a novel response. Robust levels of responding for cues were maintained on both an intermittent schedule (Random Ratio 2) and a predictable schedule (Fixed Ratio 1). Overall, we successfully established and optimized a procedure in which cues develop conditioned reinforcing properties noncontingently and elicit robust levels of behavior for cues.

1.4.2 Aim 2: Investigate the role of dopamine in the nucleus accumbens during a robust test of cocaine conditioned reinforcement

We investigated the dopaminergic system in cocaine conditioned reinforcement using New Response Acquisition by 1) measuring extracellular dopamine concentrations in the NAc core and shell and 2) locally and systemically potentiated dopamine concentrations by administration of indirect dopamine agonists. We hypothesized that dopamine levels would be greater in rats that respond for cues paired with cocaine (Paired groups) than in control groups in which cues were not paired with cocaine (Unpaired groups). Further, activating the dopamine system via indirect agonists would further drive responding to earn cues, as seen in reinstatement procedures (Lu *et al.*, 2004). Surprisingly, dopamine levels were unaltered from baseline while animals responded for cues, and there were no differences in dopamine concentrations between Paired and Unpaired groups, despite differences in levels of responding. Local and systemic administration of psychostimulants did not potentiate responding for cocaine-paired cues, contrary to our hypothesis. Overall, dopamine did

not mediate responding to earn cocaine-paired cues in the New Response Acquisition procedure.

1.4.3 Aim 3: Assess the role of the opioidergic system during acquisition of a novel operant response for cocaine-paired stimuli alone

We then sought to evaluate a role of the opioidergic system in mediating the conditioned reinforcing effects of cocaine-paired cues. We administered pharmacological treatments to target different opioid peptide and receptor families, focusing on the enkephalins and DOR. We focused on the DOR system based on previous work showing enkephalins and DOR activation promoted cocaine reinstatement (Simmons and Self, 2009). We hypothesized that indirect or direct DOR activation would increase the conditioned reinforcing properties of cocaine-paired cues and would potentiate responding to earn cues. Indeed, acute administration of an enkephalinase inhibitor, which prevents the degradation of endogenous enkephalins (Jutkiewicz, 2007; Roques, 2018), robustly enhanced responding to earn cocaine-paired cues, and this effect was blocked by a DOR selective antagonist. DOR activation alone was able to enhance cocaine conditioned reinforcement, but MOR or KOR activation was not. Overall, this study suggests cocaine conditioned reinforcement may be mediated via the DOR system.

In conclusion, the work completed in these Aims have addressed the objective of the dissertation to investigate dopaminergic and opioidergic systems in mediating the conditioned reinforcing properties of drug-paired cues. In Aim 1, we established a procedure to selectively measure the conditioned reinforcing properties of cocaine-paired cues and optimized it to elicit robust responding for cues. In Aim 2, we evaluated

a role of dopamine and demonstrated that responding for cocaine-paired cues in this procedure does not appear to be dopamine-dependent. In Aim 3, we investigated role of the opioidergic system in cocaine conditioned reinforcement and found that DOR activation, either via protected endogenous enkephalins or exogenous ligands, potentiated the conditioned reinforcing properties of cocaine-paired cues and drove responding to earn cues, suggesting the DOR system may play a role in regulating this behavior. Overall, the work discussed in this dissertation has provided novel and surprising insight into the mechanisms of cocaine conditioned reinforcement and will serve as a foundation for future investigations into neurobiological targets to prevent cue-induced cocaine craving and relapse.

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Chapter 2
Establishing a Test of Cocaine Conditioned Reinforcement

2.1 Abstract

Environmental cues acquire conditioned reinforcing properties following pairing with a primary reinforcer during Pavlovian Conditioning. Conditioned reinforcers induce behavior in animals and can support learning of a novel operant response maintained by cues alone in New Response Acquisition paradigms, which are thought to be a selective measure of the conditioned reinforcing properties of drug-paired cues. In the current study, we sought to establish a procedure of cocaine New Response Acquisition. This procedure begins with Pavlovian Conditioning in which subjects receive infusions of cocaine and either simultaneous (Paired) or separate (Random or Unpaired) presentations of a light+tone stimulus per day for consecutive days. Then, novel operant manipulanda are introduced into the chamber, and responses produce presentations of cues formerly associated with cocaine (Acquisition). We evaluated the extent to which dose of cocaine (0.1, 0.32, or 0.56 mg/kg/infusion) and the total number and pattern of pairings (10 pairings/day for 5 days, 5 pairings//day for 10 days, 20 pairings/day for 5 days, 10 pairings/day for 10 days) influenced the conditioned reinforcing properties of cues and altered responding. We compared levels of responding in Paired groups to controls that received cocaine and cues Randomly or explicitly Unpaired. Finally, we evaluated levels of responding when cues were delivered via a random ratio 2 (RR2) or fixed ratio 1 (FR1) schedule of reinforcement

during Acquisition. Overall, we optimized the procedure such that conditioning with 0.32 mg/kg/infusion for 5 pairings/day for 10 days (50 total pairings) elicited robust responding for cues earned on a RR2 schedule. These results highlight factors that influence the development of conditioned reinforcing properties of cues and demonstrate a procedure to measure cocaine conditioned reinforcement selectively.

2.2 Introduction

Cocaine use disorder is a chronically relapsing condition characterized by difficulty to terminate use and frequent relapse following periods of abstinence. One major contributor to relapse is exposure to cocaine-paired stimuli that have been associated with cocaine-use (Bonson *et al.*, 2002; Volkow and Li, 2005). Cocaine-paired cues have conditioned reinforcing properties after being paired, or associated with, delivery of primary reinforcers (i.e., drug or food), such that they can elicit drug-seeking behaviors in animals (Venniro *et al.*, 2020) and likely influence susceptibility to relapse in humans. Understanding the underlying neurobiological mechanisms of how conditioned reinforcers modify behavior may provide novel targets for developing treatments for relapse.

In preclinical studies, operant responses that produce presentation of drug-paired cues in the absence of drug delivery is often referred to or interpreted as drug-seeking behavior. Animals with a history of drug self-administration behavior will demonstrate this drug-seeking behavior in paradigms known as cue-induced reinstatement. Under these conditions, the function of the cue in maintaining responding could be due to motivational, discriminative, and/or conditioned reinforcing properties, because the operant response to deliver the cue in reinstatement was associated previously with

contingent drug-delivery (self-administration). To isolate the conditioned reinforcing properties of drug-paired cues, other behavioral paradigms are used in an attempt to directly test the ability of a cue to act as a conditioned reinforcer. In these procedures, drug-paired cues are evaluated for their ability to support new learning of a novel operant response, and thereby measure the conditioned reinforcing properties of the cue (Mackintosh, 1974; Williams, 1994).

One such procedure, often known as a New Response Acquisition assay, begins with pairing of an arbitrary cue with noncontingent delivery of a primary reinforcer, thereby forming an association between the cue and drug. Then, the ability of the cue to act as a conditioned reinforcer (i.e., acquired conditioned reinforcing properties) is tested by allowing the subject to learn a novel operant response to produce presentations of the cue. Importantly, this novel operant response has never been associated with prior drug delivery. Previous works have shown that these paradigms can elicit robust responding for conditioned reinforcers, such as water-, sucrose-, remifentanil-, or cocaine-associated stimuli (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Wolterink *et al.*, 1993; Di Ciano, 2008; Bertz and Woods, 2013; Bertz *et al.*, 2015, 2016; Robertson and Jutkiewicz, 2020, 2021). While the conditioned reinforcing effects of water/sucrose and remifentanil are relatively well studied, less is known about cocaine conditioned reinforcement. Therefore, we sought to establish a procedure to directly test the conditioned reinforcing properties of cocaine-paired cues.

The goals of the current study were to 1) establish that cocaine can develop robust conditioned reinforcing properties in the New Response Acquisition procedure and 2) optimize the paradigm to achieve maximal effects between experimental and

control groups. The work from this study provided a foundation to study the underlying neurobiological mechanisms of this behavior and may elucidate novel insight into behavioral measures to test cocaine conditioned reinforcement. Overall, this procedure will be a useful tool for identifying potential targets for developing treatments for cue-induced cocaine relapse.

2.3 Methods

2.3.1 Subjects

Adult male Sprague Dawley rats weighing > 280 g were obtained from Envigo (Haslett, MI). For all experiments, food and water was provided *ad libitum*. Animals were house in humidity- and temperature-controlled (28-30 C) environments with a 12 hour light/dark cycle (lights on at 0700). Experiments were performed during the light cycle. All experimental procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

2.3.2 Surgery

All rats underwent surgy for implantation of an intravenous catheter to allow for cocaine infusion. Surgical procedures were the same as reported in (Robertson and Jutkiewicz, 2021). Briefly, animals were anesthetized with ketamine (90 mg/kg intraperitoneally (ip)) and xylazine (10 mg/kg ip). Carprofen (5 mg/kg subcutaneously (sc)) was given pre-surgery as well as 24-hours post-surgery to control for inflammation and pain. Then, a 1 cm incision was made on the inner thigh and the femoral vein was isolated. A catheter (Micro-Renathane Tubing, MRE-040, Braintree Scientific Inc., Braintree, MA) was inserted into the vein. The catheter was passed subcutaneously to a

mesh backplate (P1 Technologies, Roanoke, VA, 313-000BM-15UP/1/SPC) equipped with a 22-gauge stainless steel tube and externalized. The backplate was sutured between the scapulae. Catheter patency was maintained by daily flushing of 0.5 ml heparinized saline (50 USP/ml).

2.3.3 Behavioral Procedure – New Response Acquisition

2.3.3.1 Pavlovian Conditioning

For all experiments, subjects were randomly assigned to either Paired or Random/Unpaired Pavlovian Conditioning groups. In the first phase of New Response Acquisition, Pavlovian Conditioning, animals were placed into operant chambers (Med Associates, St. Albans, VE) equipped with a house light on the left wall and a speaker (ENV-230, Med Associates) on the right wall, used to generate an 80-dB white noise tone. Subjects were tethered to tubing on a swivel (375/22PLS, Instech, Plymouth Meeting, PA) connected to a syringe pump (PHM 107, Med Associates) to allow for intravenous cocaine infusion. For subjects in Paired groups, an infusion of cocaine (either 0.1, 0.32, or 0.56 mg/kg/infusion in separate groups) was delivered simultaneously with the presentation of a stimulus, a combination of house light illumination + 80-dB white noise tone. Subjects control groups, either Random or Unpaired Pavlovian Conditioning, received the same number of cocaine infusions and same number of stimulus presentations as Paired groups, but infusions and stimulus presentations occurred independently according to two separate clocks.

For Random Pavlovian Conditioned groups in Figures 2.1, 2.2, and 2.3, cocaine infusions and stimulus presentations were presented on a random schedule, such that it was possible for the events to occur simultaneously or nearly simultaneously

(coincidental pairings), although this happened rarely (1-10 over the course of Pavlovian Conditioning). Other experiments utilized Unpaired Pavlovian Conditioning groups (Figures 2.4 & 2.5), in which subjects received explicitly unpaired cocaine infusions and stimulus presentations. This was done by generating two independent schedules with mismatched time intervals. Pairings of cocaine infusions and stimulus presentations occurred according to a variable time (VT) 15-min schedule, under which cocaine cue pairings occurred on average every 15-min (range: 0-30.5 min) which is similar to the half-life of intravenous cocaine (Barbieri *et al.*, 1992). For all subjects, the duration of the cocaine infusion and stimulus presentation were determined by body weight (2.0 ± 0.5s).

The current study evaluated how cocaine dose and patterns of conditioning alter behaviors to earn presentations of cocaine-paired cues. A previous study has shown that 100 pairings, but not 20 pairings, was sufficient to produce remifentanil conditioned reinforcing effects (Bertz and Woods, 2013). Therefore, the present study tested either 50 or 100 pairings that occurred over 5 or 10 days of conditioning. Four groups were evaluated: 1) 10 pairings per day for 5 days (50 total pairings) and 2) 5 pairings per day for 10, 3) 20 pairings per day for 5 days (100 total pairings), and 4) 10 pairings per day for 10 days. The duration of the sessions depended on the number of pairings in one day such that sessions with 5 infusions lasted 1.25 hours, 10 cocaine infusions lasted 2.5 hours, and 20 infusions lasted 5 hours.

2.3.3.2 Acquisition (ACQ)

In the second phase of the New Response Acquisition assay, Acquisition (ACQ), subjects were placed into the same operant chambers as Pavlovian Conditioning

starting the day after the last Conditioning session. In this phase, two novel nosepoke manipulanda (ENV-114BM, Med Associates) each illuminated with an LED light were introduced into the operant chamber. The location of the two nosepokes (one active and the other inactive) were counterbalanced between the right side and left side of the right wall. Subjects were tethered to the same tubing, swivel, and syringe pump; however, no solution was infused at any point during an ACQ session. Saline syringes were placed on the drug pump to maintain pressure on the catheter. Subjects could freely respond on both nosepokes. The first response on the active nosepoke yielded a ~2s stimulus presentations (house light+white noise stimulus from Pavlovian Conditioning). Subsequent responses on the active nosepoke produced stimulus presentations according to either a random ratio 2 (RR2) schedule of reinforcement, such that on average every 2 active nosepoke results in a cue presentation (range: 1-6) or a fixed-ratio 1 (FR1) schedule, in which every active nosepoke yielded a cue presentation. Responses on the inactive nosepoke had no scheduled consequences but were recorded. The duration of each ACQ session was 60 min and sessions occurred once per day for either 7 or 42 days.

2.3.4 Materials

Cocaine hydrochloride was obtained from either Sigma-Aldrich or the Michigan Medicine Hospital Pharmacy and diluted in 0.9% sterile saline solution. For surgical procedures, ketamine and xylazine (Dechra Pharmaceuticals, Northwich, UK) and carprofen (Zoetis, Parsippany-Troy Hills, NJ) were used. Heparin (Pfizer, New York, NY) was used for maintaining catheter patency.

2.3.5 Data Analysis

Data are often presented as the number of active and inactive responses made during the ACQ sessions. Preference scores were also calculated by subtracting the number of inactive nosepokes from active nosepokes individually and averaged within an ACQ session across conditioning types.

Statistical analyses were conducted using either Prism GraphPad 9.5.1 or SPSS. Repeated measures (RM) four-way Analysis of Variance (ANOVA) was used to examine differences in active and inactive responding as a function of conditioning history (Paired vs. Unpaired Pavlovian Conditioning groups), and dose of cocaine (0.1, 0.32, 0.56 mg/kg/infusion) as between-subjects measures. ACQ Day and response type (active vs. inactive nosepoke) were within-subjects measures.

RM three-way ANOVAs were used to examine the effects of different Conditioning patterns between Paired and Unpaired groups. ACQ Day and response type were within-subject measures. RM three-way ANOVAs were utilized for comparison of preference score between Paired and Unpaired groups and schedules of reinforcement (RR2 vs. FR1) across ACQ Days.

2.4 Results

2.4.1 Effect of cocaine dose on the acquisition of a novel response to earn cocaine-paired cues (work done by Dr. Stephen Robertson)

Previous work showed that drugs (i.e., remifentanil) produce dose-dependent increases in conditioned reinforcing effects (Bertz and Woods, 2013; Bertz *et al.*, 2016; Robertson and Jutkiewicz, 2020). To determine cocaine doses effective for developing

conditioned reinforcing properties, separate groups of animals underwent conditioning with either 0.1, 0.32, or 0.56 mg/kg/infusion of cocaine. Cocaine and cues were delivered 10 times per session for 10 consecutive sessions for all groups tested.

In Figure 2.1, cocaine-paired cues induced more active responding than inactive responding in a dose-dependent manner, revealed by a dose x response type interaction (F (2, 42) = 8.43, P = 0.001, η_p^2 = 0.29). In groups of animals in which the cocaine and cues were delivered simultaneously (Paired groups), active responses were greater than active responding in group of rats that received cocaine infusions and cue presentations randomly during Pavlovian Conditioning (Random groups). These data were supported by a conditioning type x response type interaction (F (1, 42) = 11.00, P = 0.002, η_p^2 = 0.21).

At the 0.1 and 0.32 mg/kg/infusion cocaine doses, responding between active and inactive nosepoke was similar in the Random groups across days, revealed by a response type x dose x conditioning type x day interaction (F (15.71, 330.1) = 1.73, P < 0.05). At the highest dose, 0.56 mg/kg/infusion, the Random conditioning produced more active responding as compared with that observed following conditioning with smaller cocaine doses; therefore, the difference in active responding between Paired and Unpaired groups at this dose was small.

Importantly, cocaine-paired cues tended to maintain more active responding in Paired groups than Random groups across all doses, indicated by a main effect of conditioning type that approached significance (F (2, 42) = 3.65, P = 0.06, η_p^2 = 0.08). Together, these data indicate that cues developed conditioning reinforcing properties following Paired Pavlovian Conditioning in a dose-dependent manner.

The conditioned reinforcing properties of cocaine-paired cues developed by this procedure persisted for many days. Subjects in Paired groups still responded on the active nosepoke for cues long after last cocaine exposure. Active responding maintained solely by cocaine-paired cues in Paired groups remained elevated as compared with Random groups for upwards of 40 ACQ sessions, supported by a conditioning type x response type x day interaction (F (7.86, 330.01) = 3.17, P = 0.04, η_p^2 = 0.08). The intermediate dose of 0.32 mg/kg/infusion cocaine maintained the greatest group difference in responding between Paired and Random groups. At this dose, the Paired group responded more robustly on the active nosepoke across multiple days of ACQ than the Random group. Therefore, 0.32 mg/kg/infusion cocaine was used during Pavlovian Conditioning for the remaining experiments. Overall, the effects of Paired Pavlovian Conditioning in this procedure on cocaine conditioned reinforcement is robust.

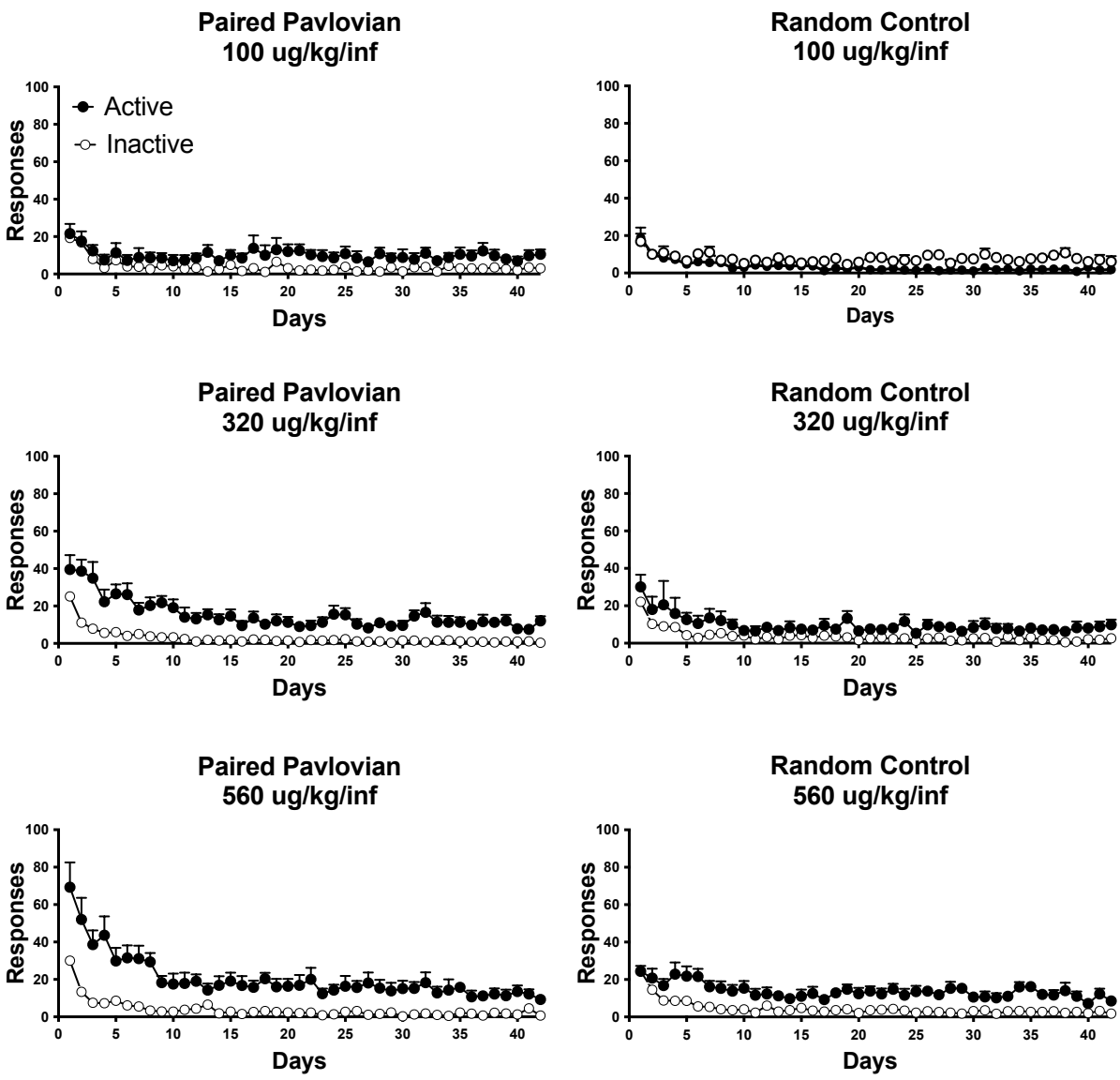

Figure 2.1 Courtesy of Dr. Stephen Robertson. Levels of active and inactive responding for Paired and Random groups with varying doses of cocaine during Pavlovian Conditioning.

2.4.2 Effect of total pairings and number of days of conditioning on the conditioned reinforcing properties of cocaine-paired cues

The number of pairings during Pavlovian Conditioning is directly related to the ability of a drug-paired cue to develop conditioning reinforcing properties (Bertz and Woods, 2013). Previous work has shown that 20 total pairings of remifentanil+cues during conditioning did not elicit as robust behavior to earn cues as did 100 total

pairings (Bertz and Woods, 2013). Presumably, more pairings should lead to greater association between cocaine and the cue and stronger conditioned reinforcing properties. Therefore, we investigated how the number and pattern of pairings during Pavlovian Conditioning influenced the ability of a cocaine-paired cue to elicit novel responding during ACQ.

In the first condition, both Paired and Random groups received 10 cocaine infusions (0.32 mg/kg/infusion) and 10 stimulus presentations a day for 5 days (50 total pairings). In these groups (Figure 2.2A&B), higher levels of active responding were observed in both groups, indicated by a main effect of response type (F (1,14) = 67.30, P < 0.0001). Overall responding decreased with time, indicated by a main effect of time (F (6, 84) = 8.938, P < 0.0001). Interestingly, there was no effect of conditioning type on responding, as there was no main effect of conditioning type nor an interaction between conditioning type x response type. Together, the cue developed conditioning reinforcing properties in both Paired and Random groups.

Next, keeping total pairings the same (50 total pairings), we altered the number of days of conditioning, such that all subjects received only 5 cocaine infusions/stimulus presentations a day for 10 days (Figure 2.2C&D). This conditioning paradigm elicited greater responding on the active nosepoke to earn presentations of cocaine-paired cues than the inactive nosepoke in both Paired and Random groups, indicated by a main effect of response type (F (1,14) = 22.65, P < 0.001). The Paired group tended to make more active responses for cues than the Random group, with a conditioning type x response type that trended towards significance (F (1,14) = 3.771, P = 0.07). Overall responding decreased over time for both groups, revealed by a main effect of time (F

(6,84) = 18.00, $P < 0.0001$). Interestingly, Paired and Random groups responded similarly for cues, as there was no main effect of conditioning type ($P = 0.08$). It is important to note that the effect of Paired Pavlovian Conditioning on active responding was robust, but variable, in this paradigm, such that high levels active responding was observed in some animals (n = 4/8 showed greater than 100 active nosepokes in a single session) while the rest responded just over 40 times on average. The variability within this group likely influences the lack of significant main effect of conditioning type and interaction between conditioning type x response type. Therefore, both groups had high levels of responding to earn cues, although the Paired group tended to make more active responses.

We then sought to determine whether increasing total cocaine+cue pairings would strengthen the conditioned reinforcing effects of the cocaine-paired cues. In Figure 2.2E&F, Paired and Random groups received 20 infusions of cocaine/stimulus presentations per session for 5 days (100 total pairings). Interestingly, despite subjects receiving more total cocaine exposure and pairings of cocaine+cue than previous groups, responding for cues was low overall. Active responding for cues was higher than inactive for both Paired and Random groups, revealed by a main effect of response type (F (1,16) = 17.63, $P < 0.001$). There was no difference in responding between Paired and Random groups, as there was a lack of significant main effect of conditioning type. Paired groups tended to make more active responses than Random groups, however, as a conditioning type x response type interaction trended towards significance (F (1,16) = 3.552, $P = 0.08$). Across time, active responding was reduced in both groups, indicated by a response type x time interaction (F (6,96) = 4.464, $P <$

0.001). Time also affected overall responding of the Paired group to a greater extent than the Unpaired group, revealed by a conditioning type x time interaction (F (6,96) = 2.661, $P < 0.05$). Overall, 20 pairings a day for 5 days did not elicit robust conditioned reinforcement in the Paired group.

Lastly, the conditioning phase was extended so that both Paired and Random groups received 10 cocaine infusions (0.32 mg/kg/infusion) and 10 stimulus presentations a day for 10 days (100 total pairings). In Figure 2.2G&H, cues maintained higher levels of responding on the active nosepoke than the inactive in both groups, indicated by a main effect of response type (F (1,15) = 23.10, $P < 0.001$). Both groups reduced responding over time, as there was a significant main effect of time (F (6,90) = 6.664, $P < 0.0001$). Overall, the Paired group had higher levels of responding than the Random group, supported by a main effect of conditioning type (F (1,15) = 5.217, $P < 0.05$) and this tended to be selective for the active nosepoke, as the interaction between conditioning type x response type trended towards significance (F (1,15) = 4.105, $P = 0.06$).

