Evaluating the differential effects of cue extinction on goal-directed and habitual cocaine-seeking behavior

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Several types of maladaptive learning and memory contribute to substance use disorders (SUDs). Pavlovian associations form between the drug and contextual and discrete stimuli, or cues, and later exposure to these cues can trigger drug cravings and promote relapse. Cue exposure therapy, the repeated exposure to cues in the absence of drugs to extinguish drug-cue associations, has been proposed as a behavioral treatment to reduce cue-induced cravings. Cue extinction, a preclinical model of cue exposure therapy, reduces cue-induced drug seeking in rats through the depotentiation of thalamo-lateral amygdala synapses. Initial experiments utilized protocols that promote goal-directed drug seeking that relies on the dorsomedial striatum (DMS) and the association between cocaine and the drug-seeking behavior, but certain conditions facilitate habitual behavior that relies on the dorsolateral striatum (DLS) and the association between cues and the drug-seeking behavior. An imbalance of goal-directed and habitual control may also contribute to compulsive drug-seeking behavior that persists despite punishment. Additionally, whereas goal-directed behavior is reduced by inhibition of the basolateral amygdala (BLA), where cue extinction has its effects, habitual behavior is not impacted by BLA inhibition, which suggests that cue extinction may not impact habitual drug seeking. Therefore, in the work presented in this dissertation, we examine how cue extinction differentially affects goal-directed and habitual drug seeking and the dorsal striatum. We find that both habitual and punishment-resistant drug seeking is dependent on DLS dopamine, and habitual drug seeking results in different patterns of protein

expression and *in vivo* calcium and dopamine activity in the DLS and DMS compared to goal-directed drug seeking. Additionally, cue extinction reduces goal-directed, but not habitual, drug seeking unless goal-directed control is pharmacologically restored in rats previously behaving habitually. Cue extinction also reduces dopamine and calcium activity during drug seeking in the DMS, but has no effects on the DLS. Together, these findings indicate that extinction of drug-cue associations does not affect habitual behavior or the underlying neural circuitry involve in promoting habitual behavior and suggest that different methods may be required to target habitual and compulsive aspects of drug-seeking behavior in the treatment of SUDs.

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Preface

I would like to express immense gratitude for several individuals who helped make the completion of this dissertation an enjoyable and fulfilling experience, as well as those that provided me with support throughout the challenges I faced during the past six years.

I would first like to thank my advisor and mentor, Dr. Mary Torregrossa, for her support and guidance. Mary provided the perfect combination of giving detailed, knowledgeable guidance along with fostering independent thought and creativity. Her extensive knowledge of the field and excellent constructive feedback gave me the support I needed to succeed, and her openness to my ideas had given me confidence in pursuing future research.

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1.0 Introduction: Maladaptive learning and memory in substance use disorders

Portions of this chapter are adapted from the following published manuscript:

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Memories related to obtaining positive outcomes promote continued reward-seeking behavior that is important for survival (Bozkurt, 2022). Learning that occurs through reward seeking allows individuals to predict the availability of rewards, routinely perform complex behaviors, orient attention toward relevant stimuli, and make decisions about which behaviors will maximize reward (Bozkurt, 2022). Drugs of abuse influence these natural reward systems, often resulting in exaggerated neural responses compared to those that occur for natural rewards such as food (Milton and Everitt, 2012). Additionally, drugs of abuse can interfere with learning and memory mechanisms, and drug-related memories can become abnormally strong and maladaptive (Milton and Everitt, 2012). In some cases, these maladaptive memories may eventually promote drug use over other important behaviors (Milton and Everitt, 2012).