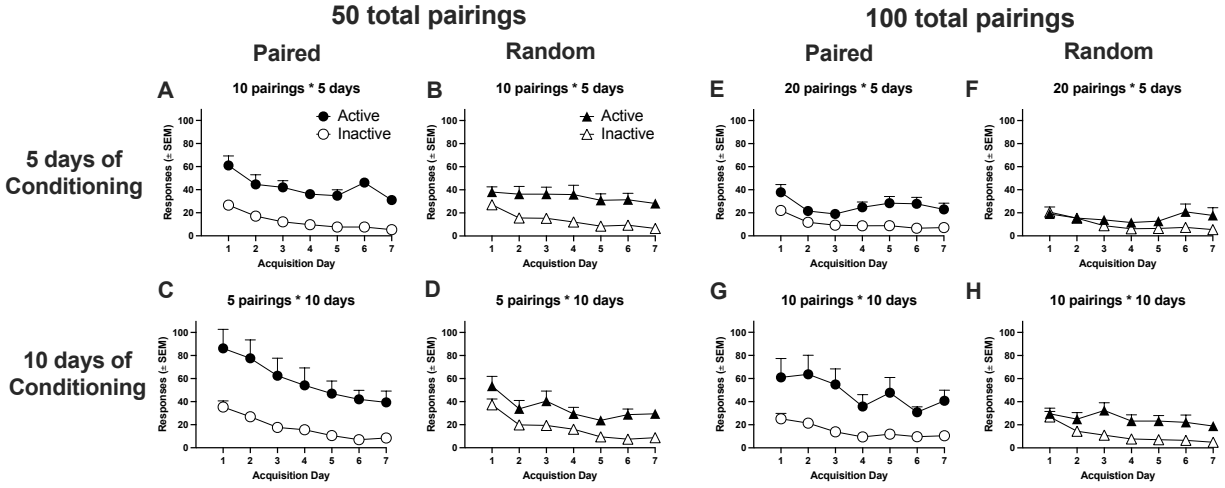

Figure 2.2 Levels of responding between Paired and Random groups across different conditioning paradigms. A&B) 50 total pairings (10 pairing/day for 5 days) yielded robust conditioned reinforcing

properties of the cocaine-paired cue, as there was a main effect of response type. C&D) Extended the duration of conditioning to 10 days, 5 pairings/day for 10 days (50 total pairings) elicited robust levels of active responding in the Paired group (C), but also yielded high levels of responding in the Random group (D). E&F) Increasing the total pairings to 100, 20 pairings/day for 5 days elicited low levels of responding. G&H) 10 pairings/day for 10 days (100 total pairings) seemed to elicit better conditioned reinforcing properties of the cue, as Paired and Random groups responding more on the active nosepoke than inactive.

2.4.3 Evaluating preference of active responding to produce cocaine-paired cues as an indicator of conditioned reinforcement

The cue should develop conditioned reinforcing properties through its association with cocaine in Paired groups selectively; yet, in many of the pairing conditions evaluated above, both Paired and Random groups responded more on the active nosepoke for cues than the inactive nosepoke. Paired groups tended to make more active responses than Random groups in certain conditions, as interactions between conditioning type x response type trended towards significance (Figure 2.2).

To further investigate the relative preference between the active and inactive manipulanda between Paired and Random groups, we calculated the preference score (active nosepokes – inactive nosepokes) for each subject individually and averaged within conditioning group. In Figure 2.3, Paired groups overall have a higher preference score than Random groups, supported by a main effect of conditioning type (F (1,59) = 12.576, $P < 0.001$). Conditioning patterns influenced preference score, revealed by a main effect of conditioning pattern (F (3,59) = 3.61, $P < 0.05$); however, there was no difference between Paired and Random groups within conditioning pattern as there was no conditioning type x conditioning pattern interaction. Across ACQ days, responding was reduced in Paired groups as compared with Random groups, supported by a conditioning type x time interaction (F (6,59) = 4.031, $P < 0.01$). Overall, the difference

in preference scores across different patterns of conditioning were not statistically significant.

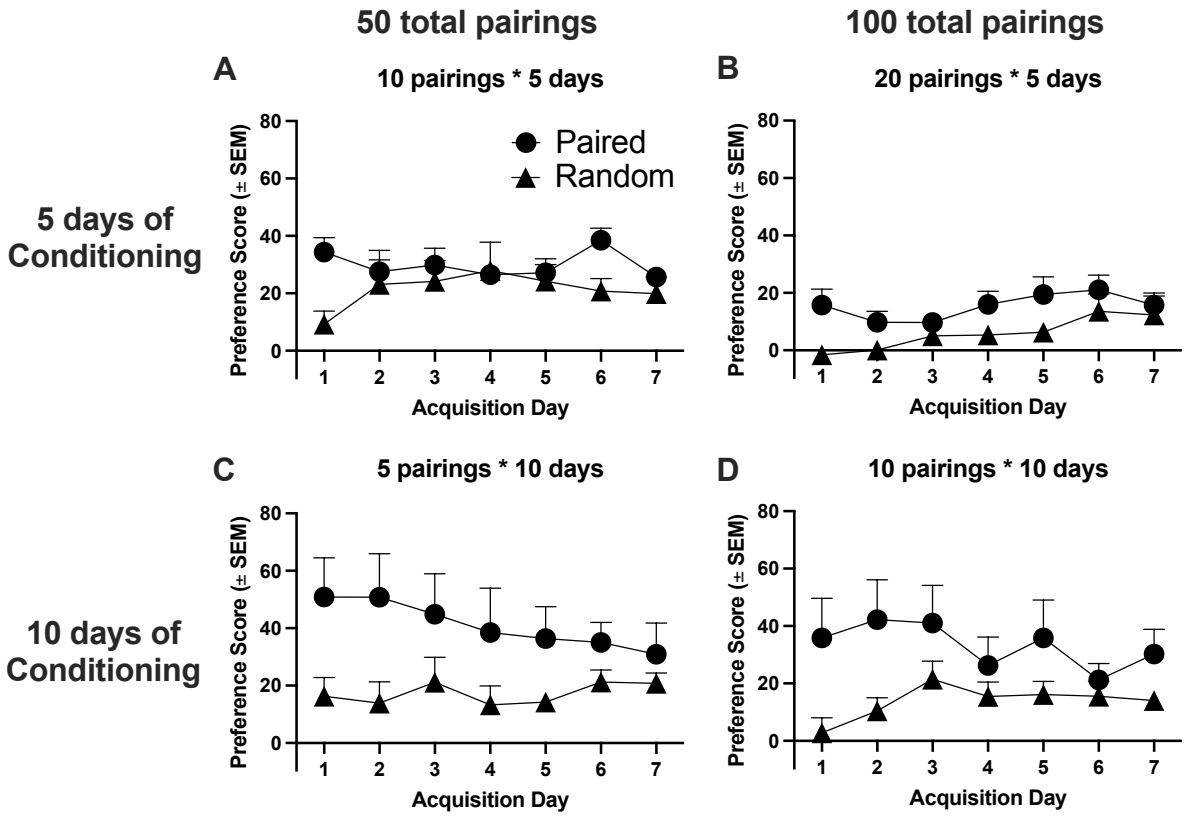

Figure 2.3 Preference scores between Paired and Random groups in various conditioning paradigms. Overall, Paired groups have a higher preference score than Random groups, but there was no influence of conditioning paradigm, as evaluated by a three-way ANOVA.

2.4.4 Optimizing differences between conditioning types with explicitly unpaired paradigm

Since there was a lack of significant group differences in levels of responding for cues between Paired and Random groups, we next sought to determine if explicitly unpairing the cocaine and cues would increase differences in active responding between the conditioning types. From our previous work (Figures 2.2 & 2.3), the conditioning paradigm of 5 pairings/day for 10 days (50 total pairings) appeared to elicit the most responding in the Paired group and the greatest preference scores, thus we

used this paradigm and adapted it to have an explicitly unpaired control condition (Unpaired group).

In Figure 2.4A, cues maintained significantly more active than inactive responses in the Paired group as well as more active responses in the Paired group as compared with the Unpaired group. A RM three-way ANOVA revealed a significant two-way interaction of conditioning type x response type interaction (F (1,13) = 17.20, P < 0.01). The Paired group showed robust levels of active responding, similar to that observed in Figure 2.2C. Overall responding decreased in both Paired and Unpaired groups over time, indicated by a main effect of time (F (6,78) = 12.91, P < 0.0001). In Figure 2.4C, the preference score for the active nosepoke was higher in the Paired group than the Unpaired group (F (1,12) = 15.64, P < 0.05). These data are discussed further in Chapter 3 of the dissertation.

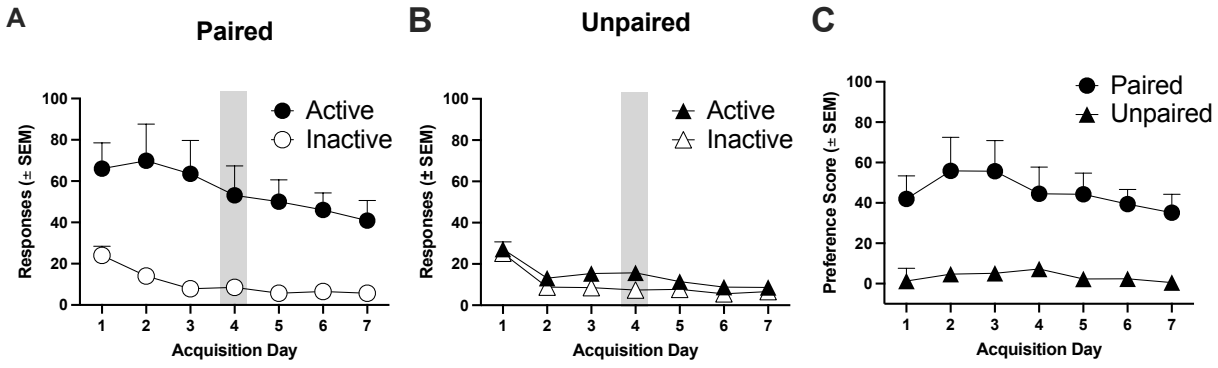

Figure 2.4 Levels of active and inactive responding between Paired and Unpaired groups following 5 pairings/day for 10 days using an explicitly unpaired paradigm. The Paired group had higher levels of active responding that inactive responding, and significantly more active responses to earn presentations of cocaine-paired cues than the Unpaired group (B). C) The Paired group maintained a higher preference for the active nosepoke than the Unpaired group across time.

2.4.5 Effects of schedule of reinforcement on novel responding to produce presentations of cocaine-paired cues

Lastly, to further maximize responding to deliver presentations of cocaine-paired cues in Paired groups selectively, we sought to determine whether schedule of reinforcement during ACQ would influence responding. Groups of rats underwent conditioning with 5 pairings/day for 10 days, and the Unpaired group received explicitly unpaired cocaine infusions and cue deliveries. Then, different groups earned cue presentations according to either RR2 or FR1 (Figure 2.5). Preference scores were calculated for comparison between conditioning types and schedules of reinforcement. Paired groups showed greater conditioned reinforcement than Unpaired groups, as they had higher preference scores, revealed by a main effect of conditioning type (F (1,28) = 16.52, $P < 0.001$). Time reduced responding in all groups, supported by a main effect of time (F (4.402,123.3) =2.512, $P < 0.05$). Time influenced responding differently between Paired and Unpaired groups, indicated by a conditioning type x time interaction (F (6, 168) = 6.632, $P < 0.0001$). It appears that active responding decreased in the Paired group while active responding was not altered in the Unpaired group across time, regardless of schedule of reinforcement. These data are discussed in more detail in Chapter 3 of the dissertation.

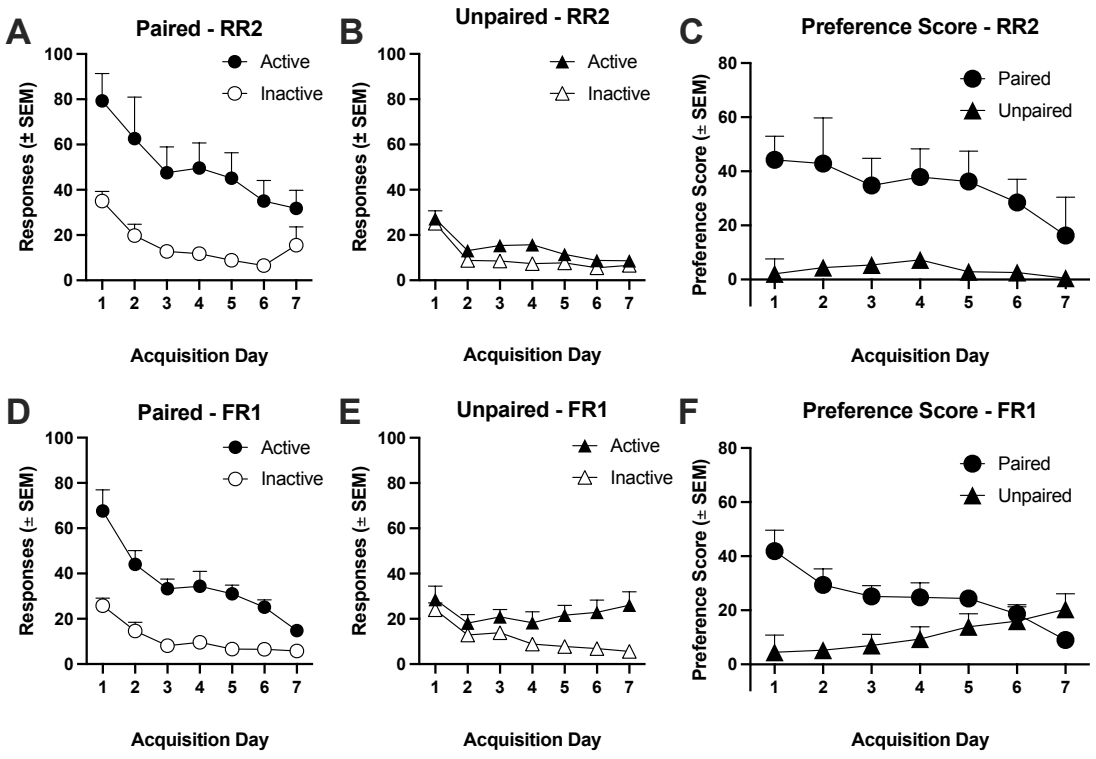

Figure 2.5 Levels of responding for cues delivered via either RR2 or FR1 schedules of reinforcement. A&C) Cocaine-paired cues delivered on a RR2 schedule maintained higher levels of active responding in the Paired group as compared with the Unpaired group. B&D) Paired subjects trained to earn cues on an FR1 schedule made more active responses for cues than Unpaired groups.

2.5 Discussion

Previous work established procedures to directly measure the conditioned reinforcing effects of either water-, sucrose-, or remifentanil-associated cues (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Wolterink *et al.*, 1993; Di Ciano, 2008; Bertz and Woods, 2013; Bertz *et al.*, 2015, 2016; Robertson and Jutkiewicz, 2020, 2021). However, cocaine-paired cues in similar paradigms have not been evaluated to the same extent (Di Ciano and Everitt, 2004*a*; Goddard and Leri, 2006; Di Ciano, 2008; Di Ciano *et al.*, 2008). The goals of this project were to establish and optimize a test of cocaine conditioned reinforcement. In the current study, cues associated with cocaine infusion dose-dependently acquired conditioned reinforcing properties using the New

Response Acquisition procedure. Cocaine-paired cues supported new learning of an instrumental response maintained by cues alone (Mackintosh, 1974; Williams, 1994). This work has replicated other studies using similar procedures to measure remifentanil conditioned reinforcement (Bertz and Woods, 2013; Bertz *et al.*, 2015, 2016; Robertson and Jutkiewicz, 2020, 2021). Since remifentanil dose had been shown to be related to the magnitude of responding maintained by conditioned reinforcers (Bertz *et al.*, 2016), we also evaluated dose of cocaine during Pavlovian Conditioning. Cocaine conditioning produced dose-dependent increases in active responding for Paired groups. However, the cue should not have formed an association with cocaine in Unpaired groups, yet for the largest dose of cocaine tested (0.56 mg/kg/infusion), active responding in the control group was high. This dose of cocaine likely resulted in weak conditioned reinforcing properties of the cue, as cocaine was delivered in the same context as cues were presented and the duration of effects of intravenous cocaine likely overlapped with cue presentations (Robertson and Jutkiewicz, 2020, 2021). The intermediate dose of 0.32 mg/kg/infusion of cocaine was thus better at parsing apart differences in the reinforcing properties of the cues between Paired and Unpaired groups and was used for the rest of the experiments.

Cocaine-paired cues acquired conditioned reinforcing properties in this paradigm and elicited robust behavior. Interestingly, Paired groups across all doses tested maintained higher levels of active responding than inactive for upwards of 40 days of ACQ. Responding maintained by cues alone often decreases over time, as the association between the cue and drug becomes weaker (Bouton *et al.*, 2021). In the current study, subjects had not been exposed to cocaine since the Conditioning phase;

therefore, all cues presented during ACQ were in the absence of drug and could have facilitated extinction. While responding decreased over time, cocaine-paired cues were able to maintain robust levels of responding for approximately 40 days. Resistance to extinction to cocaine-paired cues has also been documented in reinstatement procedures (Weiss *et al.*, 2001), supporting that drug-paired cues have the ability to elicit persistent behavior in animals.

While cocaine-paired cues maintained higher levels of active responding in the current study, one previous report failed to demonstrate conditioned reinforcing properties of cocaine-paired cues (Goddard and Leri, 2006). Two important differences between the current study and the previous study are the time interval between cocaine infusions and the dose of cocaine used during Pavlovian Conditioning. In Goddard and Leri, 2006, cocaine (either 0.5 or 1.0 mg/kg/infusion) was delivered noncontingently during Pavlovian Conditioning every 4 min within a session. Under this schedule, the cocaine-paired cue did not support higher levels of responding on the active manipulandum as compared with a manipulandum that produced a novel (non-cocaine-associated) cue. Multiple factors may have influenced this lack of effect, such as the frequency of cocaine delivery may have led to an accumulation of cocaine dose; therefore, the cue delivery may not have been discrete with the effects of cocaine and disrupted the association between cocaine+cue or failed to produce conditioned reinforcement. In the current work, we extended the time between cocaine infusions using a VT-15 min schedule, which is the approximate half-life of intravenous cocaine (Barbieri *et al.*, 1992). While some pairings did occur within the half-life of cocaine, this

schedule was sufficient for the cue to form as association with cocaine and support new learning during ACQ.

The patten of conditioning also alters the conditioned reinforcing properties of cocaine-paired cues. We expected that more pairings between cocaine+cue would produce more robust conditioned reinforcing properties; however, the current data did not support this hypothesis. It appears that the number of days of conditioning played a more important role in establishing conditioned reinforcing effects. Figure 2.2, we observed that more days of conditioning influenced responding for cues during ACQ, and not necessarily the total number of pairings. The Paired group that received 5 pairings/day for 10 days appeared to have the highest levels of active responding, despite having less total cocaine exposure and fewer total pairings than groups that received 100 pairings. Variability in this group contributed to a lack of effect of conditioning type between the Paired and the Random group, but the preference score of this group appeared to have the greatest difference (Figure 2.3). In general, 10 days of conditioning produced greater conditioned reinforcing effects than 5 days of conditioning. It is possible that more conditioning days allows for recruitment of neurobiological processes to encode the association between cocaine and the cue, such as increases in dopamine concentrations following stimulus presentations over multiple daily sessions (Dalley *et al.*, 2005; Flagel *et al.*, 2011; Saunders *et al.*, 2018).

The lack of group differences between Paired and Random groups with the varying conditioning paradigms (Figures 2.2 & 2.3) was surprising, since only in the Paired group should the cue form an association with cocaine delivery and acquire conditioned reinforcing properties. The higher levels of active responses than inactive in

Random groups likely influences the comparison between active responding between conditioning type, contributing to a lack of significant interaction between conditioning type x response type. Random groups had received the same exposure to cocaine and cue presentations; however, due to the time intervals on the independent clocks that determine when events occur during conditioning, it was possible for "incidental pairings," in which some infusions and cues were delivered simultaneously. Therefore, it is possible that a few incidental pairings across multiple days of conditioning were enough to induce weak conditioned reinforcing properties of the cue in Random groups.

We then sought to investigate whether an explicitly unpaired paradigm, in which cocaine infusions and cue presentations were separated during conditioning, would prevent the generation of weak conditioned reinforcing effects of the cues in Random groups. It is important to note that some cue presentations occurred within the half-life of cocaine, so there was still some overlap between events in Unpaired groups but no incidental pairings. Indeed, in Figure 2.4, the Unpaired group responded similarly on both active and inactive nosepokes. Overall, we were able to maximize differences in active responding for cues between Paired and Unpaired groups using an explicitly unpaired paradigm.

Lastly, another factor that contributes to the expression of conditioned reinforcement is contingency of conditioned reinforcer delivery. Previous works had adapted a form of New Response Acquisition such that subjects learned operant self-administration for contingent drug delivery, in which cues were delivered with drug, but learned a different operant response for cue delivery alone (Di Ciano and Everitt, 2004*a*; Di Ciano, 2008; Di Ciano *et al.*, 2008). The formation of the association of cues and

cocaine delivery is likely influenced by whether or not the drug (and cue) was delivered contingently (Namba *et al.*, 2018). Further, the predictability of contingent cue presentation may elicit different levels of behavior and also recruit different neurobiological processes. The differences in responding between Paired and Unpaired groups appeared slightly greater when cues were delivered via RR2 than FR1; however, this effect was not significant. Intermittent reinforcer delivery likely maintains higher levels of behavior to earn conditioned reinforcers, similar to that seen in the self-administration literature (Carr *et al.*, 2020). Responding decreased faster in Paired groups, regardless of schedule. Subjects in the Paired groups earned more cue presentations than those in Unpaired groups, so it is possible the association between cue and cocaine extinguished faster with more cue presentations. However, rates of decline appeared similar between RR2 and FR1 Paired groups, despite FR1 groups receiving twice the number of cue presentations. Further, more cue presentations earned does not always lead to extinction as seen in Figure 2.1, as cues maintained responding for almost 40 days.

Overall, we established a paradigm to test the conditioned reinforcing effects of cocaine-paired cues selectively. We demonstrated that dose, pattern of conditioning, and schedule of reinforcement influence responding for cues as shown previously (Bertz and Woods, 2013; Bertz *et al.*, 2016). We were able to optimize conditioning pattern using: 1) 5 pairings/day for 10 days conditioning paradigm and 2) explicitly unpaired conditioning controls to elicit robust group differences in responding maintained by cues. This paradigm minimizes the duration of daily conditioning sessions and does not require catheter patency beyond 10 days. The New Response

Acquisition procedure will allow us to study and better characterize neurobiological differences between Paired and Unpaired groups to explain differences in behavioral responding to earn cocaine-paired cues.

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Chapter 3
Evaluating a Role of Dopamine in Cocaine Conditioned Reinforcement Using the New Response Acquisition Procedure

3.1 Abstract

Neutral cues gain conditioned reinforcing properties following repeated pairing with cocaine delivery and/or taking behavior. The conditioned reinforcing properties of cocaine-paired cues can elicit drug-seeking behavior in animals and contribute to relapse in humans. In the current study, we sought to evaluate the role of dopamine in a stringent test cocaine conditioned reinforcement, the New Response Acquisition procedure, in which subjects learn to make a novel operant response to earn presentations of cocaine-paired cues. Following Pavlovian Conditioning, subjects that received cocaine infusions and cue presentations simultaneously (Paired groups) responded more for cues than subjects that received cocaine and cues separately (Unpaired groups). We hypothesized that responding to earn presentations of cocaine-paired cues would be associated with increases in dopamine levels in the nucleus accumbens and that pharmacologically elevating dopamine levels would further drive responding for cues. Contrary to our hypothesis, responding for cocaine-paired cues did not alter dopamine levels in either the nucleus accumbens core or shell from baseline, nor were there differences in dopamine concentrations between groups that received with Paired or Unpaired cues. Systemic or intra-nucleus accumbens cocaine administration did not enhance the conditioned reinforcing properties of cues in two

different schedules of reinforcement. Together, these data suggest that dopamine does not mediate the conditioned reinforcing properties of cocaine-paired cues as measured in the New Response Acquisition procedure. Drug-paired cues in various tests of conditioned reinforcement may rely on functionally distinct neurobiological mechanisms to elicit behavior in animals.

3.2 Introduction

Neutral stimuli, or cues, acquire conditioned reinforcing properties following repeated pairings with a primary reinforcer. Drug-paired cues elicit behaviors in animals (Venniro *et al.*, 2020) and likely contribute to relapse in humans (Bonson *et al.*, 2002; Volkow and Li, 2005). Previous work sought to understand the neurobiological mechanisms by which drug-paired cues induce drug-seeking behaviors, proposing that dopamine plays a large role in the formation of the cue and drug associations required for conditioned reinforcement (Schultz, 2006; Flagel *et al.*, 2011; Steinberg *et al.*, 2013; Nasser *et al.*, 2017; Saunders *et al.*, 2018). Briefly, phasic increases in dopamine levels within the mesolimbic dopamine pathway, primarily the nucleus accumbens (NAc), occur following presentation of stimuli previously associated with contingent or noncontingent delivery of psychostimulants (Gratton and Wise, 1994; Di Ciano *et al.*, 1998, 2008; Ito *et al.*, 2000; Weiss *et al.*, 2000; Phillips *et al.*, 2003, Di Ciano and Everitt, 2004*a*; Di Ciano, 2008; Aragona *et al.*, 2009; Parsegian and See, 2014) or sucrose (Phillips *et al.*, 1994; Datla *et al.*, 2002; Day *et al.*, 2007; Flagel *et al.*, 2011) and may precede responding maintained by these drug-paired cues. Further, administration of indirect dopamine agonists potentiated responding for drug-paired cues measured during reinstatement procedures (Weiss *et al.*, 2000; Park *et al.*, 2002; Phillips *et al.*,

2003; Nicola *et al.*, 2005; Volkow *et al.*, 2006; McGlinchey *et al.*, 2016; Halbout *et al.*, 2019; O'Neal *et al.*, 2020) while administration of dopamine receptor antagonists reduced responding for drug-paired cues (Cervo *et al.*, 2003; Saunders *et al.*, 2013). Together, these data suggest that dopamine regulates responding for drug-paired cues.

However, cues that induce reinstatement are complex because the operant responses maintained by cues have been previously associated with contingent drug delivery in self-administration procedures. Therefore, the function of the cue during reinstatement (i.e., motivational, discriminative, and/or conditioned) is unclear. In an attempt to evaluate the conditioned reinforcing properties of drug-paired cues in isolation (Mackintosh, 1974; Williams, 1994), a novel response that has never been associated with delivery of a primary reinforcer is used to produce the conditioned reinforcer (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Wolterink *et al.*, 1993; Phillips *et al.*, 1994, Di Ciano and Everitt, 2004*a*; Bertz and Woods, 2013; Robertson and Jutkiewicz, 2020).

The goal of the current study was to better understand the underlying neurobiology of the conditioned reinforcing effects of cocaine using the New Response Acquisition procedure. We hypothesized that dopamine modulates responding for cocaine-paired cues in this assay, as previously reported for cue-induced reinstatement procedures (Weiss *et al.*, 2000; Parsegian and See, 2014) and in studies described above. In the present study, we evaluated 1) levels of dopamine in the NAc core and shell using *in vivo* microdialysis and 2) local and systemic administration of indirect dopamine agonists in rats responding for cocaine-paired cues. Dopamine levels were evaluated in both NAc core and shell due to functional differences in reinforcement

behaviors between the subregions (Parkinson *et al.*, 1999; Ito *et al.*, 2000, 2004; Di Chiara, 2002; Fuchs *et al.*, 2004). Overall, this study demonstrates that cocaine-paired cues maintained high levels of responding but is not dependent on increased dopamine levels in the NAc.