Substance use disorders (SUDs) are a category of psychiatric disorders that involve the compulsive persistence of drug seeking and drug use despite negative consequences and are accompanied by chronic periods of abstinence and relapse (Bozkurt, 2022; Milton and Everitt, 2012). SUDs present a prominent social and economic burden on society, and current treatment options are limited and often only successful for small populations (Bozkurt, 2022; Daley, 2013; Milton and Everitt, 2012; SAMHSA, 2022). One of the most prominent challenges in the treatment of SUDs is intense drug cravings caused by drug-associated cues (Carter and Tiffany, 1999; Grant et al., 1996; Wang et al., 1999). Even during abstinence, exposure to cues associated with drug use

can trigger memories that elicit craving, cause physiological stress, and initiate drug-seeking behaviors that lead to relapse (Carter and Tiffany, 1999; Grant et al., 1996; Milton and Everitt, 2012; Wang et al., 1999). Uncovering the mechanisms underlying the formation and retrieval of these drug memories may reveal ways to improve treatment and prevent relapse (Torregrossa and Taylor, 2013).

Much of the extensive research focused on understanding the neural underpinnings of drug-associated memories has focused on memories formed during exposure to cocaine as the drug of abuse (Belin-Rauscent et al., 2016; Milton, 2013; Milton and Everitt, 2012; See, 2005). The neural mechanisms relevant to cocaine memories likely overlap with those of natural rewards and other drugs of abuse (Di Chiara et al., 2004; See, 2005). Despite increases in opioid use, cocaine use also continues to increase, and cocaine use disorder (CUD) remains prominent (Kerridge et al., 2019; SAMHSA, 2022). In 2021, about 0.5% of individuals age 12 or older in the United States had a diagnosed CUD (SAMHSA, 2022). Furthermore, there has been an increase in overdose deaths involving cocaine in the past decade, as well as recent increases in overdose deaths involving concomitant cocaine and heroin use (Kerridge et al., 2019; Spencer et al., 2021). Therefore, the prevalence of CUDs and the extensive literature around cocaine-related memories make cocaine an ideal drug for the study of maladaptive learning and memory that contributes to SUDs.

1.1 Rodent models of cocaine self-administration

Learning and memory mechanisms involve interactions between several neural circuits. Researchers have identified many key brain regions that are activated by drugs of abuse, examined

how neuroanatomy is altered in people with SUDs, and evaluated the impact of drug use and exposure on learning and memory (Ersche et al., 2021; Hahn et al., 2018; Maas et al., 1998; Sjoerds et al., 2013; Tricomi et al., 2009; Vaquero et al., 2017). These types of experiments provide valuable insights into interactions between drugs and learning and memory. However, studying learning and memory in animal models of drug use can provide several advantages, including the ability to control the timing and extent of drug exposure, evaluate different contributions to drug-seeking behavior, and isolate different types of associative memories that may contribute to persistent drug use (Belin-Rauscent et al., 2016; Gardner, 2000; Panlilio and Goldberg, 2007). The advent of animal models of drug self-administration, in which animals learn to perform a behavior (such as pressing a lever) to receive a drug, catalyzed the development of several ways to evaluate effects of drugs and their impact on learning and behavior (Gardner, 2000; Weeks, 1962). In these models, cocaine infusions are often accompanied by an audiovisual cue consisting of the illumination of a cue light and the playing of an audio tone, which allows for the investigation of the contribution of drug-cue associations on drug-seeking behavior (Everitt, 2014; Panlilio and Goldberg, 2007). Rodent self-administration methods continue to evolve as scientists seek to evaluate and isolate increasingly complex aspects of drug use (Belin-Rauscent et al., 2016).

1.1.1 Types of memories modeled by rodent cocaine self-administration

Several types of associative learning are involved in the establishment of rodent cocaine self-administration, and the use of these models allows for the analysis of the contribution of different learning processes in drug-related behavior (**Figure 1**) (Everitt, 2014). Drug memories often involve associations between the drug and contextual or discrete environmental cues, information about the interoceptive effects or the value of the drug, and information about

behaviors involved in seeking and obtaining the drug (Di Chiara et al., 2004; Milton and Everitt, 2012; Torregrossa et al., 2011). Drug memories can elicit cravings that promote continued use and relapse, but different types of memories may be differentially important depending on the nature, extent, and manner of drug use (Milton and Everitt, 2012). Moreover, these different types of cocaine associations may develop through distinct mechanisms and in divergent brain circuits, which is important to understand to optimize treatment development.