3.3 Methods

3.3.1 Subjects

Adult male Sprague Dawley rats weighing >280 g were obtained from Envigo (Haslett, MI) and housed in temperature- (21-23°C) and humidity-controlled environment with a 12 hour light/dark cycle (lights on at 06:30). Food and water were provided *ad libitum* throughout the entirety of the experiments. All experimental procedures were approved by the University of Michigan Institutional Care and Use Committee.

3.3.2 Surgery

Catheters were placed 7-10 days prior to cannula implantation or initiation of experiments, and cannula were implanted 2-3 days prior to the start of experiments.

3.3.2.1 Intravenous Catheter Implantation

All subjects were implanted with a catheter in the femoral vein externalized to a back mount cannula (8I313000BM14, P1 Technologies, Roanoke, VA) to allow for intravenous infusions of cocaine. Surgical procedures for catheter implantation were the same as reported in (Robertson and Jutkiewicz, 2021). Briefly, for intravenous catheter implants, subjects were anesthetized with intraperitoneal (ip) injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). Carprofen (5 mg/kg) was given subcutaneously prior to

the start of surgery as well as 24-hour post-surgery to control for postoperative inflammation and pain. After making an incision approximately 1 cm rostral to the top of the inner left thigh, the femoral vein was isolated and a catheter (Micro-Renathane Tubing, MRE-040, Braintree Scientific, Inc., Braintree, MA) was inserted into the vein. The catheter was passed subcutaneously to a mesh backplate with a 22-gauge stainless steel tube (313-000BM-15UP/1/SPC, P1 Technologies, Roanoke, VA) and was sutured (661G, Ethilon Sutures, Ethicon Inc., Raritan, NJ) to the muscle between the scapulae. Catheter patency was maintained by flushing daily with 0.5 ml heparinized saline (50 USP/ml).

3.3.2.2 Cannula Implantation

In subjects that underwent microdialysis collection, cannula (CMA 12 Guide Cannula, Harvard Apparatus, Holliston, MA) were implanted to the following coordinates (in reference to bregma): NAc Shell (NAcSh) AP: +1.7 mm ML: -0.9 mm DV: -6.0 mm or NAc Core (NAcC) AP: +1.6 mm ML: +1.6 mm DV: -5.2 mm. Cannula were secured to the skull by three metallic bone screws (0.86 mm shaft, Fine Science Tools, Foster City, CA) and acrylic dental cement (Lang Dental, Wheeling, IL). Carprofen (5 mg/kg) was given subcutaneously prior to surgery and 24- and 48-hours post-surgery.

3.3.3 Behavioral Procedure – New Response Acquisition

3.3.3.1 Pavlovian Conditioning (PAV)

For the Pavlovian Conditioning phase, subjects were placed into operant chambers (Med Associates Inc., St. Albans, VE) equipped with a house light on the left wall and a speaker (ENV-230, Med Associates) used to generate an 80-dB white noise

stimulus, on the right wall. Subjects were tethered to tubing on a swivel (375/22PLS, Instech, Plymouth Meeting, PA) and received intravenous infusions of 0.32 mg/kg/infusion of cocaine via a 10 ml syringe on a drug pump (PHM 107, Med Associates). This dose of cocaine was chosen based on previous work demonstrating that it incurred robust conditioned reinforcing properties (Chapter 2). For all subjects, the duration of the cocaine infusion and stimulus presentation were determined by body weight (2.0 ± 0.5 s).

Subjects were assigned randomly to either Paired or explicitly Unpaired (control) Pavlovian Conditioning groups. For Paired groups, an infusion of cocaine (0.32 mg/kg/infusion) was delivered simultaneously with a presentation of a stimulus, an 80-dB dual white noise + house light illumination in an operant chamber (Med Associates). Pairings of infusions and stimulus presentations occurred according to a variable time (VT) 15-min schedule (range 0.1 s-30.5 min). For Unpaired groups, subjects received infusions of cocaine and stimulus presentations that were explicitly unpaired and operated on independent schedules. All animals received 5 infusions of cocaine and 5 stimulus presentations per day for 10 days. Each session lasted 75 min (+/- 30 s).

3.3.3.2 Instrumental Acquisition (ACQ)

During the second phase of New Response Acquisition, subjects were placed into the same operant cambers as Pavlovian Conditioning; however, in this phase the chambers were also equipped with two nosepoke manipulanda (ENV-114BM, Med Associates) each illuminated with a single LED light. The location of the active nosepoke (right or left side on the right wall of the chamber) was counterbalanced across subjects. Subjects were tethered to the same tubing and swivel; however, no

cocaine was available. Rather, 10 ml saline syringes were on the drug pump to maintain pressure on the catheter and no infusions were given at any point throughout the ACQ sessions.

During ACQ sessions, two nosepoke manipulanda (ENV-114BM, Med Associates) were introduced into the operant chamber. Externalized catheters were attached to an infusion pump, but no infusions occurred. At the start of the session, LED lights illuminated both nosepokes, and subjects were able to respond freely on each nosepoke. The first response on the active nosepoke of each session resulted in a ~2s presentation of the white noise+house light stimulus. Subsequent responses on the active nosepoke resulted in presentations of the stimulus according to either a random ratio 2 (RR2) schedule of reinforcement, such that, on average, every two responses on the active nosepoke resulted in a cue presentation (range 1-6 active responses) or a fixed-ratio 1 (FR1), in which every active nosepoke yielded a cue presentation. Responses in the inactive nosepoke were recorded but had no scheduled consequences. The duration of each ACQ session was 60 min and sessions occurred once per day for 4-7 days.

For systemic drug administration studies, acute drug treatments were given immediately prior the start of the fourth ACQ session. Separate groups of animals (n=6-8) received injections of either cocaine (1.0, 3.2, 10 or 18 mg/kg intraperitoneally (ip)), amphetamine (1.0 mg/kg subcutaneously (sc)) or vehicle (saline either ip or sc). Vehicle treated groups received injections of saline either subcutaneously or intraperitoneally immediately prior to the start of ACQ sessions 4-7 in which cues were delivered via RR2 or FR1 schedules.

3.3.4 *In vivo* Microdialysis

All microdialysis experiments were conducted in awake, behaving rats (Paired NAcC: n=8, Unpaired NAcC: n=8, Paired NAcSh: n=7, Unpaired NAcSh: n=7) during the third ACQ session (ACQ3). After the second ACQ session, subjects were briefly anesthetized with isoflurane and a microdialysis probe equipped with a 2 mm semi-permeable membrane (CMA 12, Harvard Apparatus) was inserted into the cannula. Probes were connected to a dual-channel swivel (22 ga, Harvard Apparatus) then to syringes (1 ml microsyringe, Harvard Apparatus) and flushed with artificial cerebrospinal fluid (aCSF; consisting of 145 mM NaCl, 2.68 mM KCl, 1.4 mM $CaCl_2$, 1.01 mM $MgSO_4$, 1.55 mM Na_2HPO_4, 0.45 mM NaH_2PO_4, 250 mM ascorbic acid) at a flow rate of 0.3-0.5 µl/min overnight for at least 16 hours prior to the start of the experiment using a syringe pump (CMA 4004, Harvard Apparatus). The morning of experiments, flow rate was increased to 0.5 µl /min at least 1 hour prior to the start of collection. All dialysate samples were collected at 0.5 µl /min at 10-min intervals (5 µl samples). For each subject, 6 baseline samples were taken while subjects were in the home cage. Then, rats were placed in the operant chamber, the ACQ3 session started, and another 6 samples were collected throughout the 60-min ACQ3 session. Since microdialysis probe insertion decreased responding between ACQ3 and ACQ4, some subjects received noncontingent stimulus presentations during ACQ 3 to help stimulate contingent responses, however these were removed for analyses. Following the end of ACQ3, 7 µg cocaine in aCSF was retrodialyzed (Paired NAcC: n=8, Unpaired NAcC: n=8, Paired NAcSh: n=6, Unpaired NAcSh: n=5; 3 subjects in Unpaired NAcSh group received 46 µg) over 10 min into the microdialysis probe via a liquid switch (CMA 110,

Harvard Apparatus) and dialysate samples were collected for 30 min. Responding was recorded for another 60 min during and after cocaine retrodialysis (extended ACQ3). Three dialysate samples were collected over 30 min after cocaine retrodialysis to ensure increases in dopamine concentrations. At the end of the second hour of ACQ3, probes were removed and, with aCSF still flowing, placed into a recovery solution with known concentrations of various neurotransmitters to measure relative *in vitro* recovery.

3.3.5 Neurochemical Analysis

As described previously (Song *et al.*, 2012; Wong *et al.*, 2016), each dialysate sample or standard was derivatized with benzoyl chloride and quantified via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Each sample or standard was mixed with 100 mM sodium carbonate, benzoyl chloride (2% in HPLC-grade acetonitrile), and internal standard sequentially in a 2:1:1:1 volume ratio. Internal standards were prepared by derivatizing a mixture of standards with 13C-BzCl. Derivatized dialysate samples were analyzed using a Phenomenex (Torrance, CA) Kinetex C18 chromatography column (100 x 2.1 mm, 1.7 um, 100 A) held at 30°C in still air mode on a Vanquish ultrahigh-performance liquid chromatograph (ThermoFisher, Waltham, MA) interfaced to a TSQ Quantum Ultra triple quadrupole mass spectrometer (ThermoFisher). Mobile phase A was 10 mM ammonium formate and 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The gradient was as follows: initial, 5% B; 0.010 min, 19% B; 0.680 min, 26% B; 1.055 min, 75% B; 1.805 min, 100% B; 2.180 min, 100% B; 2.280 min, 5% B, 3.000 min, 5% B. The mass spectrometer was operated in positive ion mode. The spray voltage was 3000 V. The vaporizer temperature was set to 300°C and the capillary temperature was 325°C. Auxiliary gas, sheath gas, and ion

sweep gas pressures were set at 10, 50, and 0.2 respectively. The peak areas of each analyte were divided by the internal standard peak area for quantitation. Thermo XCalibur 3.0 MS software (ThermoFisher) was used to process and integrate peaks automatically, but all peaks were visually inspected to ensure proper integration.

The following analytes (parent →product ion) were analyzed for each sample: Dopamine (466 → 105), acetylcholine (146 → 87), choline (104 → 60), glutamate (252 → 105), gamma-aminobutyric acid (GABA; 208 → 105), adenosine (372 → 136), homovanillic acid (HVA; 304 → 105), 3-methoxytyramine (3-MT; 376 → 105), and norepinephrine (464 → 105).

3.3.6 Probe location verification

For microdialysis experiments, cannula placement was checked using cresyl violet staining (Figure 3.5). Upon completion of experiments, animals were deeply anesthetized with pentobarbital (70 mg/kg intraperitoneally; MWI Animal Health, Gainseville, GA) and microdialysis probe cannula were infused with fast green dye to visualize probe location. Animals were then euthanized and brains were extracted and flash-frozen with isopentane (ThermoFisher) and kept at -80°C until sliced on the cryostat (Leica Biosystems, Deerfield, IL). Slices were then stained with cresyl violet and viewed under a microscope to confirm cannula placement. Animals with cannula placements that could not be verified or were in the wrong location (n=5) were removed from the study.

3.3.7 Materials

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Cocaine hydrochloride (Michigan Medicine Hospital Pharmacy) and amphetamine (NIDA Drug Supply) were dissolved into sterile 0.9% saline solution. For surgical procedures, ketamine and xylazine (Dechra Pharmaceuticals, Northwich, UK), and carprofen (Zoetis, Parsippany-Troy Hills, NJ) were used. Heparin (Pfizer, New York, NY) was used for maintaining catheter patency.

3.3.8 Data Analysis

Data analyses were conducted separately for each experiment using Prism GraphPad 9.5.1 software. For behavioral analyses, repeated measures (RM) three-way analyses of variance (ANOVA) were conducted between conditioning type (Paired vs. Unpaired), response type (active vs. inactive), and day (ACQ session). In some experiments, preference scores were calculated by subtracting the number of inactive nosepokes from number of active nosepokes individually for each ACQ session. RM two-way ANOVAs were conducted for: preference scores (conditioning type x session), acute drug pretreatments (drug x response type), and microdialysis dialysate data within each NAc subregion (conditioning type x time). ANOVAs used Geisser-Greenhouse corrections. Raw concentrations (nM) of neurotransmitters were either transformed to percent change of baseline (%) or normalized to individual *in vitro* probe recoveries. For comparison of dopamine levels between the first 30 min of ACQ3 and extended ACQ3 post-cocaine retrodialysis, area under the curve (AUC) was calculated from normalized concentrations and analyzed (conditioning type x drug treatment). Simple linear regressions were conducted for correlational comparisons.

3.4 Results

3.4.1 Effects of Pavlovian Conditioning on responding for cocaine-paired cues

After 10 days of either Paired or Unpaired Pavlovian Conditioning, responses on active and inactive nosepokes were recorded during ACQ sessions (Fig 3.1A). In rats with microdialysis probes in the NAcC, cocaine-paired cues maintained more responding on the active nosepoke than the inactive nosepoke following Paired Pavlovian Conditioning under a RR2 schedule of reinforcement (Fig 3.1B). However, in rats that were exposed to explicitly unpaired cocaine and cue presentations, responses on the active and inactive manipulanda were similar (Fig 3.1C). In addition, cues maintained more responding on the active nosepoke following Paired Pavlovian Conditioning as compared with the Unpaired group, supported by a conditioning type x response type interaction ($F(1,38)=10.65$, $P<0.01$). Responding decreased across ACQ days for both Paired and Unpaired groups, indicated by a main effect of day ($F(3,42)=18.14$, $P<0.0001$). It is important to note that following insertion of the NAcC microdialysis probes, responding decreased between ACQ sessions 3 and 4, possibly due to the disrupting nature of the microdialysis probe insertion, similar to previous reports (Weiss *et al.*, 2000). To further examine the effects of conditioning on responding for cocaine-paired cues, preference scores (Fig 3.1D) were averaged across subjects within each conditioning group. The Paired group had a greater preference for the active manipulandum than the Unpaired group, supported by a main effect of conditioning type ($F(1,14)=10.00$, $P<0.01$). There was no main effect of day and no conditioning type x day interaction.

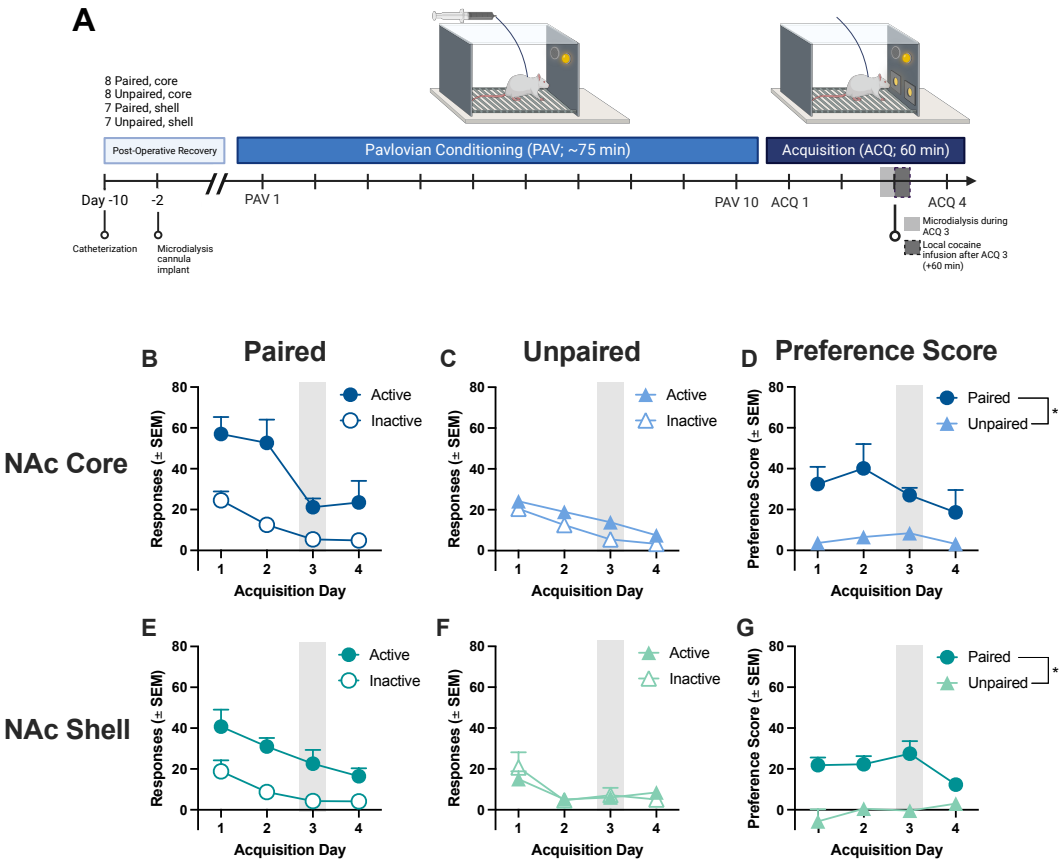

Figure 3.1 Responding on active and inactive nosepokes over four Acquisition (ACQ) sessions in subjects with a history of Paired and Unpaired Pavlovian Conditioning with either NAcC or NAcSh cannula implantations. A) Schematic of experimental design. Image made using BioRender.com. B&C) NAcC implanted subjects with a history of Paired Pavlovian Conditioning had higher active nose pokes than subjects in the Unpaired group. Main effects: Conditioning type ($F(1,38) = 11.49$, $P < 0.01$), response type ($F(1,14) = 24.81$, $P < 0.001$), and time ($F(3,42) = 18.14$, $P < 0.0001$) with interaction between conditioning type and response type ($F(1,38) = 10.65$, $P < 0.01$). D) The Paired group shows a higher preference score. Main effect of conditioning type ($F(1,14) = 10.00$, $P < 0.01$). E&F) Similarly, in NAcSh implanted groups, subjects in the Paired group also made more active nosepokes than the Unpaired group. Main effects: Conditioning type ($F(1,29) = 7.636$, $P < 0.01$), response type ($F(1,13) = 24.28$, $P < 0.001$), and time ($F(3,39) = 11.53$, $P < 0.0001$) with interaction between conditioning type and response type ($F(1,29) = 34.75$, $P < 0.0001$). G) The Paired group has a higher preference score. Main effect of conditioning type ($F(1,12) = 51.60$, $P < 0.0001$).

In subjects with microdialysis probes in the NAcSh, cocaine-paired cues supported higher levels of active responding than inactive responding in the Paired group (Fig 3.1E). Active responding was higher in the Paired condition than Unpaired group, in which levels of responding on both manipulanda were similar, indicated by a conditioning type x response type interaction ($F(1,29)=34.75$, $P<0.0001$). For both

Paired and Unpaired groups, responding decreased over time (main effect of time: $F(3,39)=11.53$, $P<0.0001$). Further, the Paired group had a higher preference score (Fig 3.1G), indicated by a main effect of conditioning type ($F(1,12)=51.60$, $P<0.0001$), but there was no main effect of time or conditioning type x time interaction. Overall, cues that were paired with cocaine infusions maintained higher levels of active responding than cues in the Unpaired groups, indicating that the cue had acquired robust conditioned reinforcing properties selectively in Paired groups following implantations in either NAcC or NAcSh.

3.4.2 Dopamine levels in the NAc core or shell during responding for cocaine-paired cues in an ACQ session

Previous research has shown that dopamine levels are elevated in reward circuitry while responding for drug-paired cues (Weiss *et al.*, 2000; Parsegian and See, 2014), suggesting dopamine may mediate this behavior. To evaluate dopamine during ACQ, dopamine levels were measured via microdialysis in Paired or Unpaired groups with probes aimed at the NAcC or NAcSh. We hypothesized that dopamine levels would increase while responding cocaine-paired cues in both the NAc core and shell in Paired groups, but there would be no changes in dopamine levels in rats in Unpaired groups. There were no significant changes in dopamine levels across the 60-min ACQ3 session in either the NAc core or shell in Paired and Unpaired groups, as indicated by no main effects of conditioning type or time and no conditioning type x time interaction (Fig 3.2A&H). These findings were surprising considering the robust effect of Paired vs. Unpaired conditioning on active responding as described in Figure 3.1.

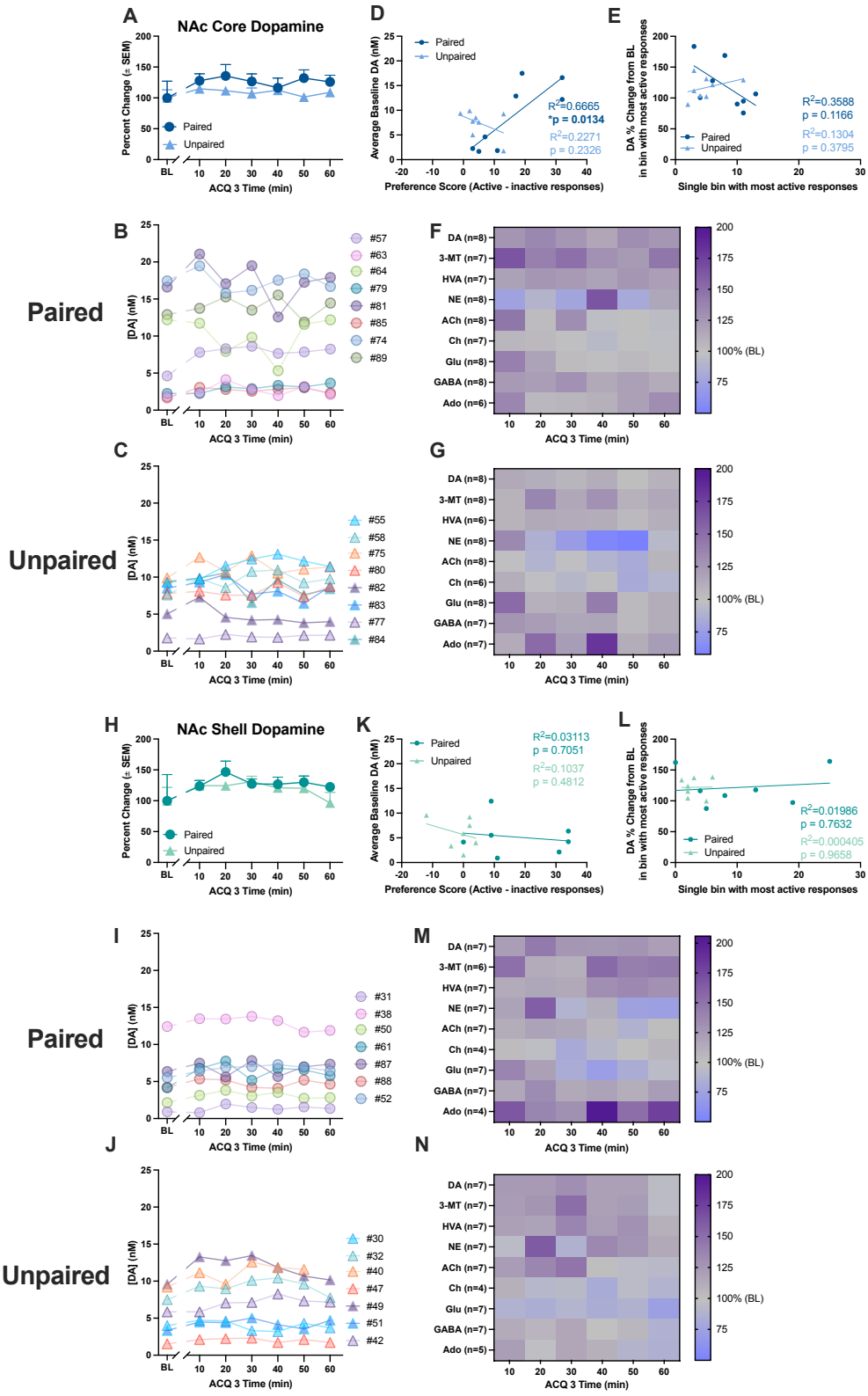

Figure 3.2 A) In subjects with NAcC implants, there are no changes in dopamine levels from baseline (BL) in either the Paired (n=8) for Unpaired (n=8) groups. No main effects of conditioning type nor time.

B&C) Individual traces of dopamine concentrations in the NAcC (nM) throughout the 60-min ACQ session measured in 10 min bins. D) BL dopamine levels in the NAcC of Paired subjects correlate with preference score (*P < 0.05), but there is no correlation between increases in dopamine in single 10-min bins in which subjects make the most active responses during the ACQ session (E). F&G) Levels of other neurotransmitters in the NAcC are unchanged from BL (no main effect of time) and do not differ between groups, except for 3-MT (main effect of conditioning type: $F(1,13) = 5.483$, $P < 0.05$). H) Dopamine levels in the NAcSh did not change from BL in either Paired (n=7) or Unpaired (n=7) groups across ACQ3. No main effects of conditioning type nor time. I&J) Individual dopamine concentrations shown. K&L) NAcSh BL dopamine is not correlated with preference score nor is dopamine higher than BL in a single time bin in which animals make the most responses. M&N) Levels of other neurotransmitters in the NAcSh do not differ between groups, nor do concentrations change from BL over time, except for choline (main effects of time: $F(2.092,12.55) = 4.974$, $P < 0.05$). BL = baseline, DA = dopamine, 3-MT = 3-methoxytyramine, HVA = homovanillic acid, NE = norepinephrine, ACh = acetylcholine, Ch = choline, Glu = glutamate, GABA = gamma aminobutyric acid, Ado = Adenosine

Individual differences in dopamine concentrations may be masked when data are transformed to percent change from baseline (BL). To investigate further the individual patterns of dopamine levels across the ACQ session, dopamine concentrations (nM) per subject were evaluated across the 60-min ACQ session (Fig 3.2B&C, H&I). BL dopamine levels were similar between conditioning types in the NAcC (Paired: 8.7 ± 2.4 nM; Unpaired: 7.4 ± 1.0 nM) and NAcSh (Paired: 5.1 ± 1.4 nM; Unpaired: 5.9 ± 1.2 nM). While no discernible patterns emerged (i.e., no increasing levels of dopamine across the 60 min session), some subjects in the Paired group show higher BL dopamine concentrations in the NAcC than others (Fig 3.2B). Therefore, we performed comparisons between BL dopamine concentrations and responding in ACQ3. In Figure 3.2D, BL dopamine levels in the NAcC only were positively correlated only with the preference score of Paired subjects (simple linear regression: $R^2=0.67$, $P<0.05$), suggesting increased dopamine levels may predict more responding for cues. To assess if dopamine is elevated during specific periods of high responding, we evaluated the correlation between change in dopamine concentration from BL and the number of responses in the single 10-min period of ACQ3 with the most active responses on an individual basis. Changes in dopamine concentrations did not correlate with the 10-min

time bin with the most responding per individual subject (Fig 3.2E). Unlike that observed in the NAcC, there was no significant correlation between BL dopamine levels in the NAcSh and the preference score for either Paired or Unpaired group (Fig 3.2K). In the time bin in which subjects make the most active responses for cues, dopamine percent change from BL was not correlated with higher responding (Fig 3.2L). Furthermore, bouts of responding per subject, as shown in cumulative records, were not correlated with changes in dopamine concentrations in 10 min bins across ACQ3 (Figure 3.3 & 3.4). Together, these data suggest that elevated dopamine levels in the NAcC may not mediate or induce responding but may be related to greater overall active responding in rats with a history of cue-paired conditioning.

Other neurotransmitters, including acetylcholine, choline, glutamate, GABA, adenosine, homovanillic acid, 3-MT, and norepinephrine, were collected and analyzed in the same dialysate samples. Similar to that seen with dopamine, there were no main effects of time in any neurotransmitter analyzed in either NAc core (Fig 3.2F&G) or shell (Fig 3.2M&N) and no time x conditioning type interactions in either Paired or Unpaired groups during ACQ3. There were significant main effects of conditioning type for NAcC 3-MT and NAcSh choline only, but no significant post-hocs. Overall, these findings suggest that responding for cocaine-paired cues did not alter these neurotransmitters in the NAc.

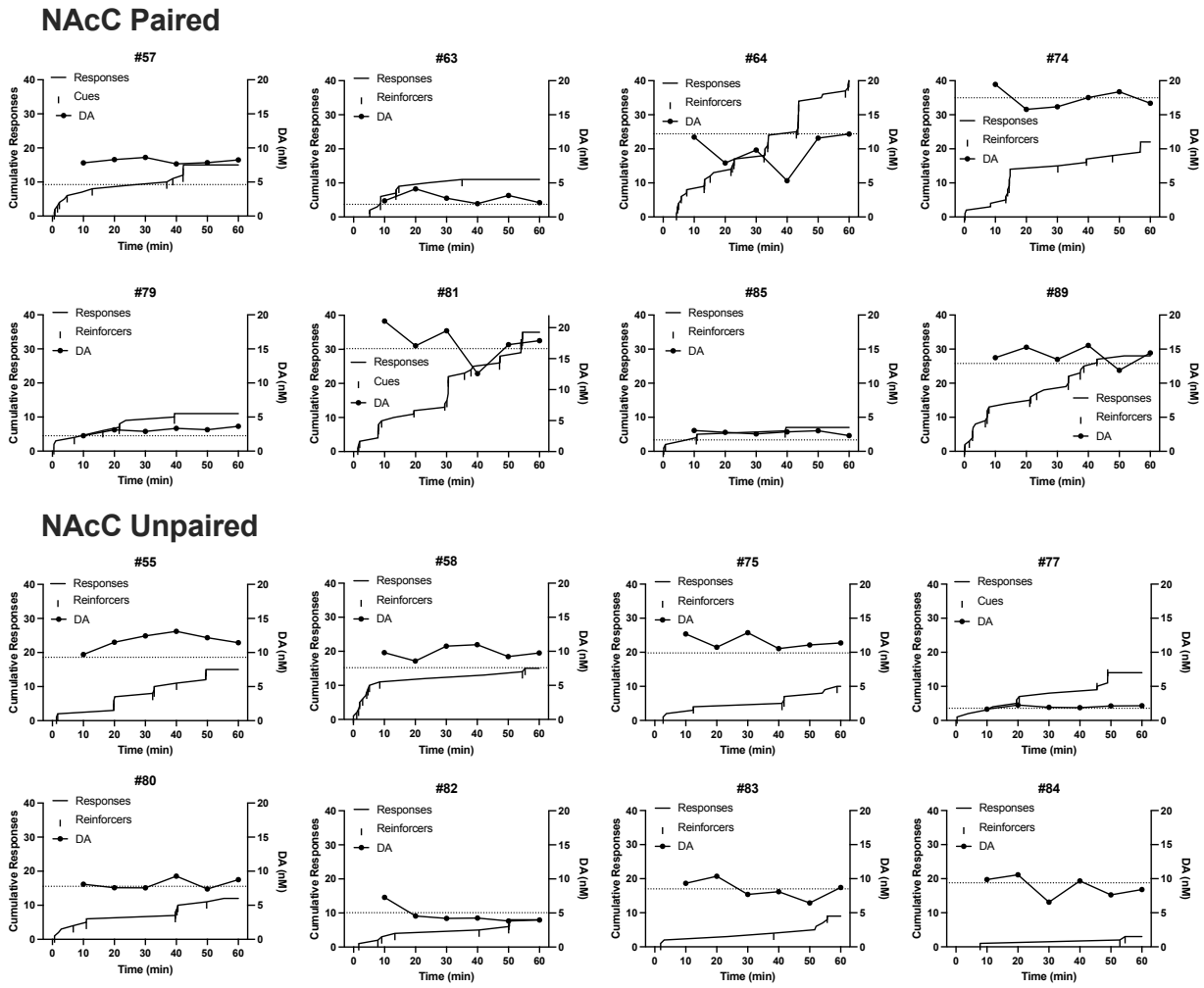

Figure 3.3 Cumulative records of active responding during ACQ3 in subjects that underwent microdialysis collection of dopamine (nM) in the NAcC. Dotted lines represent the average BL dopamine concentration per subject

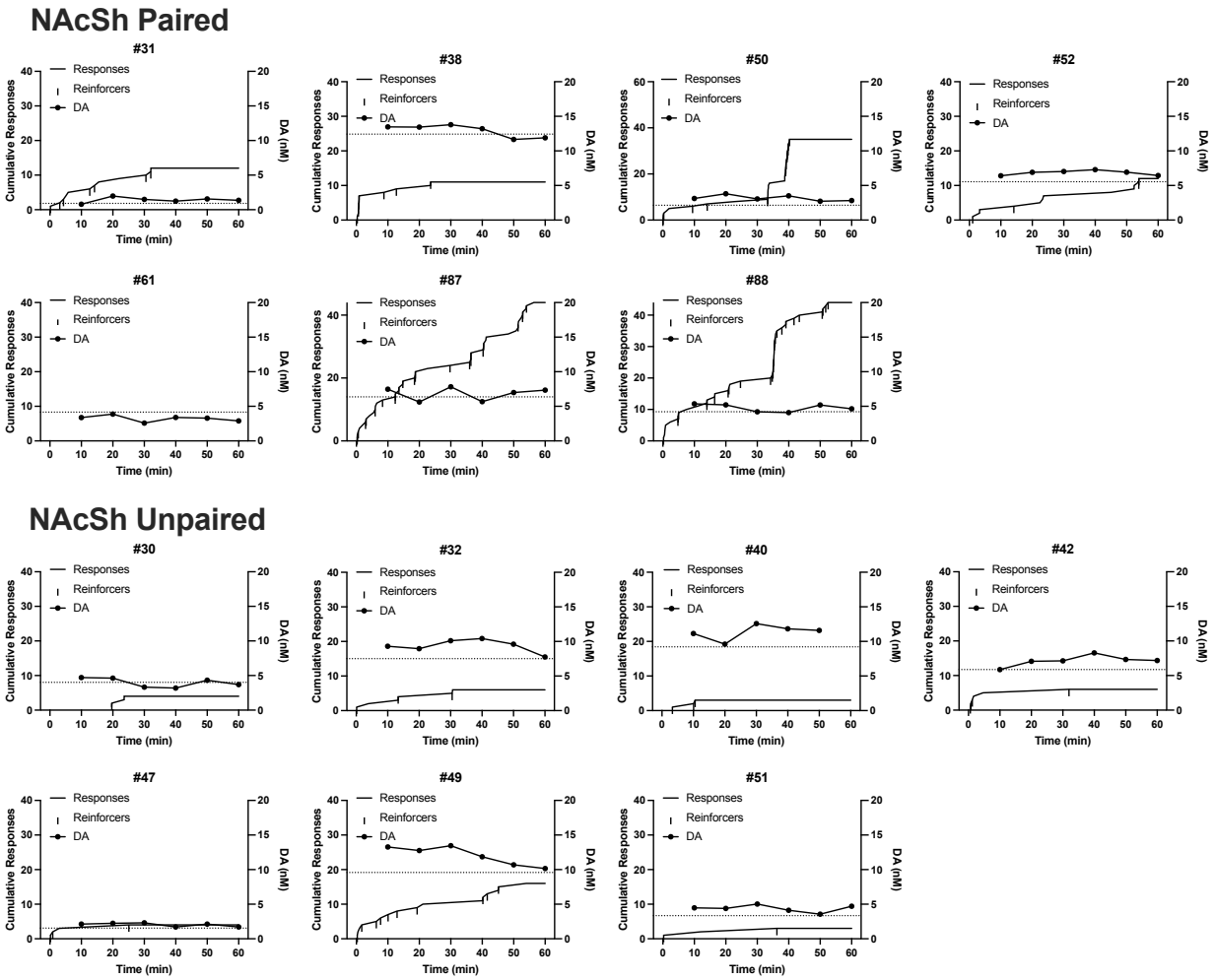

Figure 3.4 Cumulative records of active responding during ACQ3 in subjects that underwent microdialysis collection of dopamine (nM) in the NAcSh. Dotted lines represent the average BL dopamine concentration per subject.

3.4.3 Effects of local and systemic administration of indirect dopamine agonists on responding for cocaine-paired cues

Although dopamine levels in the NAc core and shell were unchanged while responding for cues, increasing dopamine levels via administration of indirect dopamine agonists may increase responding for cues as shown previously (Neisewander *et al.*, 1996; Park *et al.*, 2002; Lu *et al.*, 2004; Tang *et al.*, 2005). Following the end of the 60-min ACQ3 session, cocaine was retrodialyzed into the NAc over 10 min then dopamine

was collected for 30 min while responses were recorded for an additional 60 min (extended ACQ3 session). As expected, local cocaine infusion enhanced dopamine concentration in Paired and Unpaired groups in both NAcC and NAcSh (Fig 3.5A&E; main effect of drug: NAcC: $F(1,14)=28.77$, $P<0.0001$; NAcSh: $F(1,9)=48.68$, $P<0.0001$). Heatmaps comparing percent change from BL for all neurotransmitters analyzed following cocaine retrodialysis are shown in Figure 3.5. Within the NAcC, levels of dopamine and 3-MT are increased following cocaine infusion, indicated by main effects of time (Dopamine: $F(1.63,22.82)=26.64$, $P<0.0001$; 3-MT: $F(2.003,26.03)=44.23$, $P<0.0001$). Similar effects of time were seen for dopamine, 3-MT, and NE in the NAcSh (Dopamine: $F(2.025,18.23)=5.658$, $P<0.05$; 3-MT: $F(1.395,11.16)=10.85$, $P<0.01$; NE: $F(1.358,12.22)=28.06$, $P<0.0001$). No interactions between drug x conditioning type were observed for any neurotransmitter analyzed.

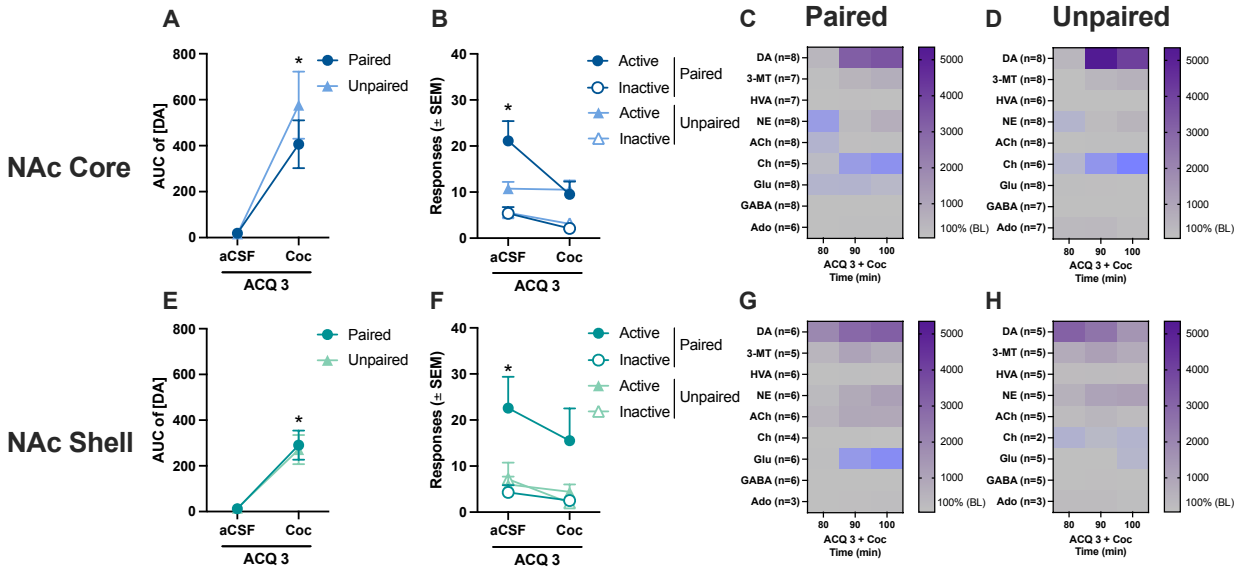

Figure 3.5 Cocaine infusion into the NAc core or shell and responding in the extended ACQ3 session. A) In the NAcC, within 30 min after cocaine infusion (AUC values), dopamine concentrations are significantly increased as compared with the first 30 min of the prior ACQ3 session (Paired n=8; Unpaired n=8). Main effect of drug ($F(1,14) = 28.77$, $^*P < 0.0001$) only. B) Despite increased dopamine concentrations following cocaine retrodialysis, active responding decreased in Paired animals or was unaltered in Unpaired subjects. Main effects: Response type ($F(1,14) = 29.69$, $P < 0.0001$), drug ($F(1,14) = 11.89$, $P < 0.01$), with conditioning type x drug ($F(1,14) = 5.825$, $P < 0.05$) and drug x conditioning type x response

type interactions (F (1,14) = 8.495, P < 0.05). Post-hocs show active responding is greater in Paired groups than Unpaired groups during ACQ3 (*P <0.05). C&D) Levels of other neurotransmitters in the NAcC throughout the first 30 min post cocaine infusion (percent change of baseline). E) In the NAc shell (Paired n=6; Unpaired n=5), cocaine infusion increased dopamine levels (only main effect of drug: F (1,9) = 48.68, *P < 0.0001), but reduced active responding for cues in both Paired and Unpaired groups (F). Main effects: Response type (F (1,12) = 8.53, *P < 0.05), drug (F (1,12) = 6.08, P < 0.05), with interactions between conditioning type x response type (F (1,6) = 6.07, P < 0.05) and drug x conditioning type x response type interaction (F (1,6) = 6.953, P < 0.05). Post-hocs show active responding is higher in Paired group than Unpaired group during ACQ3 (*P <0.05). G&H) NAcSh neurotransmitters following cocaine retrodialysis (percent change of baseline). aCSF = artificial cerebrospinal fluid Coc = Cocaine Retrodialysis

Although local administration of cocaine into the NAcC increased dopamine levels, active responding decreased after cocaine retrodialysis in the Paired group but was unaltered in the Unpaired group as compared with responding prior to cocaine retrodialysis (Fig 3.5B), supported by a drug x conditioning type interaction, ($F(1,14)=5.825$, $P<0.05$) and a three-way interaction between drug x conditioning type x response type ($F(1,14)=8.495$, $P<0.05$). Active responding in both Paired and Unpaired groups decreased after cocaine infusion into the NAcSh (Fig 3.5F), yet Paired subjects maintained higher levels of active nosepokes than Unpaired before and after cocaine retrodialysis, supported by a conditioning type x response type interaction ($F(1,6)=6.07$, $P<0.05$) and a three-way interaction between drug x conditioning type x response type ($F(1,6)=6.953$, $P<0.05$). Together, these data demonstrate that cocaine retrodialysis increased dopamine concentrations in the NAc but did not increase responding for cocaine-paired cues as predicted.

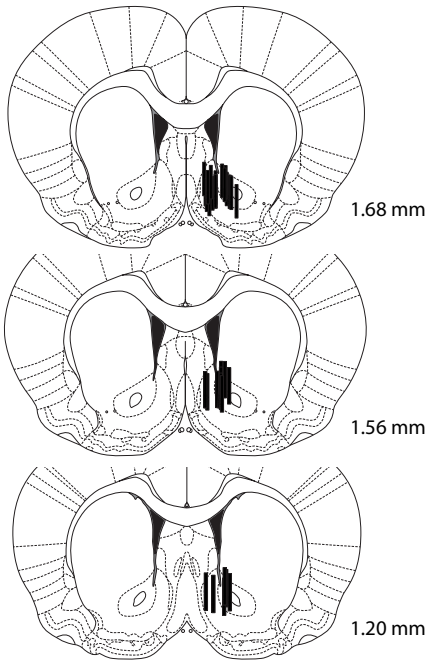

Figure 3.6 Schematic showing relative placements of microdialysis probes in either the left NAc core or shell. Placements were verified histologically according to Paxinos & Watson, 2004 (Paxinos and Watson, 2004).

Indirect dopamine agonists, cocaine (1.0, 3.2, 10 or 18 mg/kg) or amphetamine (1.0 mg/kg), or saline, were administered acutely to separate Paired groups immediately prior to the start of ACQ4. These doses of indirect dopamine agonists have been shown to increase dopamine levels in the NAc (Chen and Reith, 1994; Parsons *et al.*, 1998; Frank *et al.*, 2008) and induce reinstatement behaviors in previous literature (Neisewander *et al.*, 1996; Park *et al.*, 2002; Lu *et al.*, 2004; Tang *et al.*, 2005). Subjects in the Paired group maintain higher responding for cocaine-paired cues under a RR2 schedule than Unpaired animals, supported by a significant interaction between conditioning type x response type ($F(1,13)=7.596$, $P<0.05$). Responding decreased over time, to a greater extent in the Paired group, revealed by a conditioning type x time interaction ($F(6,78)=3.951$, $P<0.01$). As shown in Figure 3.7A&E, vehicle treatments immediately before ACQ4 did not alter responding following Paired or Unpaired

conditioning. Neither cocaine nor amphetamine treatments, at any dose tested, increased the average number of active or inactive responses for cocaine-paired cues in Paired subjects (Fig 3.7B&F) or Unpaired subjects (data not shown).

With the RR2 schedule, not every operant response is reinforced with a cue presentation during ACQ and therefore, may not be necessarily predictive of a cue presentation. Thus, we sought to investigate how a more predictable schedule of reinforcement might alter behavior, such as an FR1 schedule of reinforcement often used in reinstatement assays (Weiss *et al.*, 2000; Cervo *et al.*, 2003; Fuchs *et al.*, 2004; Berglind *et al.*, 2006; Bastle *et al.*, 2012; Saunders *et al.*, 2013). In Figure 3.7C&G, cocaine-paired cues were presented on an FR1 schedule and maintained more active than inactive responses and more active responses than cues not paired with cocaine infusions, as supported by a conditioning type x response type interaction ($F(1,14)=9.225$, $P<0.01$) and response type x conditioning type x day interaction ($F(6,84)=7.532$, $P<0.0001$) as seen previously (Bertz and Woods, 2013). Subsequently, a group of Paired subjects received cocaine (18 mg/kg) prior to the start of ACQ4. Cocaine administration did not increase average levels of active or inactive responding (Fig 3.7D&H) as compared with vehicle or responding on ACQ3.

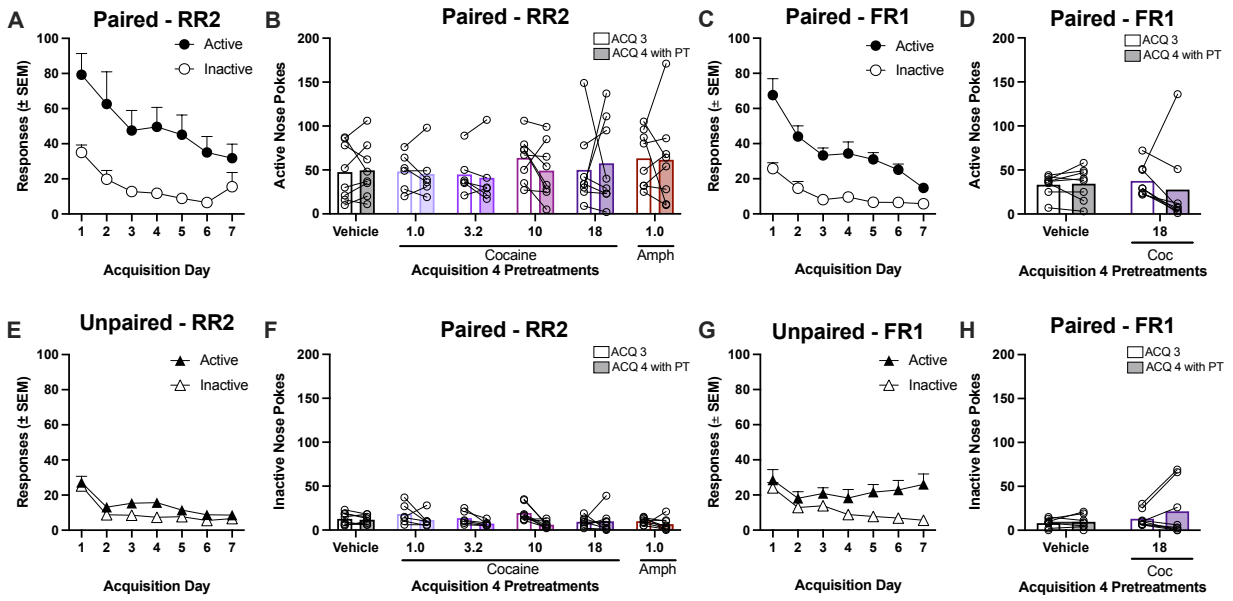

Figure 3.7 Systemic pretreatments of psychomotor stimulants prior to ACQ4 with either RR2 or FR1 schedules of reinforcement (n = 6-8/group). A&E) Paired animals make more active nosepokes to earn presentations of cocaine-paired cues than Unpaired animals on a RR2 schedule of reinforcement. Main effects: Conditioning type ($F(1,13) = 10.30$, $P < 0.01$), response type ($F(1,13) = 12.55$, $P < 0.01$), and time ($F(6,78) = 25.40$, $P < 0.0001$). Interactions: Conditioning type x response type ($F(1,13) = 7.596$, $P < 0.05$), and conditioning type x time ($F(6,78) = 3.951$, $P < 0.01$). B&F) Acute cocaine (1.0, 3.2, 10 or 18 mg/kg ip) or amphetamine (1.0 mg/kg sc) pretreatments did not alter active or inactive responding for cues as compared with vehicle. No main effects of drug. C&G) Cocaine-paired cues increased active responding on an FR1 schedule as compared with Unpaired groups (conditioning type x response type interaction: $F(1,14) = 9.225$, $P < 0.01$) and active responding decreased over time. Main effects: Response type ($F(1,14) = 61.35$, $P < 0.0001$), and time ($F(3.354,46.96) = 25.14$ $P < 0.0001$). Additional interactions: Conditioning type x time ($F(6,84) = 7.671$, $P < 0.0001$), and conditioning type x response type x time ($F(6,84) = 7.532$, $P < 0.0001$). D&H) An acute cocaine pretreatment (18 mg/kg ip) did not alter responding for cues under an FR1 schedule (no main effect of drug). RR2 = random ratio 2, FR1 = fixed ratio 1, PT = pretreatment

3.5 Discussion

Drug-paired cues acquire conditioned reinforcing properties and can elicit behavior in animals. It is commonly thought that dopamine in reward circuitry contributes to the expression and modulation of cue-induced reinstatement (Weiss *et al.*, 2000; Park *et al.*, 2002; Cervo *et al.*, 2003; Phillips *et al.*, 2003; Nicola *et al.*, 2005; Volkow *et al.*, 2006; Saunders *et al.*, 2013; Parsegian and See, 2014; McGlinchey *et al.*, 2016; Halbout *et al.*, 2019; O'Neal *et al.*, 2020). However, a number of studies have shown that increases in dopamine levels in the NAc do not always coincide with high

levels of responding for drug-paired cues (Neisewander *et al.*, 1996; Bradberry *et al.*, 2000; Ito *et al.*, 2000; Di Ciano *et al.*, 2001). These studies suggest that the drug-paired cue in reinstatement has a complex function and may differentially recruit the dopaminergic system. Therefore, the goal of the present study was to characterize the role of dopamine in the conditioned reinforcing properties of cocaine-paired cues using the New Response Acquisition procedure.

In the current study, cocaine elicited conditioned reinforcing properties such that only cues formerly paired with cocaine infusion facilitated the acquisition of a novel response (Bertz and Woods, 2013; Bertz *et al.*, 2015, 2016; Robertson and Jutkiewicz, 2020, 2021). This is consistent with previous data demonstrating that cocaine-paired cues can maintain behavior even in the absence of cocaine infusion. Contrary to our hypothesis, dopamine levels in the NAc were not altered in subjects responding for cocaine-paired cues, and indirect dopamine agonists did not increase responding for cues. Overall, these data suggest that dopamine likely does not mediate the conditioned reinforcing properties of cocaine-paired cues as measured in this procedure. However, there was some evidence that dopamine may modulate conditioned reinforcement in some subjects, as suggested by previous studies highlighting the importance of individual differences in reward-related behaviors (Homberg *et al.*, 2004; Flagel *et al.*, 2011; Robinson *et al.*, 2015; Shaw *et al.*, 2021). BL dopamine concentrations in the NAcC were elevated in some individuals and positively correlated with greater preference scores, yet dopamine levels were unchanged during the 10 min period when active responding was the highest (Fig 3.2E). Additionally, individual differences emerged with systemic psychostimulant treatment, such that 18 mg/kg cocaine and 1.0

mg/kg amphetamine enhanced active responding for cocaine-paired cues in a subset of animals (n=2 and n=1, respectively) but did not potentiate responding for cues in all rats tested (Fig 3.7). There may be differential involvement of the dopaminergic system on an individual basis, such that dopamine levels influence responding for cues in some animals, potentially due to stronger conditioning, a function of learning (Dalley *et al.*, 2005; Flagel *et al.*, 2011).

One explanation for the lack of involvement of dopamine in the conditioned reinforcing effects of cocaine evaluated here is that microdialysis may not be sensitive enough to measure localized or phasic changes in dopamine on an individual basis. Other techniques (i.e., voltammetry) would be required to investigate how phasic dopamine signals in the NAc may differ between groups while responding for cues (Schultz, 2006; Aragona *et al.*, 2009; Flagel *et al.*, 2011). Further, the neurobiological underpinnings of cocaine conditioned reinforcement may involve: 1) other neurotransmitters or peptides not analyzed or 2) brain regions other than the NAc, such as the amygdala, where dopamine has been shown to modulate drug-seeking (Weiss *et al.*, 2000, Di Ciano and Everitt, 2004*b*; Schmidt *et al.*, 2005; Berglind *et al.*, 2006).

Previous studies demonstrated that activation of dopamine receptors by exogenous ligands increased responding for conditioned reinforcers (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Wolterink *et al.*, 1993; Phillips *et al.*, 1994; Park *et al.*, 2002; Cervo *et al.*, 2003, Di Ciano and Everitt, 2004*a*; Lu *et al.*, 2004; Tang *et al.*, 2005; Goddard and Leri, 2006; Di Ciano, 2008; Di Ciano *et al.*, 2008; Saunders *et al.*, 2013; Bertz *et al.*, 2015). Therefore, we expected to see similar effects of indirect dopamine agonists on responding for cocaine-paired stimuli in this procedure. First, we

found that retrodialysis of cocaine (7 µg) during an extended ACQ3 session produced robust increases in dopamine (5000-6000%) in both NAc subregions but did not potentiate responding for cues (Fig 3.6B&F). Second, systemic cocaine and amphetamine at doses that have been shown to induce reinstatement (Neisewander *et al.*, 1996; Park *et al.*, 2002; Lu *et al.*, 2004; Tang *et al.*, 2005) did not potentiate the conditioned reinforcing properties of the cue, as active responding in Paired groups was not significantly different from vehicle treated subjects (Fig 3.7). Together, these data demonstrate that indirect dopamine agonists known to increase dopamine levels and responding for cocaine-paired cues in other procedures failed to increase the conditioned reinforcing properties of cocaine-paired cues in the New Response Acquisition procedure. While these data were unexpected, one possible explanation is that the stimulant properties of these drugs (Bedingfield, 1998) interfered with the acquisition or learning of a novel behavior. However, we think this is unlikely because prior work administered larger doses of cocaine intra-NAc and showed increased operant responding during reinstatement (Park *et al.*, 2002). Further, we tested a wide range of doses that increased cue-induced reinstatement (Lu *et al.*, 2004), including doses that should not stimulate locomotor activity or induce stereotypy (Carr *et al.*, 2020).

Another possible explanation between the current study and previous studies showing cocaine stimulates responding for cues is in the schedule of reinforcement. Under a RR2 schedule of reinforcement, cocaine administration prior to ACQ did not alter responding for cues. To evaluate responding to earn cocaine-paired cues under a more predictable schedule of reinforcement often used in reinstatement assays,

separate groups were trained to earn cues on an FR1 schedule (Weiss *et al.*, 2000; Cervo *et al.*, 2003; Fuchs *et al.*, 2004; Berglind *et al.*, 2006; Bastle *et al.*, 2012). Systemic cocaine administration did not enhance responding for cocaine-paired cues under an FR1 schedule in this procedure. Overall, schedule of contingent cue delivery does not seem to influence the recruitment of the dopaminergic system in this procedure, yet for other tests of conditioned reinforcement dopamine may influence responding for cues, possibly due to the complexity of the cue from prior contingent drug delivery (Di Ciano and Everitt, 2004*a*; Di Ciano, 2008; Di Ciano *et al.*, 2008).

Together, these data suggest that the role of dopamine in mediating the conditioned reinforcing properties of drug-paired cues is not straightforward. Dopamine levels in the NAc may play a more critical and complex role in the formation of cocaine-cue associations (Schultz, 2006; Winterbauer and Balleine, 2007; Flagel *et al.*, 2011; Jeong *et al.*, 2022; Kutlu *et al.*, 2022) during the Pavlovian Conditioning phase, rather than in mediating conditioned reinforcing effects of cocaine in the Acquisition phase. Importantly, there is evidence that the underlying neurobiology mediating cocaine conditioned reinforcement measured in subjects with a history of non-contingent or contingent drug delivery is functionally distinct (Namba *et al.*, 2018) such that dopamine may mediate the motivational aspects for potential drug delivery (Berridge, 2007) rather than conditioned reinforcing effects alone. Overall, the current study highlights the ability of cocaine-paired cues to maintain high levels of behavior in this procedure, independent of dopamine. Future studies will continue to investigate underlying neurobiology of conditioned reinforcement to provide insight for novel treatments to prevent relapse.

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Chapter 4
Opioidergic Mechanisms of Cocaine Conditioned Reinforcement

4.1 Abstract

One contributor to relapse is the ability of environmental cues that have been associated with drug-taking behavior to evoke drug-seeking behaviors. Drugs that act on the opioidergic system, such as mu opioid receptor (MOR) and delta opioid receptor (DOR) agonists, can increase responding maintained by drug-paired cues. We sought to evaluate whether opioid receptor ligands that induce drug-seeking behavior in reinstatement assays also increase responding for cocaine-paired cues in a stringent test of cocaine conditioned reinforcement (New Response Acquisition). This procedure begins with Pavlovian Conditioning in which subjects receive five infusions of cocaine (320 µg/kg/inf) and either simultaneous (Paired) or separate (Unpaired) presentations of a light+tone stimulus per day for 10 days. Then, novel operant manipulanda are introduced into the chamber, and responses produce presentations of cues formerly associated with cocaine (Acquisition). On the fourth day of Acquisition, treatments were administered acutely before the start of the session and responding was evaluated. Subjects in the Paired group make more active responses for cue presentations than Unpaired subjects. The enkephalinase inhibitor RB101 (10 mg/kg), and the DOR agonists SNC80 (3.2 mg/kg) and deltorphin II (10 µg icv), enhance responding for cocaine-paired cues in Paired subjects in this procedure, and these effects were blocked by a DOR selective antagonist. Neither morphine nor a kappa opioid receptor

(KOR) agonist enhanced behavior in this procedure. Therefore, cocaine conditioned reinforcement may be modulated by delta opioid receptor activation.

4.2 Introduction

Cocaine use disorder is a chronic disease characterized by frequent relapses to cocaine use after attempts at or periods of abstinence. Multiple factors can induce conditions that increase the likelihood of relapse, such as exposure to stress, drug, or environmental stimuli that have been associated with cocaine-taking behaviors (Bonson *et al.*, 2002; Volkow and Li, 2005). After repeated associations or pairings with a drug or primary reinforcer, neutral environmental stimuli or cues develop conditioned reinforcing properties, such that behaviors producing presentations of the drug-paired stimuli occur even in the absence of the primary reinforcer. Conditioned reinforcers may induce drug-seeking behavior in animals and likely play a role in drug craving and relapse.

Neurobiological mechanisms mediating the conditioned reinforcing effects of drug-paired cues are thought to involve dopamine (Wise, 2004) and glutamate (Kalivas and McFarland, 2003). Endogenous and exogenous opioid ligands have been shown to modulate dopamine and glutamate release within reward-related pathways (Mongi-Bragato *et al.*, 2018; Rysztak and Jutkiewicz, 2022) and may also play a role in conditioned reinforcement (for review, see: Pellissier *et al.*, 2018).

There is evidence that neuroadaptations in the endogenous opioid system occur following cocaine exposure to promote cocaine reinforcement. For example, repeated cocaine may increase expression of endogenous enkephalin peptides, which may contribute to the primary reinforcing effects of cocaine (Sun *et al.*, 2020), cocaine sensitization (Mongi-Bragato *et al.*, 2016, 2021), motivation for cocaine

(Gutiérrez-Cuesta *et al.*, 2014), and/or cue-induced cocaine-seeking behaviors (Burattini *et al.*, 2008). Further, expression of endogenous opioid peptides may change after periods of cocaine abstinence, such that endogenous opioid peptides are upregulated in certain brain regions, i.e., the ventral pallidum, to contribute to cocaine-seeking behaviors (Tang *et al.*, 2005; Kupchik *et al.*, 2014).

Consistent with evidence that changes in the opioidergic system are associated with reward-related behaviors, manipulating the opioidergic system can alter cue-controlled behaviors. Reducing opioid receptor signaling by administration of an antagonist (Burattini *et al.*, 2008) or genetic deletion of receptors (Gutiérrez-Cuesta *et al.*, 2014) attenuated cue-induced cocaine reinstatement. Conversely, increasing endogenous enkephalins or activating mu or delta opioid receptors (MOR & DOR, respectively) promotes responding maintained by cocaine-paired cues (Phillips *et al.*, 1994; Simmons and Self, 2009). It is possible that the different opioid peptides or receptor types have overlapping functions within reinforcement behaviors. Both MOR and DOR systems may be critical for the formation of drug-cue associations which are critical for the development of conditioned reinforcement (Skoubis *et al.*, 2005; Le Merrer *et al.*, 2011; Bertran-Gonzalez *et al.*, 2013; Gutiérrez-Cuesta *et al.*, 2014) while MORs alone directly or indirectly contribute to the primary reinforcing effects of MOR agonists and cocaine (Ward *et al.*, 2003; Schroeder *et al.*, 2007; Charbogne *et al.*, 2014).

In measuring cocaine-seeking behaviors, researchers have utilized reinstatement procedures in which manipulation of the opioidergic system has altered cue-induced reinstatement of an extinguished response that had previously resulted in cocaine delivery (Burattini *et al.*, 2008; Simmons and Self, 2009; Gutiérrez-Cuesta *et al.*, 2014).

The studies implicate a role of the opioidergic system in the conditioned reinforcing properties of cocaine-paired cues; however, because the operant responses maintained by cues were typically associated with prior contingent drug delivery, the function of the cue during reinstatement is complex and likely has motivational, discriminative, and/or conditioning reinforcing properties (Mackintosh, 1974; Williams, 1994). Understanding the underlying neurobiology of the conditioned reinforcing effects of drug-paired cues will lead to better insight on how cues elicit behavior in animals; therefore, we use a procedure that utilizes a novel operant response to produce cues and has never been associated with delivery of cocaine in an attempt to isolate conditioned reinforcement (Mackintosh, 1974; Williams, 1994).

The purpose of the current study is to evaluate a role of the opioidergic system in modulating behavior maintained by cocaine-paired cues in the New Response Acquisition procedure. We hypothesized the endogenous enkephalins, primarily acting via DORs, may be responsible for the expression of cocaine conditioned reinforcement, based off of DOR system mediation of behavior for cocaine-paired cues as described above (Phillips *et al.*, 1994; Simmons and Self, 2009; Gutiérrez-Cuesta *et al.*, 2014). This work provides novel insights into the role of the opioidergic system as a modulator of the conditioned reinforcing effects of cocaine-paired cues.

4.3 Methods

4.3.1 Subjects

Adult male Sprague Dawley rats weighting at least 280 g were used for all experiments. Rats were obtained from Envigo (Haslett, MI) and housed in a humidity- and temperature-controlled (21-23°C) room with a 12-hour light/dark cycle (06:30 lights

on). Food and water were provided ad libitum throughout the entire duration of experiments. All experimental procedures were approved by the University of Michigan Institutional Care and Use Committee.

4.3.2 Surgery

4.3.2.1 Intravenous catheter implantation

Surgical procedures for catheter implantations were the same as reported in (Robertson and Jutkiewicz, 2021). Briefly, subjects were anesthetized with intraperitoneal (ip) injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). Carprofen (5 mg/kg) was given subcutaneously (sc) prior to the start of surgery as well as 24-hour post-surgery to control for postoperative inflammation and pain. An incision (1 cm) was made ventral from the top of the inner left thigh, and the femoral vein was isolated. A catheter (Micro-Renathane Tubing, MRE-040, Braintree Scientific, Inc., Braintree, MA) was inserted into the vein and passed subcutaneously to a mesh backplate equipped with a 22-gauge stainless steel tube (313-000BM-15UP/1/SPC, P1 Technologies, Roanoke, VA) and sutured to the muscle between the scapulae. Rats were flushed daily with 0.5 ml heparinized saline (50 USP/ml) to maintain catheter patency. Post-operative recovery lasted for at least 7 days prior to the start of experiments or intracranial cannula implantation.

4.3.2.2 Cannula Implantation

In a subset of animals that were to receive intracerebroventricular (ICV) injections, guide cannula were implanted 7-10 days following catheter implantations. Briefly, subjects were anesthetized as described above. Then, guide cannula

(C313GRL/SPC, cut 3.5 mm below pedestal, P1 Technologies) were placed to the following coordinates (in reference to bregma): AP: -0.8 mm ML: +1.5 mm DV: -2.8 mm. Cannula were secured to the skull by two metallic bone screws (0.86 mm shaft, Fine Science Tools, Foster City, CA) and acrylic dental cement (Lang Dental, Wheeling, IL). Carprofen (5 mg/kg sc) was given 24- and 48-hour post-surgery to control for inflammation and pain. Post-operative recovery lasted for 2-3 days prior to the start of experiments.

4.3.3 Behavioral Procedure – New Response Acquisition

4.3.3.1 Pavlovian Conditioning (PAV)

Behavioral procedures were as previously described in Chapter 3. Subjects were assigned randomly to either Paired or explicitly Unpaired (control) Pavlovian Conditioning groups. For the Pavlovian Conditioning phase, subjects were placed into operant chambers (Med Associates Inc., St. Albans, VE) equipped with a house light on the left wall and a speaker (ENV-230, Med Associates) used to generate an 80-dB white noise stimulus, on the right wall. Subjects were tethered to tubing on a swivel (375/22PLS, Instech, Plymouth Meeting, PA). For subjects in Paired groups, an infusion of cocaine (0.32 mg/kg/infusion) was delivered simultaneously with a presentation of a stimulus, an 80-dB dual white noise + house light illumination. This dose of cocaine was chosen based on previous work demonstrating that it incurred robust conditioned reinforcing properties, as described in Chapter 2. For all subjects, the duration of the cocaine infusion and stimulus presentation were determined by body weight (2.0 ± 0.5 s). Pairings of infusions and stimulus presentations occurred according to a variable time (VT) 15-min schedule (range 0.1 s-30.5 min). For the Unpaired group, subjects

received infusions of cocaine and stimulus presentations that were explicitly unpaired and operated on independent schedules such that the two events never occurred simultaneously. However, cues were often delivered within 15 min of a cocaine infusion; therefore, there was overlap between cues and the physiological effects of cocaine. All animals received 5 infusions of cocaine and 5 stimulus presentations per day for 10 days. Each session lasted 75 min (+/- 30 s).

4.3.3.2 Instrumental Acquisition (ACQ)

During the second phase of New Response Acquisition, Acquisition (ACQ), subjects were placed into the same operant cambers as Pavlovian Conditioning; however, in this phase the chambers were also equipped with two nosepoke manipulanda (ENV-114BM, Med Associates) each illuminated with a single LED light. The location of the active nosepoke (right or left side on the right wall of the chamber) was counterbalanced across subjects. Subjects were tethered via the same tubing and swivel connected to a syringe filled with saline. No infusions were given at any point throughout the ACQ sessions. At the start of the session, subjects were able to respond freely on either nosepoke. The first response on the active nosepoke of each session resulted in a ~2s presentation of the white noise+house light stimulus only. Subsequent responses on the active nosepoke resulted in presentations of the stimulus according to a random ratio 2 (RR2) schedule of reinforcement, such that on average every two active nosepokes resulted in a cue presentation (range 1-6 active nosepokes). Responses in the inactive nosepoke were recorded but had no scheduled consequences. The duration of each ACQ session was 60 min and sessions occurred once per day for 7 days.

4.3.4 Pharmacological Manipulations

Separate groups of animals received different pharmacological manipulations prior to the start of specific ACQ sessions and responding on nosepokes for cues was evaluated. For opioid receptor antagonist studies, Paired groups received repeated administration of the nonselective antagonist naltrexone (NTX; 10 mg/kg sc) or vehicle immediately prior to ACQ sessions 4-7 or the DOR selective antagonists naltrindole (NTI; 3.2 mg/kg sc), naltriben (NTB; 0.32 mg/kg sc), RTI-5589-25 (RTI-25; 10 mg/kg ip), acutely prior to ACQ session 4. NTI and NTB were given as 30 min pretreatments prior to the start of ACQ4. Control groups received vehicle (either saline sc or ip or 10% ethanol, 10% castor oil and 80% sterile water (ip)) immediately prior to ACQ4.

For studies with enkephalinase inhibitors or opioid receptor agonists, separate groups received the enkephalinase inhibitor RB101 (10 mg/kg iv) or its vehicle (10% ethanol, 10% castor oil, 80% sterile water iv) with or without a 30 min pretreatment of NTI (3.2 mg/kg sc). Other groups received a delta opioid receptor (DOR) agonist SNC80 (3.2 mg/kg sc) or its vehicle (3% acid in sterile water sc) with or without 30 min pretreatments of NTI (3.2 mg/kg sc, 30 min before SNC80/vehicle) or NTB (0.32 mg/kg sc 30 min before SNC80/vehicle). Either the mu opioid receptor agonist (MOR) morphine (3.2 mg/kg sc), or the kappa opioid receptor (KOR) agonist spiradoline (1.0 mg/kg sc), were given 15 min or 30 min prior to the start of ACQ4, respectively.

4.3.4.1 ICV Injections

Deltorphin II, a peptide DOR agonist, was given ICV prior to the start of ACQ4. Subjects were removed from the home cage and dummy cannula were removed. ICV injections were manually infused using a 10 µl Hamilton syringe equipped with internal

cannula injectors to extending 2 mm past the end of the guide cannula. Once the needle was inserted into the cannula, 5 µl volume infusions were given over 60 seconds and then the needle was left inserted for an additional 60 seconds to allow for diffusion of injection. Separate groups of animals received either deltorphin II (10 µg in 5 µl) or its vehicle (10% DMSO in sterile water) with or without a 30 min pretreatment of NTI (3.2 mg/kg sc). Administration of DOR agonists can produce convulsions (Dripps *et al.*, 2020), and a subset of animals (n=4) exhibited pre-convulsive behaviors (i.e., freezing, teeth chattering, loss of muscle tone) during the 60 seconds wait time post-infusion and were removed from the experiment.

4.3.5 ICV cannula location verification

After conclusion of ICV experiments, cannula placement was checked using methylene blue injection. Briefly, subjects were deeply anesthetized with pentobarbital (70 mg/kg ip) and 5 µl of methylene blue dye were infused into the icv cannula over 60 seconds and dye was allowed to diffuse for an additional 60 seconds. Then, subjects were rapidly euthanized, and the brain was sliced to view diffusion of dye across ventricles. Subjects that did not have diffusion throughout the ventricles or were unable to verify cannula location were removed from the experimental analyses (n=8).

4.3.6 Materials

Cocaine hydrochloride and morphine sulfate were obtained from Michigan Medicine Hospital Pharmacy and dissolved into sterile 0.9% saline solution. NTX, NTI hydrochloride, NTB hydrate, and spiradoline (U-62066), and deltorphin II were purchased from Sigma Aldrich (St. Louis, MO). RTI-5589-25 was a generous gift from

F.I Carroll, RB101 was a gift from Bernard Roques, and SNC80 was given by Kenner Rice. For surgical procedures, ketamine and xylazine (Dechra Pharmaceuticals, Northwich, UK), and carprofen (Zoetis, Parsippany-Troy Hills, NJ) were used. Heparin (Pfizer, New York, NY) was used for maintaining catheter patency.

4.3.7 Data Analysis

Data analyses were conducted separately for each experiment using Prism GraphPad 9.5.1 software or SPSS (Version 28.0.0.0). For comparing effects of pharmacological manipulations, repeated measures (RM) three-way ANOVAs were conducted between response type (active vs. inactive nosepokes), session (ACQ day), and drug treatment. For treatments also given to Unpaired groups, four-way ANOVAs were used for added comparison between conditioning types (Paired vs. Unpaired).

4.4 Results

4.4.1 Opioid receptor antagonists reduce responding to earn presentations of cocaine-paired cues

In subjects that underwent Paired Pavlovian Conditioning (Paired group), cocaine-paired cues develop conditioned reinforcing properties, as demonstrated by higher levels of responding on the active nosepoke than the inactive nosepoke (Figure 4.1A), supported by a main effect of response type (F (1,14) = 32.36, P < 0.0001). To investigate whether endogenous opioid tone contributes to the conditioned reinforcing effects of cocaine-paired cues in this assay, pretreatments of naltrexone (NTX; 10 mg/kg), a non-selective opioid receptor antagonist, were given to a Paired group immediately prior to the start of ACQ sessions 4-7. Repeated NTX appeared to reduce

active responding for cues across sessions as compared with vehicle; however, active responding was not different from vehicle as the three-way interaction between antagonist x response type x ACQ day did not reach significance. No pretreatment of NTX was given prior to ACQ 8, and while active responding did not differ significantly from that on ACQ 4-6, responding following termination of NTX treatments increased.

Previous studies reported DOR activation increases responding maintained by cocaine-paired cues (Phillips *et al.*, 1994; Simmons and Self, 2009); therefore, to evaluate a potential role of DOR in this procedure, various DOR selective antagonists were administered acutely prior to the start of ACQ 4. In Figure 4.1B, acute administration of opioid receptor antagonists either did not alter or reduced active responding for cues, indicated by antagonist x ACQ day (F (4,35) = 2.685, *P < 0.05) and response type x ACQ day interactions (F (1,35) = 11.47, P < 0.01). Post-hocs indicate acute NTX reduced active responding on ACQ4 as compared with the day prior without drug (ACQ 3). Similarly, RTI-5989-25 (RTI-25; 10 mg/kg), a potent DOR antagonist (Carroll and Dolle, 2014), reduced active responding following acute administration; however, the canonical DOR selective antagonists naltrindole (NTI; 3.2 mg/kg) and naltriben (NTB; 0.32 mg/kg) did not significantly reduce responding for cues across days. Antagonists did not significantly alter inactive responding. These data suggest that either activation of DORs, and likely other ORs together by endogenous opioid peptides, may contribute to responding for cocaine-paired cues in this procedure.

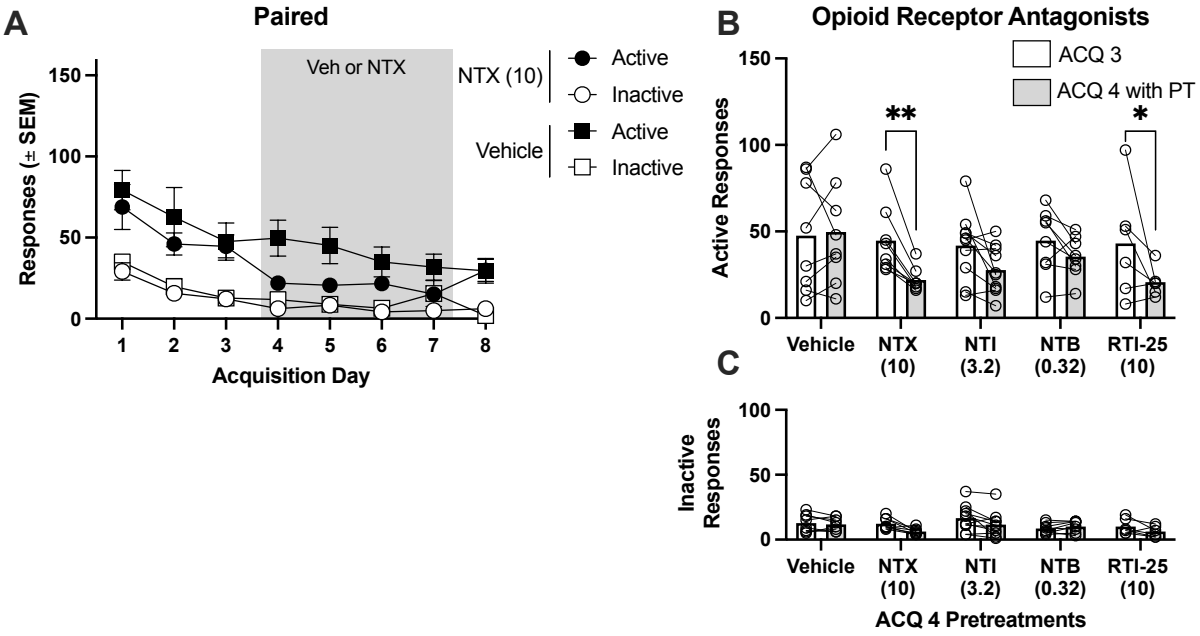

Figure 4.1 Opioid receptor antagonists reduce responding on the active nosepoke that produces presentations of cocaine-paired cues. A) Naltrexone (NTX; 10 mg/kg) seemed to reduce active responding for cues across 4 ACQ sessions (ACQ 4-7) as compared with levels of responding prior to NTX treatment. B) NTX and RTI-25 (10 mg/kg) reduce active responding with acute pretreatment as compared with responding on the day prior. The DOR selective antagonists naltrindole (NTI; 3.2 mg/kg) and naltriben (NTB; 0.32 mg/kg) did not reduce responding with acute pretreatment on ACQ 4. Overall, responding was reduced across ACQ sessions (Response type x ACQ day interaction). C) Levels of inactive responding were unchanged following various pretreatments of opioid receptor antagonists. ACQ = Acquisition, PT = pretreatment, NTX = naltrexone, NTI = naltrindole, NTB = naltriben

4.4.2 Increasing enkephalin levels further drives responding for cocaine-paired cues

In order to investigate whether increased endogenous opioid tone could potentiate responding for cocaine-paired cues, we administered a dual enkephalinase inhibitor, RB101 (10 mg/kg iv), acutely prior to the start of the ACQ4 session. RB101 inhibits both the aminopeptidase N (APN) and neutral endopeptidase (NEP) enzymes to prevent the cleavage and degradation of endogenous enkephalins (Jutkiewicz, 2007; Roques, 2018). All animals underwent Paired Pavlovian Conditioning and showed high levels of responding to earn presentations of cocaine-paired cues across the first 3 ACQ sessions, supported by a main effect of response type (F (1,10) = 72.65, P < 0.0001).

Acute administration of RB101 (Figure 4.2A) significantly enhanced active responding for cues on ACQ 4 as compared with vehicle treatment (10:10:80 iv), revealed by a three-way interaction between drug x response type x ACQ days (F (6,60) = 4.084, P < 0.01). Enkephalins bind to both MORs and DORs; therefore, we sought to determine whether or not DORs might be mediating the effects of RB101 to enhance conditioned reinforcement. In Figure 4.2B, RB101 increased responding as compared with vehicle on ACQ4 (day x agonist interaction: F (1,20) = 11.673, P < 0.01) and potentiated active responding selectively as compared with levels of responding on ACQ 3 (response type x day x agonist interaction: F (1,20) = 10.056, P < 0.01). The DOR antagonist NTI (3.2 mg/kg) administered prior to RB101 completely blocked the effects of RB101 to enhance active responding for cocaine-paired cues, supported by a response type x day x antagonist interaction (F (1,20) = 5.003, P < 0.05) and a three-way interaction between response type x day x agonist x antagonist (F (1,20) = 4.272, P = 0.05). NTI alone did not significantly alter levels of responding for cues as compared with vehicle or with the day prior to pretreatment (ACQ3), consistent with Figure 4.1B. No pretreatments significantly altered levels of inactive responding (Figure 4.2C).

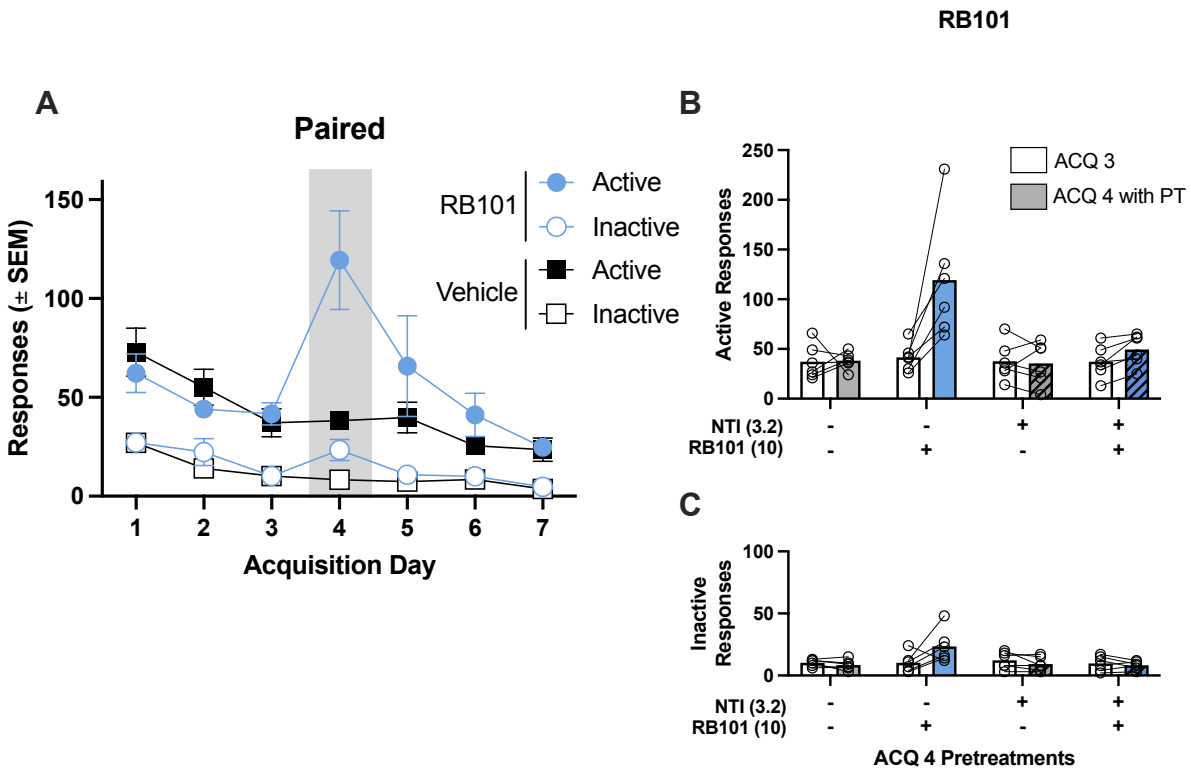

Figure 4.2 Enkephalins protected from enzymatic breakdown potentiated the conditioned reinforcing effects of cocaine-paired cues, which was attenuated by a DOR antagonist. A) Acute administration of RB101 (10 mg/kg i.v.) on ACQ4 enhanced responding on the active nosepoke to earn presentations of cocaine-paired cues. B) The effects of RB101 to enhance active responding were blocked by an acute pretreatment of NTI (3.2 mg/kg), and NTI alone did not alter levels of responding as compared with vehicle nor the day prior to administration. C) Levels of inactive responding were unchanged following all pretreatments. ACQ = Acquisition, PT = pretreatment, NTI = naltrindole

4.4.3 DOR agonists potentiate conditioned reinforcing properties of cocaine-paired cues

While findings from Figure 4.2 suggested that protected enkephalins potentiated responding through activation of DORs, enkephalins are not entirely selective for DORs (Gomes *et al.*, 2020). Commonly used MOR and KOR antagonists, such as βFNA and norBNI, were not ideal for this study because selective antagonist effects do not develop for 24-72 hours after administration (Melief *et al.*, 2011). Therefore, we evaluated whether or not a MOR and KOR agonist, morphine (3.2 mg/kg) or spiradoline

(1.0 mg/kg), respectively, could increase responding to a similar extent as seen with RB101 (Figure 4.2). The doses of MOR and KOR agonists were used because they have been shown to be discriminable in rats without complete behavioral suppression (Holtzman *et al.*, 1991; Holtzman, 2000; Hutcheson *et al.*, 2000). Acute administration of either the MOR or KOR agonist significantly reduced active responding for cocaine-paired cues, revealed by a response type x ACQ day interaction (F (6,66) = 10.59, P < 0.0001). Post-hocs showed active responding decreased on ACQ4 following either morphine or spiradoline as compared to ACQ1-3, such that levels did not differ from levels of responding on the inactive nosepoke (***P's < 0.001). Active responding returned to pre-morphine or spiradoline levels on the following ACQ session (ACQ5). Together, these data suggest that activation of DOR, not MOR or KOR, by endogenous enkephalins likely contributes to the conditioned reinforcing effects of cocaine-paired cues and is sufficient to increase responding.

Therefore, we investigated whether direct DOR activation increased responding to earn presentations of cocaine-paired cues in New Response Acquisition in a similar manner to that observed with RB101. The prototypical DOR agonist, SNC80, has been shown to be a useful tool for probing DOR-mediating behaviors, such as locomotor stimulating and antidepressant like effects (Jutkiewicz *et al.*, 2008; Dripps *et al.*, 2020). To determine whether DOR activation enhanced the conditioned reinforcing effects of cocaine specifically, SNC80 (3.2 mg/kg sc) was administered acutely to groups of rats that underwent Paired or Unpaired Pavlovian Conditioning (Figure 4.3). As expected, cocaine-paired cues maintain higher levels of active responding in the Paired group selectively, supported by a response type x conditioning type interaction (F (1,26) =

36.786, $P < 0.001$). Acute administration of SNC80 robustly increased overall responding in both the Paired group and the Unpaired group, revealed by a main effect of drug (F (1,26) = 5.002, $P < 0.05$). Further, SNC80 increased active responding to a greater extent than inactive responding, indicated by a significant three-way interaction between response type x ACQ day x drug (F (3.476,90.366 = 8.176, $P < 0.001$). SNC80-induced enhancement of active responses for cocaine-paired cues tended to be greater for the Paired group over the Unpaired group, as supported by a four-way interaction between drug x ACQ day x conditioning type x response type that approached significance ($P = 0.08$) but drug x conditioning type interaction was not significant. Overall, activation of DORs by SNC80 potentiated active responding to earn cocaine-paired cues in both Paired and Unpaired groups.

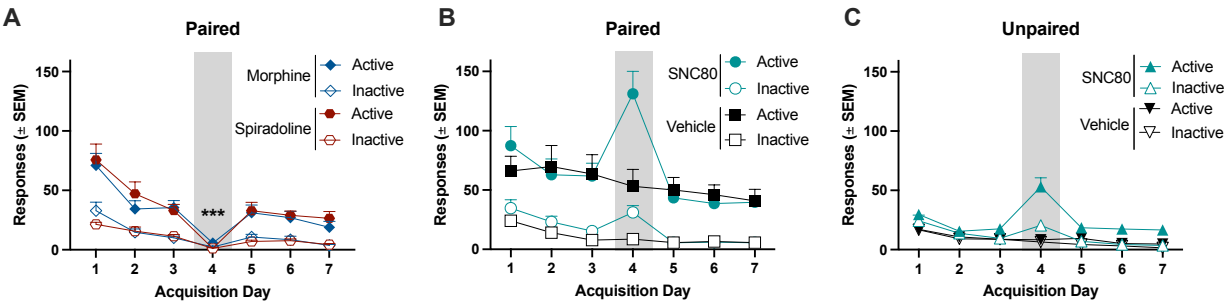

Figure 4.3 DOR agonists increase responding for cocaine-paired cues. A) Both morphine (3.2 mg/kg sc) and spiradoline (1.0 mg/kg sc) reduced active responding but had no effect on inactive responding. B) Acute administration of SNC80 (3.2 mg/kg sc) increases active responding for cocaine-paired cues to a greater extent than inactive responding. C) SNC80 also enhanced active responding on ACQ4 in subjects that underwent Unpaired Pavlovian Conditioning.

4.4.4 Other DOR agonists stimulate responding for cocaine-paired cues through a DOR selective mechanism

To further demonstrate that SNC80 increases responding for cocaine-paired cues through DOR activation, pretreatments of either NTI (3.2 mg/kg) or NTB (0.32 mg/kg) were given acutely 30 min prior to SNC80 (3.2 mg/kg) administration in separate

groups of Paired subjects (Figure 4.4A&C). In Figure 4.4A, both NTI and NTB antagonized the SNC80-induced increase in responding to earn cues, revealed by drug x day (F (3,24) = 15.01, P < 0.0001) and three-way drug x response type x day (F (3,24) = 13.17, P < 0.0001) interactions. Post-hoc tests confirmed SNC80-induced levels of active responding were higher than vehicle treatments on ACQ4 and increased from the day prior (ACQ3), while active responding with NTI or NTB alone was similar to vehicle treatment.

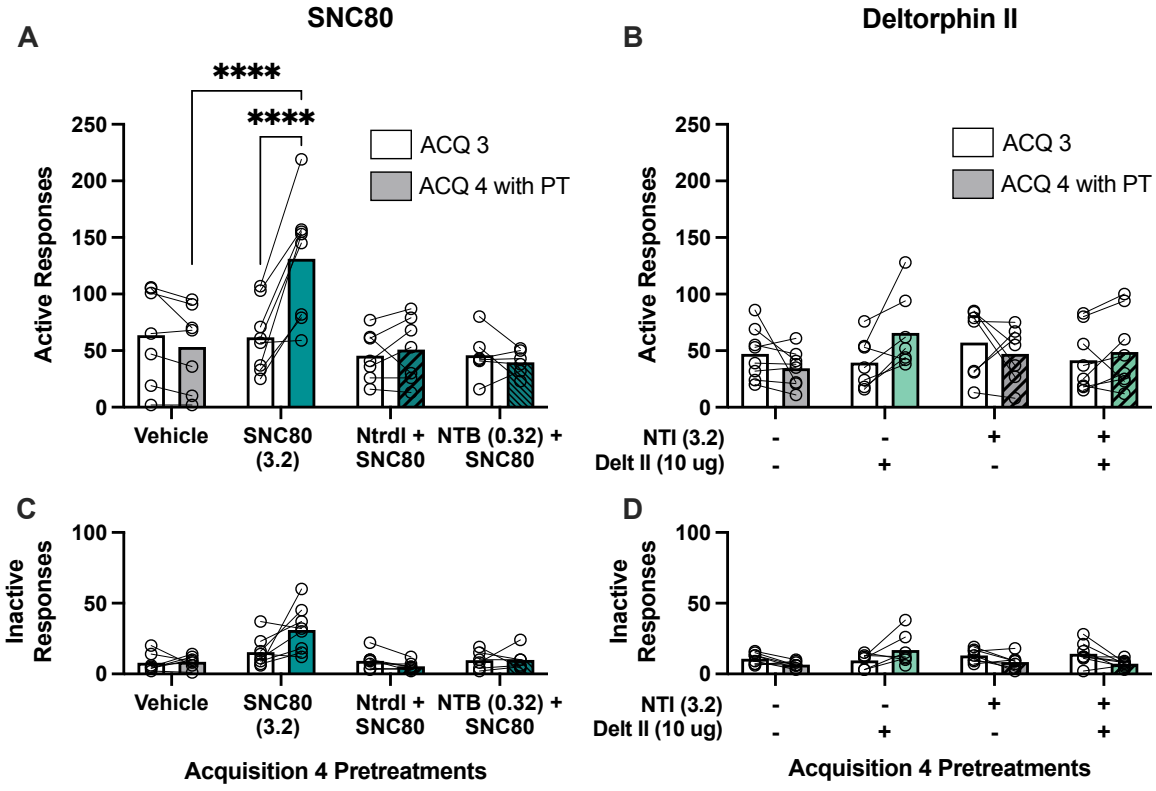

Figure 4.4 The effects of SNC80 and deltorphin II to enhance responding for cocaine-paired cues is mediated via DOR. A) Acute pretreatment of either NTI or NTB blocked the effects of SNC80 to increase levels of active responding. B) Administration of a different DOR agonist, deltorphin II, also increases active responding to earn cocaine-paired cues.

To corroborate these findings, we sought to replicate the results of SNC80 with an additional DOR agonist. Paired groups received acute administration of a peptide DOR agonist, deltorphin II (10 µg) centrally, via ICV injection (Figure 4.4B&D).

Deltorphin II significantly stimulated active responding for cocaine-paired cues without increasing inactive responses, supported by a response type x agonist x day interaction (F (1,26) = 5.885, $P < 0.05$). Levels of active responding following deltorphin II treatment were higher than vehicle on ACQ4, indicated by an agonist x day interaction (F (1,26) = 9.779, $P < 0.01$). Pretreatment of the DOR antagonist NTI (3.2 mg/kg) partially prevented the deltorphin II-induced increase in active responding; however, the effects of NTI were not significant as there was no response type x day x antagonist interaction or a four-way interaction between response type x day x antagonist x agonist.

4.5 Discussion

The opioidergic system has been shown to modulate the ability of cocaine-paired cues to drive drug-seeking behaviors in reinstatement (Tang *et al.*, 2005; Burattini *et al.*, 2008; Simmons and Self, 2009; Gutiérrez-Cuesta *et al.*, 2014). Therefore, the goal of this study was to evaluate the extent to which activation of opioid receptors by endogenous opioid peptides or exogenous opioid ligands modifies the conditioned reinforcing effects of cocaine-paired cues in the New Response Acquisition procedure. Since it is technically difficult to measure endogenous enkephalins *in vivo* while responding for cues (Conway *et al.*, 2022; Rysztak and Jutkiewicz, 2022), we evaluated the role of endogenous opioid peptides in two ways: 1) administration of OR antagonists and 2) preventing the breakdown of endogenous enkephalins by administration of an enkephalinase inhibitor. In the current study, cocaine elicited conditioned reinforcing effects such that cues delivered simultaneously with cocaine infusion during Pavlovian Conditioning supported new learning of an operant response to produce cues alone, similar to previous works (Bertz and Woods, 2013; Bertz *et al.*, 2015, 2016; Robertson

and Jutkiewicz, 2020, 2021). Inhibiting opioid receptors with naltrexone or DORs with RTI-25 attenuated responding for cues in this procedure, suggesting endogenous opioid peptides may be involved. Further, activating DORs, either via protected enkephalins or via exogenous agonists, potentiated the conditioned reinforcing effects of cocaine-paired cues, as demonstrated by increased levels of active responding. Overall, these data suggest that following formation of cocaine+cue associations, the endogenous opioid system may be responsible for mediating behavior increased by cocaine-paired cues.

Repeated treatment of the antagonist NTX appeared to slightly reduce active responding for cues (Figure 4.1A), presumably by blocking endogenous activation by endogenous opioid peptides. Previous work has shown a reduction in cue-induced reinstatement following opioid receptor blockade or knockout (Burattini *et al.*, 2008; Gutiérrez-Cuesta *et al.*, 2014), supporting that opioid receptor activation can influence behavioral responding maintained by cocaine-paired cues. NTX did not robustly decrease responding; however, it is possible that administration of NTX on an earlier Acquisition session may have had a greater effect in blocking responding for cues. The data in the current study suggest that endogenous opioid peptides possibly play a role in maintaining responding for drug-paired cues.

Prior work has implicated a role of DORs in mediating responding for cues and drug-seeking behaviors (Phillips *et al.*, 1994; Simmons and Self, 2009; Gutiérrez-Cuesta *et al.*, 2014); therefore, we sought to evaluate if selectively antagonizing DORs would reduce responding. DOR antagonists were administered prior to ACQ and yielded mixed results. RTI-25 has been shown to be a potent DOR antagonist *in vitro* as well *in*

vivo (Jutkiewicz *et al.*, 2007; Carroll and Dolle, 2014) and reduced active responding following acute administration, while NTI and NTB did not (Figure 4.1B). The doses of NTI and NTB were chosen because these are considered DOR-selective doses *in vivo*; therefore, we did not use larger doses, which may have been more effective. Opioid antagonists were not given to Unpaired groups (controls) because levels of active responding were low (Figure 4.3). The cue did not develop conditioned reinforcing properties in Unpaired groups; therefore, responding is not high enough for antagonists to presumably suppress.

Therefore, to further test if levels endogenous opioid peptides influence the conditioned reinforcing properties of cocaine-paired cues, we administered an enkephalinase inhibitor to prevent breakdown of enkephalins. Acute RB101 robustly increased responding for cocaine-paired cues as compared with vehicle (Figure 4.2A&B) and was antagonized by pretreatment of NTI (3.2 mg/kg). Protected enkephalins alone were able to enhance cocaine conditioned reinforcement via a DOR dependent mechanism. It is important to note; however, that different endogenous opioid peptides may contribute to similar behaviors in overlapping functions, therefore additional peptides, such as beta-endorphin may also be engaged in this behavior (Simmons and Self, 2009; Rysztak and Jutkiewicz, 2022). Beta-endorphin is sensitive to APN degradation; therefore, it is possible that RB101 directly protects beta-endorphins (Noble *et al.*, 2008) and/or enkephalins, which contribute to responding for cues. Future work is required to determine relative roles of beta-endorphin and enkephalins, particularly either met- or leu-enkephalins, by measuring peptides *in vivo* during conditioning and while responding for cues. Overall, the effects of NTX and RB101 in

this behavior implicate involvement of endogenous opioid peptides potentially acting via DORs.

Because opioid peptides bind to multiple opioid receptor types, we sought to determine if selective activation of each receptor type would increase responding, mimicking the effects of protected opioid peptides. The DOR agonists SNC80 and deltorphin II increased the conditioned reinforcing properties of cocaine-paired cues (Figure 4.3 & 4.4). While we hypothesized that activation of DORs would increase responding for cues in Paired groups selectively, it is possible that SNC80 increased responding in the Unpaired controls by potentiating weak conditioned reinforcing properties of cues (Robertson and Jutkiewicz, 2020, 2021). The Unpaired Pavlovian Conditioning is meant to control for learning the association between cocaine and cue; however, some cue presentations certainly overlapped with the physiological effects of cocaine. Additionally, cues were delivered in the same context (operant chamber) as cocaine delivery. Together, the Unpaired paradigm does not completely separate cue presentations from cocaine delivery. Therefore, it is not surprising that SNC80 increased active responding for cues in the Unpaired group and indicates that DOR activation is sensitive enough to promote responding for cues with weak conditioned reinforcing properties. Another rationale for SNC80-induced increases in responding in Unpaired groups and in inactive responding in Paired groups may be increased generalized locomotor activity (Jutkiewicz *et al.*, 2008). However, it is unlikely that SNC80-induced increases in active responding for cues in both Paired and Unpaired groups is due to the locomotor stimulating effects of DOR agonists because: 1) RB101 does not stimulate locomotor activity at 10 mg/kg iv (Jutkiewicz *et al.*, 2006) but did

induce active responding for cocaine-paired cues in the current study, 2) the dose of deltorphin II is similar to a dose that stimulated locomotor activity (Longoni *et al.*, 1991) yet deltorphin II selectively increased active responding, and 3) prior work administered psychostimulants at doses that increase generalized locomotion, yet they did not alter responding for cocaine-paired cues in this paradigm, as described Chapter 3. Overall, these data support that activation of DORs, presumably by enkephalins, potentiates cocaine conditioned reinforcement.

Alternatively, both the MOR agonist morphine and KOR agonist spiradoline attenuated responding for cues (Figure 4.3). MOR agonists have been shown to induce cocaine reinstatement and augment reinforcement (de Wit and Stewart, 1981; Stewart *et al.*, 1984; Phillips *et al.*, 1994; Tang *et al.*, 2005), yet in our study morphine had an opposite effect, such that responding maintained by cues was robustly decreased following acute morphine administration. The effects of morphine and spiradoline on attenuating responding for cues are likely due to behavioral disruption and/or sedation. The doses of morphine and spiradoline were chosen based on their ability to produce discriminative stimulus effects (Holtzman *et al.*, 1991; Holtzman, 2000; Hutcheson *et al.*, 2000) while minimizing undesirable effects, such as rate suppression, although these effects can still occur. Therefore, more work needs to be done to better understand a role, if any, of MORs and KORs in cocaine conditioned reinforcement in this assay.

The neurobiological processes by which a conditioned reinforcer can elicit behavior still requires further exploration. Dopamine levels in the nucleus accumbens (NAc) are thought to support learning the association between primary reinforcers and stimuli (Schultz, 2006; Flagel *et al.*, 2011) and augmenting the dopaminergic system can

induce responding for cocaine-paired cues in reinstatement procedures (Park *et al.*, 2002; Cervo *et al.*, 2003; Saunders *et al.*, 2013); however, altering dopamine levels during ACQ did not potentiate cocaine conditioned reinforcement in the New Response Acquisition procedure (Chapter 3). In the current study, morphine, which has been shown to indirectly increase dopamine in the NAc (Johnson and North, 1992), reduced responding for cues. Together, these findings suggest increased extracellular dopamine alone is not sufficient to alter expression of cocaine conditioned reinforcement, but further work will be required to better understand potential opioidergic-dopaminergic interactions in this behavior.

Further, endogenous opioid peptides in multiple different brain regions may be involved in the conditioned reinforcing effects of cocaine-paired cues. Intra-NAc administration of various opioids peptides potentiated reinstatement behaviors (Phillips *et al.*, 1994; Simmons and Self, 2009). The ventral pallidum has also been proposed to drive drug-seeking behaviors in an opioid dependent manner, such that selective stimulation facilitates, while local administration of MOR antagonists block, cocaine reinstatement (Tang *et al.*, 2005; Kupchik *et al.*, 2014; Heinsbroek *et al.*, 2017, 2020). Overall, the opioidergic system may modulate conditioned reinforcement via interactions with other neurotransmitters systems in addition to the dopaminergic system, such as the cholinergic system (Bertran-Gonzalez *et al.*, 2013; Laurent *et al.*, 2014), within multiple brain regions in the mesolimbic dopamine pathway.

Our results support the activation of DORs may contribute to the conditioned reinforcing properties of cocaine-paired cues as measured in the New Response Acquisition paradigm. Protected enkephalins increased responding to earn cues via a

DOR dependent mechanism; however, in this study administration of either NTI or NTB alone did not reduce responding. Therefore, more work needs to be done to determine whether there are neurobiological changes in enkephalin peptide release or in DOR expression/function following Paired Pavlovian Conditioning to better contextualize our results. To that end, there may be differences in levels of enkephalins between Paired and Unpaired groups in reward circuitry. Increased enkephalin concentrations in Paired groups may contribute to the enhanced the behavioral expression of cocaine conditioned reinforcement. Future studies should directly measure enkephalins during tests of cocaine conditioned reinforcement; however, technical limitations render *in vivo* enkephalin measurements difficult (Conway *et al.*, 2022; Rysztak and Jutkiewicz, 2022). Overall, these studies highlight the DOR system as a crucial modulator of the reinforcing properties of cocaine-paired cues and future studies will continue to evaluate these mechanisms to provide novel insight for treatments to prevent relapse.

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Chapter 5
Discussion

The objective of this dissertation was to investigate the neurobiological mechanisms underlying the conditioned reinforcing properties of cocaine-paired cues. Specifically, the dopaminergic and opioidergic systems were evaluated using multiple neurochemical and pharmacological methods to determine relative roles in cocaine conditioned reinforcement. Data discussed in this dissertation demonstrate that the conditioned reinforcing properties of cues may be mediated via opioidergic, but not dopaminergic, mechanisms in the New Response Acquisition procedure.

The opioidergic system has been shown to mediate cue-controlled responding such that administration of an enkephalinase inhibitor to protect enkephalin levels or administration of delta opioid receptor agonists induced cocaine reinstatement (Simmons and Self, 2009), suggesting the DOR system potentially regulates conditioned reinforcement. Therefore, we hypothesized that enkephalins acting via DORs modulate responding for cocaine-paired cues in the New Response Acquisition procedure (Phillips *et al.*, 1994; Burattini *et al.*, 2008; Simmons and Self, 2009; Gutiérrez-Cuesta *et al.*, 2014). Our original goal was to measure levels of endogenous opioid peptides, specifically met-enkephalin, *in vivo* in the nucleus accumbens (NAc) of subjects responding to earn presentations of cocaine-paired cues. Unfortunately, technical limitations left us unable to reliably measure enkephalins *in vivo*. Therefore, we probed the role of endogenous opioid peptides and opioid receptors by

pharmacological manipulation of these targets. Part of the original study design was to measure dopamine as a positive control in addition to enkephalin. We expected dopamine concentrations to be increased in the NAc of subjects that underwent Paired Pavlovian Conditioning while responding to earn cocaine-paired cues, based off of numerous studies have shown that dopamine concentrations in the NAc are associated with reinstatement (Weiss *et al.*, 2000; Owesson-White *et al.*, 2008; Sunsay and Rebec, 2014) and that activating the dopaminergic system pharmacologically can enhance responding for cues (Park *et al.*, 2002; Lu *et al.*, 2004; Bossert *et al.*, 2013). Our results were contrary to our initial hypotheses. Dopamine concentrations in either the NAc shell or core were not associated with responding for cues during Acquisition. We investigated the role of dopamine further by pharmacological manipulation via administration of indirect agonists and neither intra-NAc nor systemic cocaine altered behavior. Together, these data support that dopamine is not a driver of responding maintained by cocaine-paired cues in this procedure. Interestingly, a handful of studies have shown that dopamine is not increased despite high levels of responding for drug-paired cues (Neisewander *et al.*, 1996; Bradberry *et al.*, 2000; Ito *et al.*, 2000; Di Ciano *et al.*, 2001), which highlight the complexity of dopamine in regulating these behaviors and are consistent with our results.

These findings led us to investigate the opioidergic system in cocaine conditioned reinforcement using pharmacological methods. Consistent with our hypotheses, activation of the DOR system potentiated the conditioned reinforcing effects of cocaine-paired cues and increase active responding for cues. It is important to note that all of the work discussed in this dissertation was conducted in male rats;

therefore, interpreting these results is limited to one sex and future work will need to substantiate these findings by investigating conditioned reinforcement in females, as sex differences in behaviors to earn drug-paired cues have been observed (Bertz *et al.*, 2016). Overall, the work completed for this dissertation has added novel insight into the mechanisms by which cocaine-paired cues elicit and maintain behavior in New Response Acquisition procedures.

5.1 Evaluation of the dopaminergic system in cocaine conditioned reinforcement

One potential explanation for the lack of effect of dopamine in the current work is the time resolution of the technique used to evaluate dopamine concentrations. Microdialysis is often used to measure volumetric changes in concentrations rather than to detect small, phasic fluctuations in dopamine concentrations. Other techniques, such as voltammetry, have been used to elucidate dopamine dynamics on a rapid timescale and are useful for time-locking dopamine to specific behaviors (Phillips *et al.*, 2003; Dalley *et al.*, 2005; Day *et al.*, 2007; Owesson-White *et al.*, 2008; Sunsay and Rebec, 2008; Aragona *et al.*, 2009; Flagel *et al.*, 2011; Mohebi *et al.*, 2019). Voltammetry and microdialysis data seem to complement each other, such that phasic increases in dopamine correlate with increased volumetric concentrations in dialysate (Vander Weele *et al.*, 2014; Hamid *et al.*, 2016). We chose microdialysis in order to be able to measure other neurotransmitters simultaneously, and previous work suggested this method would be sufficient to reliably detect differences in dopamine concentrations in these types of behavioral procedures (Weiss *et al.*, 2000). Further, studies that demonstrated dopamine is not correlated with responding for cues used smaller timescales and found results consistent with the current work. In Bradberry et al., 2000,

dopamine was measured via microdialysis in two-min bins, having improved time resolution from 10-min bins, but dopamine was not elevated while animals responded for drug-paired cues alone. Further, phasic changes in dopamine were evaluated in cue-induced reinstatement using chronoamperometry and dopamine efflux was not altered in the NAc despite responding for cues (Di Ciano *et al.*, 2001). After initial experiments, we sought to increase the temporal resolution by measuring dopamine in two-min bins during Acquisition and preliminary results suggested that dopamine was still not altered from baseline while responding for cues on this timescale (Figure 5.1).

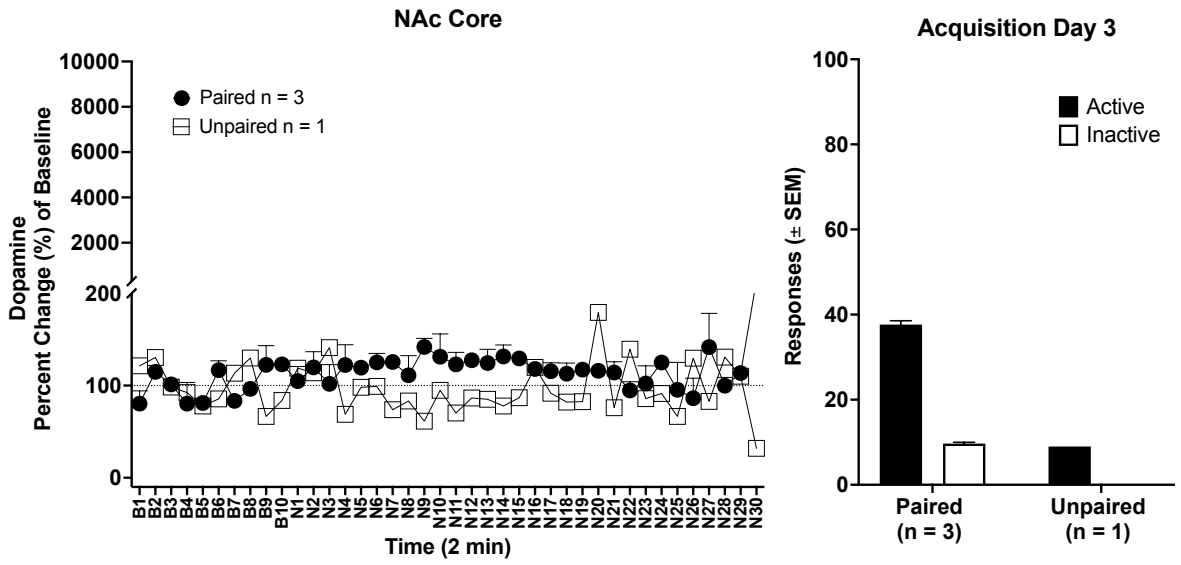

Figure 5.1 Dopamine concentrations in the NAc core measured in two-min bins via microdialysis. Preliminary data show that while subjects in the Paired group responded more than the one subject in the Unpaired group, dopamine levels were not robustly different between conditioning types. B = baseline measurements N = New Response Acquisition measurements during ACQ3

Another explanation for our results is that dopamine levels could be associated with responding to earn presentations of cocaine-paired cues during Acquisition but in brain regions other than the NAc, and future studies could examine this via microdialysis. The amygdala has been shown to mediate the formation of cue+drug associations and also responding for cues during reinstatement behaviors (Weiss *et al.*,

2000; See *et al.*, 2003; See, 2005; Berglind *et al.*, 2006). Dopamine in the amygdala (Weiss *et al.*, 2000; Berglind *et al.*, 2006) may be influential for responding for cues in this paradigm, but other neurotransmitters such as acetylcholine (Squire and Davis, 1981; See *et al.*, 2003; See, 2005) have been implicated in these behaviors and could be evaluated as well. The ventral pallidum (Smith *et al.*, 2009) is also a region of interest in reward-related behaviors and is directly downstream of D2-expressing medium spiny neurons (MSNs) projecting from the NAc (Figure 1.2). Therefore, investigating neurotransmitter dynamics within the ventral pallidum during Acquisition may provide new insight into the neurobiological differences between Paired Pavlovian Conditioning and responding for cues as compared with control conditions.

Consistent with the current data, there are multiple studies that do not show robust increases in dopamine concentrations in the mesolimbic pathway despite increased operant responding for cues (Neisewander *et al.*, 1996; Bradberry *et al.*, 2000; Ito *et al.*, 2000; Di Ciano *et al.*, 2001). One possible explanation for differential roles of dopamine across assays may arise from individual differences in responding for cues. For example, motivational aspects of cues are dependent on dopamine in some individuals but not others (Flagel *et al.*, 2011). Our findings observed individual differences in levels of responding, such that systemic cocaine and amphetamine administration increased responding for cocaine-paired cues in some rats (n=2 and n=1, respectively) and further, baseline dopamine concentrations in the NAc core of some subjects were correlated with higher preferences scores during Acquisition (Chapter 3). This may indicate there are individual differences in the role of dopamine in the conditioned reinforcing effects of cocaine and future work should continue to

characterize individual differences in this procedure. For example, within Paired groups, subjects the highest levels of responding may have increased breakpoints to earn cues alone on a progressive ratio schedule as compared with average responders. These behaviors are indicative of motivation (Gutiérrez-Cuesta *et al.*, 2014) and may be sensitive to alterations in the dopaminergic system, which would suggest there are neurobiological differences in the dopamine system between individuals that are high responders and average responders. Future experiments to parse apart the neurobiological differences in individuals with the highest levels of responding for cues may give more insight into how the strength of the conditioned reinforcing effects of drug-paired cues is encoded.

In assays of conditioned reinforcement, manipulation of the dopaminergic system in altering cue-maintained behaviors provides additional information about whether or not dopamine drives the reinforcing properties of drug-paired cues. Therefore, we administered pretreatments of cocaine and amphetamine and monitored changes in responding. While we originally predicted that cocaine would increase responding for cues at similar doses that induce reinstatement (Park *et al.*, 2002; Lu *et al.*, 2004), neither local or systemic cocaine, nor amphetamine, altered responding for cues as compared with vehicle treated rats. This finding was surprising given the robust literature showing cocaine reliably induces reinstatement and can elevate responding maintained by cocaine-paired cues alone. We confirmed that systemic cocaine (10 mg/kg ip) did not alter responding in an Unpaired control group (Figure 5.2D), indicating that that dose of cocaine did not alter responding for cues that should not have

conditioned reinforcing properties, which might have been due to undesirable effects such as locomotor stimulation.

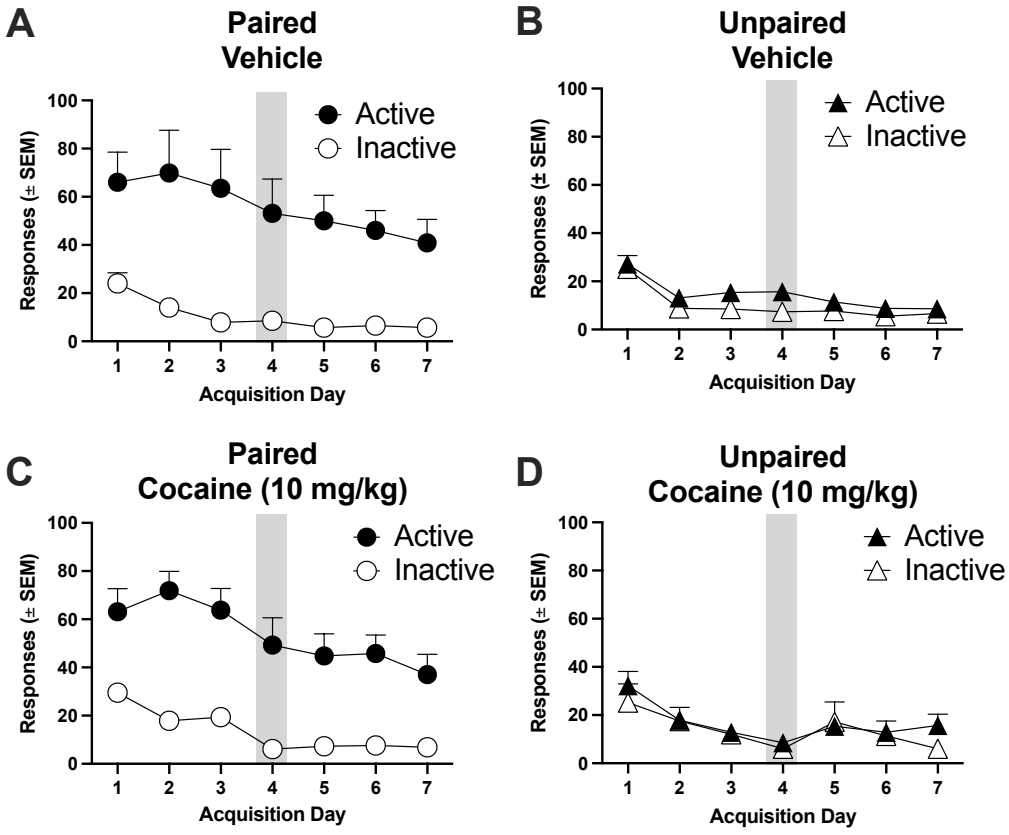

Figure 5.2 Acute systemic cocaine administration (10 mg/kg ip) did not alter responding in explicitly Unpaired (control) groups (n=8/group). All groups earned cues under a RR2 schedule of reinforcement. Cocaine was administered on ACQ4 (gray vertical bar) immediately prior to the start of the session.

Because the lack of effect of cocaine administration on responding for cocaine-paired cues was unexpected, we sought to further investigate if and how cocaine might alter responding for cues by administering it repeatedly. Cocaine (18 mg/kg ip) was given repeatedly across four Acquisition sessions in Paired groups that earned cues either via RR2 or FR1 schedules of reinforcement. In Figure 5.3, cocaine reduced responding on the first administration in RR2 trained subjects, which is different than what we had observed previously (Chapter 3 Figure 3.7). In the prior experiment, acute cocaine (18 mg/kg) reduced responding in a subset of Paired subjects trained on RR2

(n=3) and in the majority of rats trained on FR1 (n=7). The finding in Figure 5.3 is preliminary, with a small n; however, it is possible these subjects are more similar to the subset of animals in which cocaine had reduced responding previously. With repeated administration, the effect of cocaine on reducing responding on an FR1 schedule appears sustained. Overall, cocaine was unable to potentiate responding for cues after repeated administration regardless of schedule of reinforcement, further suggesting dopamine is not mediating cocaine conditioned reinforcement as measured by the New Response Acquisition procedure, contrasting reinstatement literature.

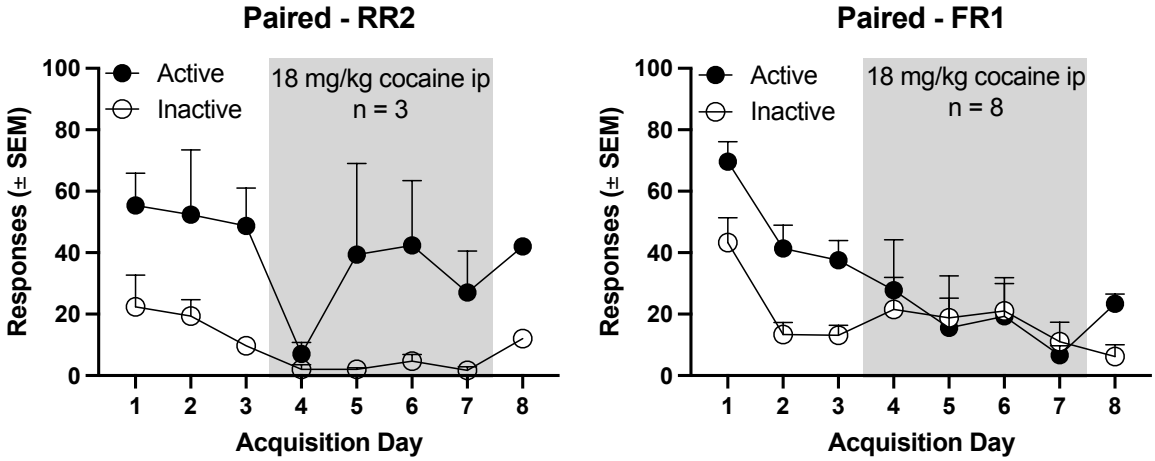

Figure 5.3 Repeated administration of cocaine (18 mg/kg) prior to Acquisition in which cues were earned either via RR2 or FR1 schedules of reinforcement.

While administration of indirect agonists did not potentiate the conditioned reinforcing effects of cocaine-paired cues in this procedure, previous studies evaluated conditioned reinforcement using direct dopamine agonists to alter behavior, such that agonists can potentiate responding for cues in similar paradigms to New Response Acquisition (Cador *et al.*, 1991; Wolterink *et al.*, 1993; Phillips *et al.*, 1994). In Bertz et al., 2015, the D2/D3 agonist pramipexole selectively increased active responding for remifentanil-associated cues in a similar procedure; however, this effect was delayed

and was observed following 6 days of repeated administration (Bertz *et al.*, 2015). We continued to evaluate this phenomenon by administering various dopamine receptor agonists acutely to rats in Paired groups to determine if responding would be altered, similar to the studies described above and in reinstatement literature. We administered the D2 agonist quinpirole (0.56 mg/kg sc), which has been shown to elevate responding for cocaine-paired cues alone (Collins and Woods, 2009), in rats that underwent Paired Pavlovian Conditioning. Interestingly, quinpirole substantially reduced active responding for cocaine-paired cues in preliminary results (Figure 5.3). It was observed that quinpirole administration seemed to be behaviorally suppressive, such that rats were visibly lethargic. The reduction in responding following quinpirole appears to be consistent with Bertz, et al., 2015, such that upon the first administration of pramipexole responding is decreased. These findings are distinct from reinstatement studies that show indirect or direct dopamine agonists can stimulate responding for cues upon the first administration, further suggesting these two behavioral procedures recruit different neurobiological processes. Subjects may become tolerant to the rate suppressing effects of D2 agonists following repeated administration, and this could be studied further in New Response Acquisition. Further, we evaluated how administration of the D1 agonist SKF-81297 would influence responding for cues in rats in Paired groups. D1 agonists have been shown to increase responding for water- (Wolterink *et al.*, 1993) or sucrose-paired cues (Phillips *et al.*, 1994) and induce cocaine reinstatement (Bachtell *et al.*, 2005), although in certain paradigms administration of D1 agonists has reduced cue-induced reinstatement (Alleweireldt *et al.*, 2002). During Acquisition, SKF-81297 (1.0 and 3.2 mg/kg) slightly reduced active responding for cues in preliminary results (Figure

5.4). Again, these results were surprising because previous literature suggested that D1 agonists would increase responding for cues in similar assays. Together, these preliminary data using direct dopamine agonists may suggest that dopamine may be involved in responding for cues in the New Response Acquisition procedure; however, potentially as a negative regulator. Agonists may alter the balance between D1 vs. D2-MSN output (McGinty, 2007; Clark and Bracci, 2018) to modify responding for cues. Moreover, the preliminary findings are likely due to behavioral suppression which would need to be addressed in this assay in future studies. Overall, the mechanisms by which D2 or D2/D3 agonists alter behavior for cues may be different between reinstatement and conditioned reinforcing behaviors, supporting the idea that the underlying neurobiology is functionally distinct.

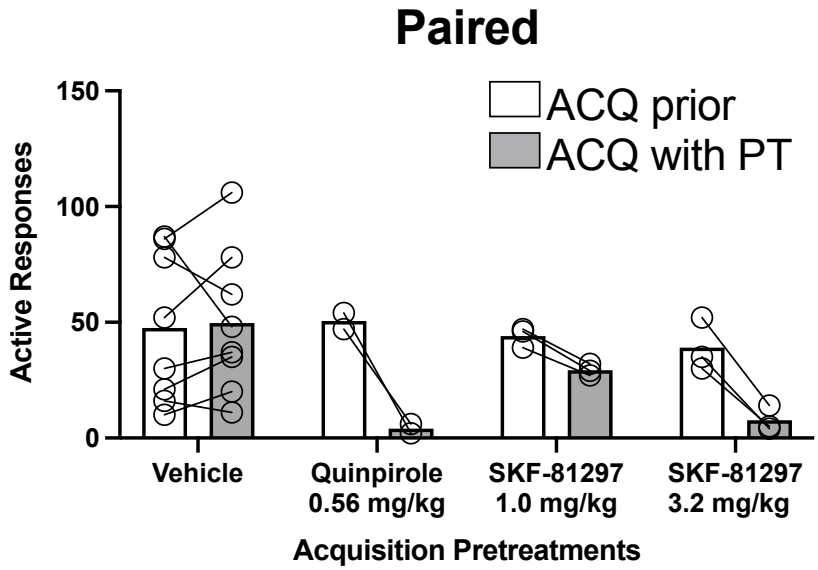

Figure 5.4 D1 or D2 agonists attenuated responding for cocaine-paired cues in New Response Acquisition when given as acute treatments prior to Acquisition.

One possibility for the lack of effect of cocaine pretreatments in the current work is that we administered cocaine, amphetamine, or D1 or D2 agonists on the fourth day of Acquisition, after the instrumental response to earn cues had already been acquired.

Previous studies demonstrating indirect or direct dopamine agonists increase responding for conditioned reinforcers often administer agonists on the first exposure to the novel manipulandum (Taylor and Robbins, 1984, 1986; Cador *et al.*, 1991; Phillips *et al.*, 1994; Bertz *et al.*, 2015). It is possible that early administration of indirect agonists enhance dopamine concentrations to facilitate the learning of the instrumental response to produce cues, and therefore, potentiate responding (Salamone, 1992; Schultz, 2006). This could be tested in by measuring dopamine concentrations or administering systemic cocaine on the first day of Acquisition using the current paradigm. If responding for cues is higher with cocaine treatment on ACQ1 as compared with vehicle treated Paired groups, this would suggest dopamine is important for learning the operant response and/or the association between operant response and cue presentation. While instrumental learning is an important aspect of the behavior to earn cues during Acquisition, it does not necessarily explain the ability of cues alone to maintain behavior for extended periods of time once the response is learned.

Another explanation for the lack of effect of dopamine on cocaine conditioned reinforcement in this procedure is drug-paired cues acquire different properties depending on the assay used and recruit distinct dopaminergic neurobiology. The function of the cue in New Response Acquisition is distinct from that during reinstatement assays in which the cue likely has motivational value. Apart from learning, dopamine has other crucial functions such as encoding motivation (Robinson and Berridge, 2001; Berridge, 2007; Peciña and Berridge, 2013). Motivation contributes to drug-seeking behaviors as measured by cue-induced reinstatement. The cue during reinstatement may act as a predictive or discriminative stimulus to potential drug

availability, driving reinstatement of responding. Therefore, reinstated responding is complex, and may not be to earn the cue alone but for potential drug. The differences in the role of dopamine between reinstatement and New Response Acquisition procedures is likely due to the history of contingent drug self-administration.

Therefore, the contingency of drug and cue delivery potentially recruits the dopaminergic system differentially during subsequent tests of conditioned reinforcement. This can be examined using New Response Acquisition procedures that utilize contingent drug-delivery prior to learning a different, novel operant response for cues. The behavior to earn cues during Acquisition appears to be the similar between animals with a history of contingent vs. non-contingent drug delivery; however, the neurobiological substrates in driving this behavior may be functionally distinct. Cocaine infusion elicits phasic dopamine increases in the NAc regardless of contingency, yet when cocaine is delivered contingently, dopamine signals are time-locked to the operant response (Stuber *et al.*, 2005). Therefore, cues may develop stronger reinforcing properties when delivered contingently due to potentially greater recruitment of the dopaminergic system in encoding the association between cue and cocaine. Indeed, pharmacologically manipulating the dopaminergic system with indirect agonists increased responding on a novel manipulandum for conditioned reinforcers in animals with a history of self-administration (Di Ciano, 2008). One potential explanation for a role of dopamine in responding for conditioned reinforcers following contingent drug delivery is that the function of the cue in Acquisition may include motivational aspects due to general overlap between an instrumental response and drug delivery. In Paired groups that received drug noncontingently, via Pavlovian Conditioning, cocaine-paired cues

reinforce active responding without the presumption that drug may be delivered via that response. These distinct functions of drug-paired cues may support differences in underlying dopaminergic mechanisms, such that conditioned reinforcing properties alone are not completely dependent on dopamine.

Overall, investigating the role of the dopaminergic system in conditioned reinforcement includes methods to measure dopamine and associate it with behavior or to modulate dopamine and determine its relationship in driving behaviors maintained by drug-paired cues. The latter can be done via pharmacological methods as well as more selective methods such as optogenetics or chemogenetics in future studies. It is important to note that each of these measures has caveats, for example stimulation of specific neurons with optogenetics releases all of the contents of the cell, not dopamine selectively. Therefore, these measures are most informative when combined to ideally demonstrate similar or corroborative findings. Moreover, there should be a match between measured dopamine concentrations and manipulation of dopamine in cue-maintained behaviors. For example, recent work used optogenetics to selectively inhibit VTA dopamine neurons during presentation of a sucrose-paired cue and observed reductions in conditioned cue approach behavior (Iglesias *et al.*, 2023), corroborating earlier studies associating dopamine with the behavior (Flagel *et al.*, 2011). When investigating the underlying neurobiology of cue-controlled behaviors in measures of conditioned reinforcement using these techniques, it is important to distinguish the function of the cue in the behavioral assay such that the underlying neurobiological processes are likely distinct between functions. In conclusion, assays in which drug-

paired cues acquire conditioned reinforcing properties selectively can be used to elicit robust behavior maintained by cues but in a dopamine-independent manner.

5.2 Evaluation of the opioidergic system in cocaine conditioned reinforcement

The endogenous opioid system has been shown to play a role in regulating the reinforcing properties of cocaine-paired cues (Phillips *et al.*, 1994; Burattini *et al.*, 2008; Simmons and Self, 2009; Gutiérrez-Cuesta *et al.*, 2014; Mongi-Bragato *et al.*, 2018; Rysztak and Jutkiewicz, 2022). Chapter 4 of this thesis determined whether endogenous enkephalins and DORs were involved in responding for cues by administering various pharmacological treatments during Acquisition. The enkephalinase inhibitor RB101 acutely enhanced responding for cocaine-paired cues, and this effect was mediated via DORs. Further, acute activation of DORs via agonist administration promoted responding for cues. Overall, these findings have added novel insight into the ability of the DOR system to influence the conditioned reinforcing properties of cocaine-paired cues in this procedure.

The endogenous opioid peptides enkephalins may mediate the ability of cues to elicit and maintain behavior. Therefore, our original hypothesis was that levels of enkephalins would be elevated in the NAc of animals that underwent Paired Pavlovian Conditioning as compared with Unpaired control animals while responding for cues. Unfortunately, we were unable to measure enkephalins *in vivo* using microdialysis due to technical limitations (Al-Hasani *et al.*, 2018; Conway *et al.*, 2022). We sought alternative methods to measure enkephalins using commercially available ELISA kits. Using two different met-enkephalin ELISA kits, results from brain tissue homogenate samples were not reliable. Samples were diluted, yet the resulting met-enkephalin

concentrations did not match dilution factors (Figure 5.5). Thus, we investigated the enkephalinergic system during Acquisition via enkephalinase inhibitors.

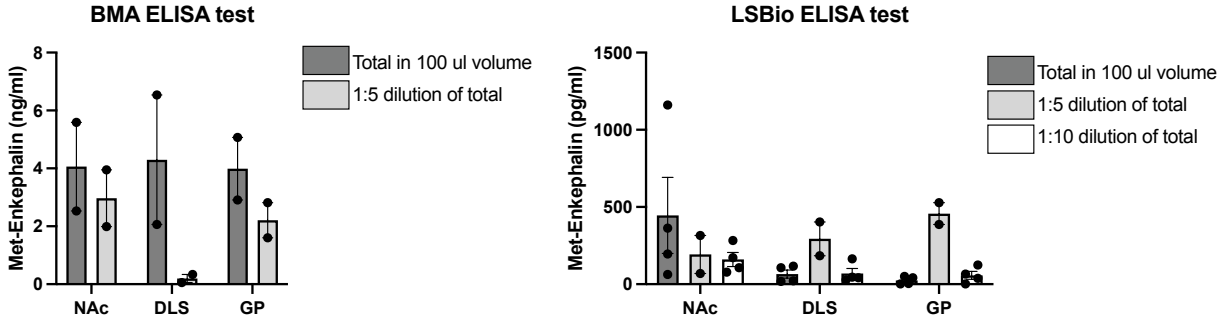

Figure 5.5 Met-enkephalin measurements of brain tissue homogenate using commercially available ELISA kits. Neither kit reliably measured dilutions of total met-enkephalin.

Enkephalinase inhibitors are an indirect way to manipulate endogenous enkephalin levels. We administered the dual enkephalinase inhibitor RB101, which inhibits both the aminopeptidase N (APN) and neutral endopeptidase (NEP) enzymes to prevent the cleavage and degradation of endogenous enkephalins (Jutkiewicz *et al.*, 2007; Jutkiewicz and Roques, 2012; Roques, 2018). RB101 (10 mg/kg) acutely enhanced responding for cocaine-paired cues and in separate groups of Paired rats this effect was blocked by the DOR antagonist naltrindole (3.2 mg/kg). Unfortunately, we had limited amount of compound; therefore, we were unable to test the effects of RB101 in control groups of animals that underwent explicitly Unpaired Pavlovian Conditioning. While we predict that RB101 administration would selectively enhance the conditioned reinforcing properties of cues in Paired groups, it is possible that RB101 would also slightly/moderately enhance (to a lesser extent) responding for cues in Unpaired groups, as seen with the DOR agonist SNC80. This would indicate that RBR101 either 1) enhances even weak conditioned reinforcing properties of unpaired cues or 2) increases responding for cues in general, not specific to drug-paired cues. The latter

result would be a caveat to our results suggesting enkephalins mediated conditioned reinforcement specifically.

One caveat to using enkephalinase inhibitors as a tool to investigate enkephalins in behavior is that these compounds are not necessarily selective for enzymes breaking down enkephalins alone. Enkephalinase inhibitors may bind to other enzymes and prevent the breakdown of other peptides, such as cholecystokinin or substance P (Rysztak and Jutkiewicz, 2022). Early experiments for this dissertation used a water-soluble enkephalinase inhibitor, PL37, as a tool for protecting enkephalin concentrations. Despite its preferable solubility, PL37 did not readily cross the blood brain barrier and needed to be administered centrally, via ICV injections. Preliminary studies sought to characterize PL37 in DOR-mediated behaviors, such as locomotor stimulation. In preliminary work, PL37 dose-dependently increased locomotor activity, similar to the locomotor stimulating effects of DOR agonists (Figure 5.6A). The effect was partially blocked by pretreatment of the DOR antagonist naltrindole (3.2 mg/kg sc), suggesting that increased enkephalins are binding to DORs to alter locomotion. Further, the neurochemical profile of PL37 was analyzed to investigate the downstream changes in neurotransmission in the NAc shell following protected extracellular enkephalins. Interestingly, administration of 23 µg of PL37 into the NAc shell robustly enhanced levels of dopamine, acetylcholine, GABA, glutamate, and adenosine from baseline (Figure 5.6B-F). However, pretreatments of naloxone systemically (3.2 mg/kg) or locally into the NAc shell (10 µg) only antagonized the PL37-induced increases in GABA, glutamate, and adenosine levels, but not PL37-induced increases in dopamine and acetylcholine levels. These data suggest that PL37-induced protected peptides alters

neurotransmission in the NAc shell via opioid receptor-dependent and -independent mechanisms. PL37 was further investigated in New Response Acquisition, which we had predicted to be opioid-dependent and enkephalin mediated. In separate experiments, PL37 prior to Acquisition did not reliably increase responding for cocaine-paired cues at any dose tested (Figure 5.6G). It is possible that the lack of effects of PL37 on responding for cues, as compared with the effects of RB101 administration, is due to greater off target increases in dopamine and acetylcholine in the mesolimbic dopamine pathway. This is consistent with our findings from Chapter 3 that dopamine does not mediate responding for cocaine-paired cues in this procedure and that increased dopamine concentrations in the NAc may reduce responding in Paired groups. Overall, while enkephalinase inhibitors are useful tools for implicating endogenous enkephalins in certain behaviors, it is important to note undesirable effects, including increasing levels of other peptides and not altering enkephalin release.

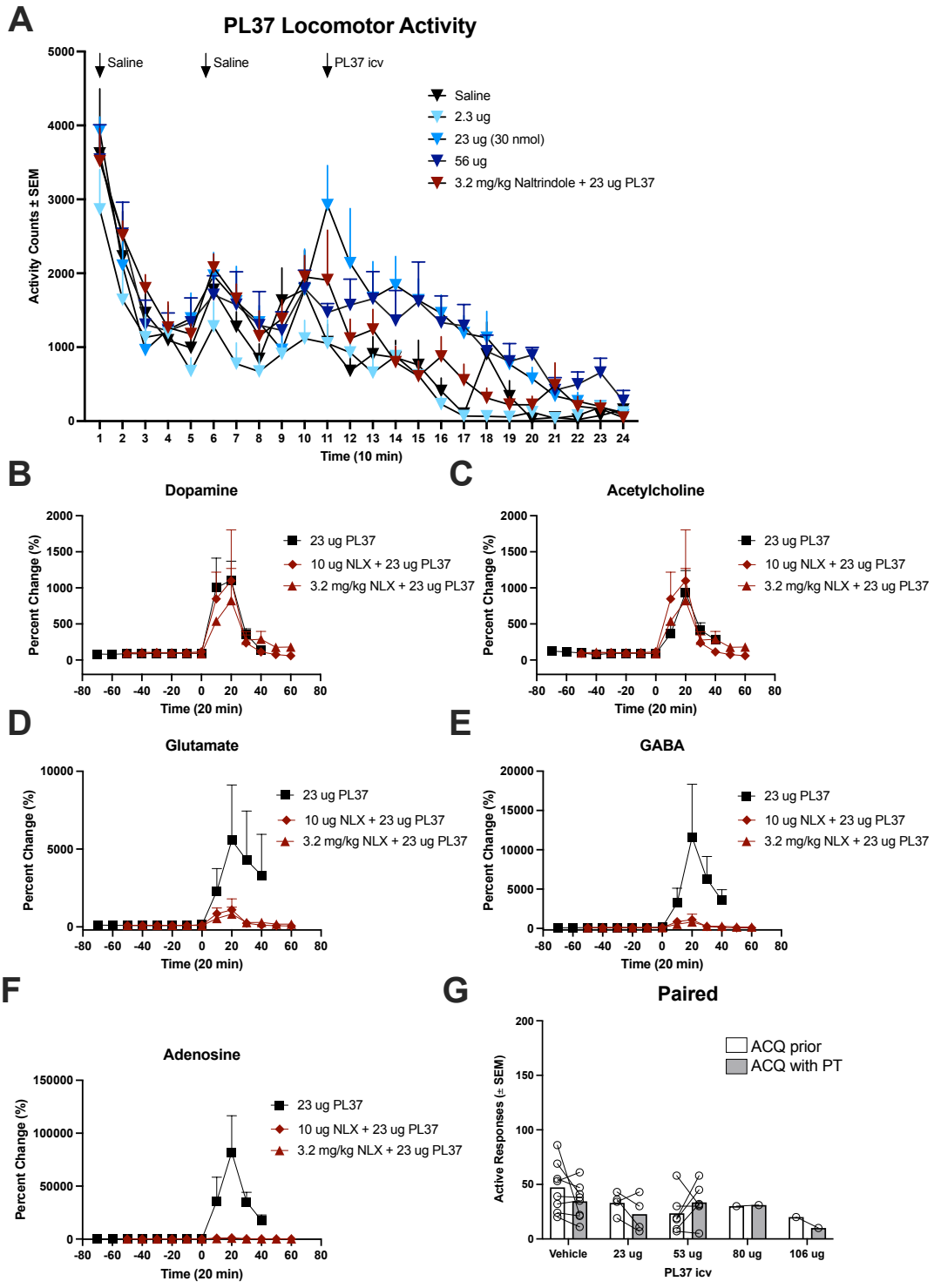

Figure 5.6 Effects of the enkephalinase inhibitor PL37 on neurotransmission, locomotor activity, and responding for cocaine-paired cues. A) PL37 dose-dependently increases locomotor activity, which is partially blocked by naltrindole. B-F) PL37 increases levels of dopamine, acetylcholine, glutamate, GABA, and adenosine in the NAc core. Certain neurotransmitters are increased via opioid receptor-dependent or -independent mechanisms. G) Acute administration of PL37 (ICV) did not enhancing responding to earn presentations of cocaine-paired cues in Paired groups during Acquisition.

Enkephalins preferentially bind to DORs; therefore, we sought to characterize how DOR activation contributes to the ability of cocaine-paired cues to maintain behavior during Acquisition. The nonpeptidic DOR agonist SNC80 and the peptide agonist deltorphin II both increased active responding for cues in Paired groups (Chapter 4). To determine whether the response-enhancing effects of SNC80 in Paired groups during Acquisition are long-lasting, preliminary studies administered SNC80 (3.2 mg/kg) repeatedly over four consecutive Acquisition sessions. In Figure 5.7, SNC80 acutely enhanced active responding for cocaine-paired cues upon the first administration on ACQ4, consistent with Chapter 4 Figure 4.3. The following day, SNC80 appears to potentiate responding, although to a lesser extent. By the third and fourth administration of SNC80, the response-enhancing effects have dissipated. The effects of repeated SNC80 administration on responding for cues appears to occur on a different timescale than that for the locomotor stimulating properties of SNC80, such that following a single administration of SNC80, tolerance develops to the locomotor stimulation (Jutkiewicz *et al.*, 2008). This work requires further experiments to determine how repeated SNC80 treatments alter responding in Unpaired groups.

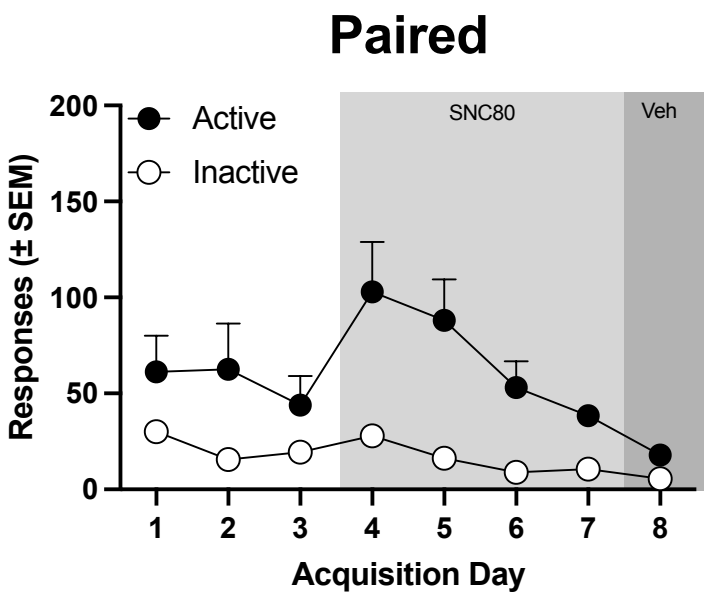

Figure 5.7 The response-enhancing effects of SNC80 in Paired groups dissipates following repeated treatments.

While our data suggest that DOR activation mediates conditioned reinforcement, there are some caveats to this interpretation. Acute SNC80 administration increased active responding in Paired groups, but also increased inactive responding in this group as well as active responding in the Unpaired group (Figure 4.3). The preference score was increased in Paired groups following SNC80, but the ratio of active responses to total responses was unaltered following SNC80 treatment and was about 0.8 on ACQ3 and ACQ4 (data not shown). While we hypothesize DORs mediate conditioned reinforcement, the increase in behavior following SNC80 could be explained by other effects. One alternative explanation is that SNC80 stimulates locomotor activity to enhance responding for cues, although we do not think this is the case as explained in Chapter 4.5. Another explanation is that SNC80 may disrupt learning or memory of the operant continencies, as it was administered on the fourth ACQ session in animals without an extensive history of operant responding. Subjects may still be learning to

respond on the active nosepoke to produce cues delivered on an intermittent schedule on ACQ4. Disruption of learning following SNC80 administration may explain the increase in inactive responding in Paired groups. We could evaluate this by administering SNC80 during Acquisition sessions that facilitate the learning of active responses for cues. First, SNC80 could be administered in later Acquisition sessions when subjects have more experience with operant responding for cues. Indeed, preliminary data suggest SNC80 (3.2 mg/kg) increased active responding for cues to a greater extent than inactive responses when given on the seventh day of ACQ (data not shown). Further, SNC80 could be administered to groups trained to earn cues on a more predictable schedule, such as FR1, or in groups that have enhanced discriminability of the nosepokes by illumination of the active nosepoke alone. We would predict SNC80 to increase active responding to a greater extent than inactive, supporting that SNC80 is not disrupting learning during Acquisition.

Another way DOR agonists may alter learning in New Response Acquisition is by contributing to the association between cue+drug during Pavlovian Conditioning, or for the contingency of the instrumental response for cues during Acquisition. Previous work has shown the DOR system modulates other processes likely involved in behaviors of conditioned reinforcement, including learning (Pradhan *et al.*, 2011; Pellissier *et al.*, 2018). Expression of DORs on cholinergic interneurons in the NAc is increased following Pavlovian Conditioning, and DORs have been shown to be required for the formation of drug+context associations (Skoubis *et al.*, 2005; Le Merrer *et al.*, 2011; Bertran-Gonzalez *et al.*, 2013; Gutiérrez-Cuesta *et al.*, 2014). The role of DORs in learning the associations between cocaine and cues could be tested by administering

naloxone or naltrindole prior to the start of each conditioning session in Paired groups. If subjects do not robustly respond for cues or do not show preference for the active response during Acquisition, that would suggest DORs are important for the development of conditioned reinforcing effects of the cue. Similarly, future work could administer naloxone or naltrindole on the first exposure to novel operant manipulanda (ACQ1) to reduce responding for cues, indicating that opioid receptors are critical for instrumental learning. Overall, the expression of conditioned reinforcement during Acquisition likely involved multiple processes and future work should characterize the role of DORs in regulating learning.

The role of opioid receptor activation in the behavior to earn cocaine-paired cues requires further characterization in the following ways. To identify which brain regions mediate the effects of RBR101 and DOR agonists, future studies should administer naloxone or deltorphin II locally and measure alterations in responding. These experiments will be crucial to inform further experiments to measure opioid peptides *in vivo*. There are many other pharmacological treatments that would add information to the role of the opioidergic system in this behavior and inform future experiments. Further characterization of the DOR system via administration of other DOR antagonists would add to the current work. For example, naltrindole isothiocyanate, an irreversible antagonist, may be better at blocking endogenous opioid ligands at DORs and reducing behavior. We would predict such an effect to be permanent, continuing for multiple Acquisition sessions. Additionally, because enkephalins also bind to MORs, future directions will further investigate a potential role of MORs by completing a full dose-response curve of morphine prior to Acquisition to determine if lower doses stimulate

responding for cocaine-paired cues. KOR antagonists may also prevent the binding of dynorphin and stimulate responding for cues, further implicating a role of this system in addition to DOR. Additionally, administration of an opioid ligand with complex pharmacology, such as buprenorphine, would be interesting to determine how relative effects at multiple opioid receptors simultaneously might alter behavior to earn cocaine-paired cues. Administration of ORL1 agonists would also implicate this opioid receptor, despite not binding canonical endogenous opioid peptides. ORL1 agonists have been shown to decrease cocaine reinstatement; therefore, they may also reduce responding for cues in this assay (Hillhouse *et al.*, 2021). Lastly, altering enkephalin concentrations via different methods, such as induction of chronic pain, may alter the conditioned reinforcing properties of cues and might enhance responding for cues in this procedure.

Our results suggest that the endogenous opioid system may regulate robust behavior to earn cues in Paired groups, yet we were unable to determine if neuroadaptations occur in opioid peptide release or opioid receptor number and/or function following Paired Pavlovian Conditioning. Alterations in peptide release, either enkephalins or endorphins, should be measured *in vivo* while both Paired and Unpaired groups respond for cues. Based off of findings from Chapter 4, we would predict that extracellular concentrations of enkephalins are higher in Paired groups than Unpaired groups, which would suggest enkephalins are associated with the behavior to earn cues. Higher concentrations of enkephalins in Paired groups could explain behavioral differences between the conditioning types by altering reward-related circuitry (described below). Alternatively, peptide release may not be altered in Paired groups following conditioning, but rather DOR or MOR expression or function are enhanced.

Following Paired Pavlovian Conditioning, DORs could be upregulated such that when enkephalins are released while subjects respond for cues, the downstream signaling output is enhanced. This hypothesis could be tested via ligand autoradiography to visualize the number of DOR or MOR binding sites, and receptor function could be evaluated using GTPgammaS for comparison between Paired and Unpaired groups. Future studies should continue to investigate the mechanisms by which the opioidergic system contributes to and mediates the conditioned reinforcing properties of cocaine-paired cues.

5.3 Proposed Mechanism of Cocaine Conditioned Reinforcement

Understanding the underlying neurobiological mechanisms by which cocaine-paired cues drive behaviors may provide insight into potential targets for preventing drug-craving and relapse in humans. The data presented in this dissertation suggest that the enkephalinergic system likely plays a role in regulating the conditioned reinforcing properties of cocaine-paired cues and responding to earn these cues as measured in New Response Acquisition. Based on the findings in this dissertation, we propose the effects of enkephalins potentially occur via the following mechanism which should be probed in future studies (Figure 5.8). 1) Enkephalins are released in brain regions within the mesolimbic dopamine circuit either via projection neurons (origin unknown) or via local release by D2-MSNs (Rysztak and Jutkiewicz, 2022). 2) Enkephalins bind to DORs expressed on cholinergic interneurons (Bertran-Gonzalez *et al.*, 2013; Laurent *et al.*, 2014), inhibiting acetylcholine release. 3a) Less acetylcholine binds to excitatory muscarinic acetylcholine receptors (mAChRs; M1) expressed on D2-MSNs (Ztaou and Amalric, 2019), reducing activation of these neurons. Decreased

activity of D2-MSNs reduces GABA release in the ventral pallidum, downstream of the NAc and part of the indirect MSN pathway, and augments the reinforcing properties of cocaine-paired cues and increases responding. Consistent with this proposed mechanism, optogenetic or chemogenetic inhibition of D2-MSNs in the NAc increases cue-induced reinstatement (Heinsbroek *et al.*, 2017; O'Neal *et al.*, 2020). Interestingly, enkephalin concentrations are thought to be increased in the ventral pallidum following repeated cocaine exposure (Tang *et al.*, 2005; Kupchik *et al.*, 2014; Heinsbroek *et al.*, 2017). This may arise from 4) enkephalinergic cell bodies within the ventral pallidum (Heinsbroek *et al.*, 2020) that are disinhibited due to reduced GABA release from lower activity of D2-MSNs. Additionally, less acetylcholine release (2) would also reduce binding to inhibitory mAChRs (M4) expressed on D1-MSNs. 3b) Less inhibition of D1-MSNs might increase activity of these neurons projecting to the VTA, as part of the direct MSN pathway, to also augment cue-maintained behaviors (Laurent *et al.*, 2014; O'Neal *et al.*, 2020). Future work would be required to further characterize relative roles of D1- vs. D2-MSNs in conditioned reinforcement. For example, experiments to determine if D1-MSN increases in activity occur simultaneously with D2-MSN reductions in activity to alter cue-maintained behaviors, or how the balance between D1- and D2-MSN outputs might be altered to add insight to this proposed mechanism.

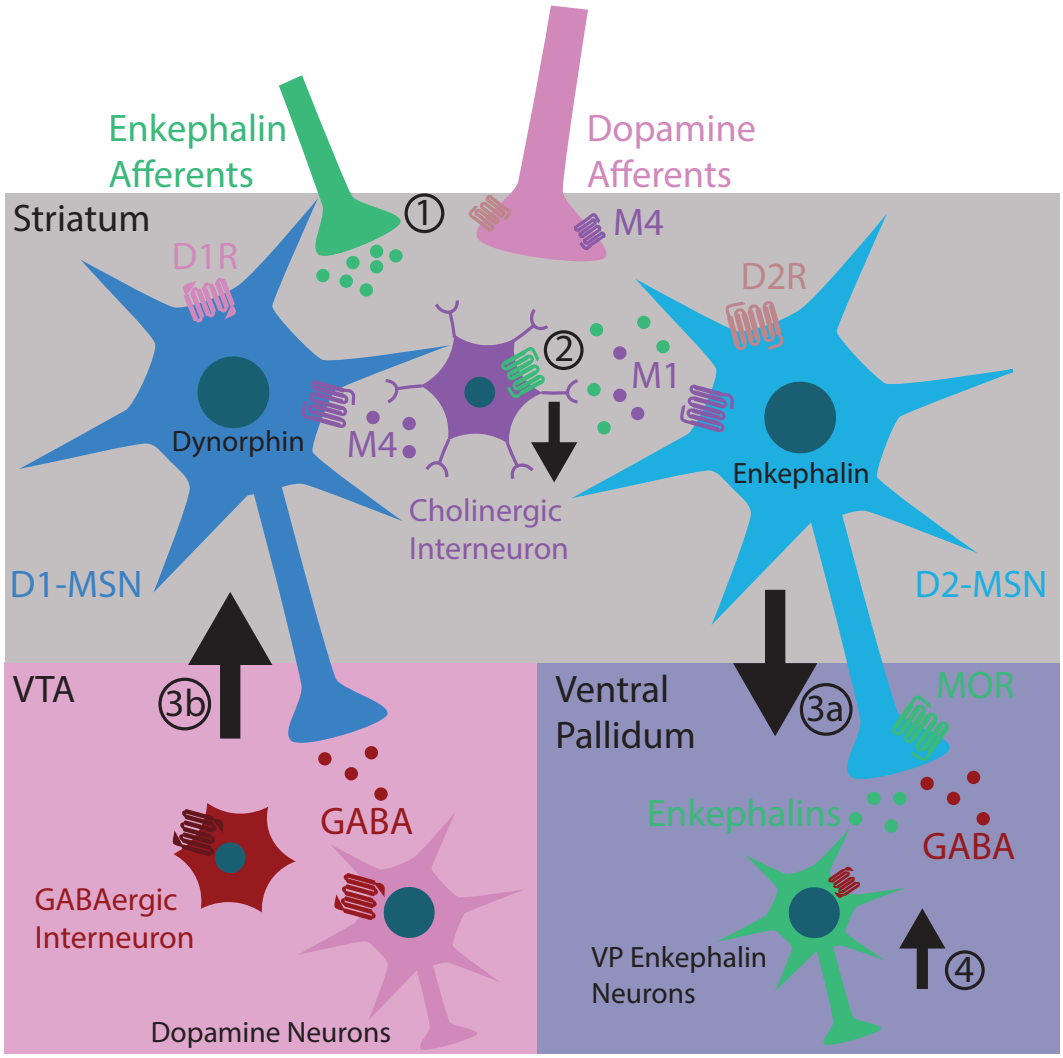

Figure 5.8 Proposed mechanism of DOR mediated responding for cocaine-paired cues in New Response Acquisition.

The work presented in this dissertation supports this proposed mechanism in the following ways. First, in Chapter 3, dopamine concentrations are unaltered in the NAc shell or core despite Paired groups responding more to earn presentations of cocaine-paired cues than Unpaired groups. Second, local infusion of cocaine into the NAc core or shell at concentrations that increase dopamine did not potentiate responding for cues. Together, these data support that the mechanism of conditioned reinforcement in this procedure likely does not require robust activation of either D1 or D2 receptors by

dopamine, although this would have to be confirmed by administration of selective dopamine receptor antagonists locally. Additionally, acetylcholine concentrations should be lower in Paired groups than Unpaired groups due to inhibition of cholinergic interneurons by DORs following binding of enkephalins. While there were no significant differences in extracellular acetylcholine concentrations in either the NAc core or shell of Paired groups as compared with baseline concentrations (Chapter 3), we sought to further test this relationship by manipulating the cholinergic system prior to Acquisition (Figure 5.9). Surprisingly, administration of nicotine, which has been shown to induce cocaine reinstatement (Bechtholt and Mark, 2002; Nunes *et al.*, 2019) and increase responding for conditioned reinforcers (Collins and Woods, 2009; Guy and Fletcher, 2014), significantly reduced responding for cocaine-paired cues in New Response Acquisition. The nicotinic receptor antagonist mecamylamine had no effect on responding, but interestingly, acute administration of the nonselective mAChR antagonist scopolamine (0.32 mg/kg) robustly increased active responding following acute, but not following repeated administration (Figure 5.9). These data support our hypothesized mechanism, such that administration of scopolamine inhibits D2-MSNs to promote cue-controlled behaviors. Further, preliminary experiments sought to investigate whether mAChRs are downstream of DORs in producing this effect, by administering naltrindole (3.2 mg/kg) prior to scopolamine. Naltrindole did not block the effects of scopolamine on enhancing cocaine conditioned reinforcement, supporting that mAChRs are either not upstream of DORs or activity of these receptors occur on an independent pathway. These data further confirm that conditioned reinforcement measured by New Response Acquisition likely has distinct underlying neurobiology from

reinstatement as scopolamine has been shown to reduce reinstatement (Nunes *et al.*, 2019).

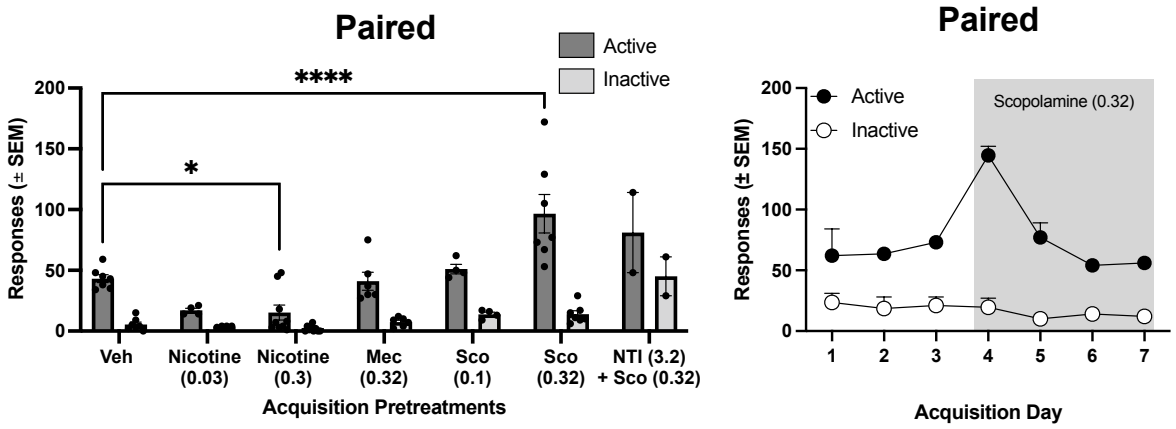

Figure 5.9 Inhibiting mAChRs with scopolamine robustly increased the conditioning reinforcing properties of cocaine during Acquisition. Acute nicotine reduced responding for cues. Pretreatment of naltrindole did not block the effects of scopolamine, suggesting mAChRs are downstream of DORs in this mechanism. The effects of scopolamine are rapidly dissipated following repeated adminstration. Veh = vehice, Mec = mecamylamine, Sco = scopolamine, NTI = naltrindole

Future directions should test this proposed mechanism by measuring levels of endogenous opioid peptides *in vivo* while animals responding for cues to provide an association between enkephalins and conditioned reinforcement. Further, studies will have to confirm the primary brain region mediating this effect by manipulating neurotransmitter systems of interest, via administration of naltrexone, enkephalinase inhibitors, or scopolamine locally and measuring expected alterations in responding for cues. Lastly, other experiments to selectively augment enkephalin levels in the striatum (or other brain regions) by optogenetic activation of enkephalinergic cells would also implicate a role of enkephalins in responding for cocaine-paired cues in New Response Acquisition.

5.4 Conclusions

The conditioned reinforcing properties of cocaine-paired cues established following Pavlovian Conditioning appear to be modulated by the opioidergic system, but not the dopaminergic system. Dopamine concentrations in either the NAc core or shell are not altered between groups of animals that show differential responding to earn presentations of cocaine-paired cues during Acquisition. Further, manipulation of the dopaminergic system via indirect and direct agonists did not potentiate conditioned reinforcement and did not increase responding for cues. Rather, activation of opioid receptors, specifically DORs, by protected levels of enkephalins or direct agonists was able to drive further responding for cues. Future work should continue to investigate the underlying neurobiological processes of the behavior controlled by cocaine-paired cues in both sexes, including potential contributions of the opioid system. Alternatively, future directions could continue to examine how dopamine is involved in the learning of the association between cues+cocaine as well as the instrumental contingency for cues. This could be done by administering pharmacological pretreatments during Pavlovian Conditioning or by measuring dopamine concentrations on the first day of Acquisition, as preliminary data demonstrated that dopamine concentrations in the NAc shell are not robustly increased from baseline in Paired groups on ACQ session 2 (data not shown). Overall, the work presented in this dissertation has provided novel insight into the mechanisms of conditioned reinforcement and should contribute to identifying novel targets to prevent relapse in humans. It is possible that the reward circuitry thought to be responsible for the primary reinforcing effects of cocaine or other drugs of abuse are distinct from circuitry mediating the conditioned reinforcing properties of drugs of abuse.

The possibility of pharmacologically targeting other neuromodulatory systems, independent of dopamine, in reducing the ability of cues to elicit behavior would greatly aid in efforts to hinder relapse and prolong abstinence in people with substance use disorders.

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