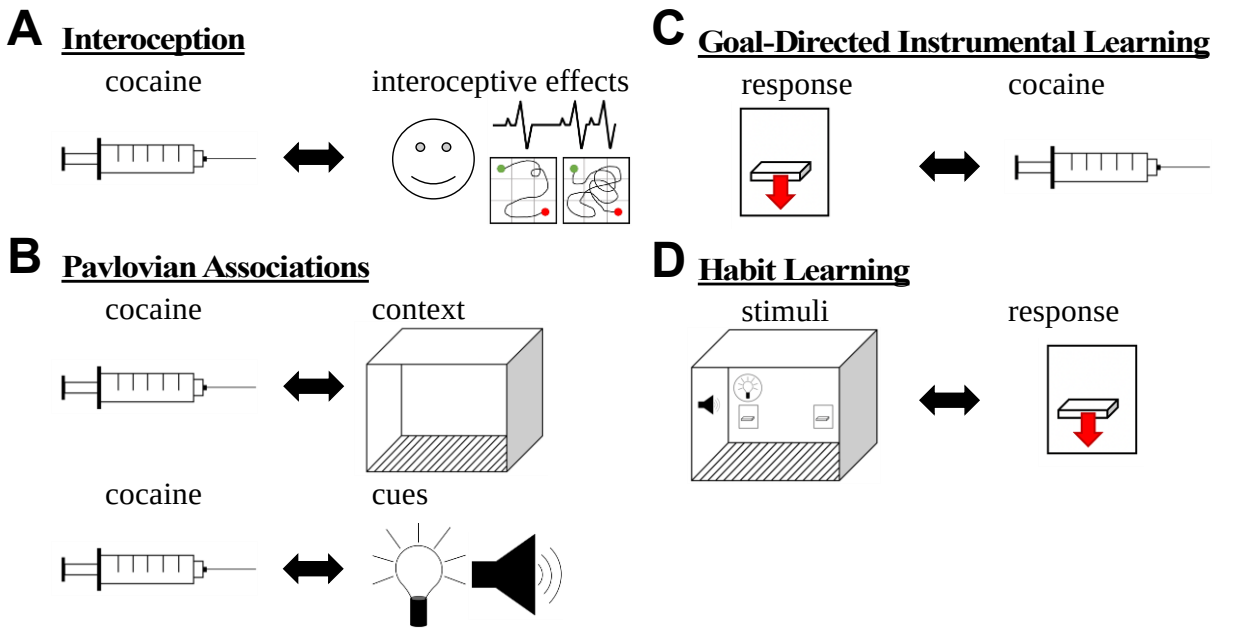

Figure 1: Cocaine memories

Rodent cocaine self-administration models the formation of several types of memories related to drugs and drug-seeking behavior. Interoception involves associations between cocaine and its reinforcing and interoceptive effects, including increased heart rate and blood pressure, increased locomotor activity, and activation of brain regions associated with reward (**A**). Pavlovian learning results in associations between cocaine and discrete cues and/or the context in which cocaine is received (**B**). Goal-directed instrumental learning concerns the association between the instrumental response and cocaine (**C**) and habit learning concerns associations between contextual and discrete environmental stimuli (or cues) and the instrumental response (**D**).

1.1.1.1 Interoception and reward

Due to its direct effects on synaptic dopamine levels in brain regions associated with reward, cocaine is highly reinforcing (Hyman et al., 2006). Cocaine also produces several physiological effects, referred to as interoceptive effects, and associative memories between the use of cocaine and these rewarding/interoceptive effects are formed (**Figure 1A**) (Mihindou et al., 2011). Cocaine-primed reinstatement, in which cocaine is injected by an experimenter rather than self-administered, describes the phenomenon by which previously extinguished cocaine seeking is reinstated by the association between cocaine's reinforcing effects and the internal state produced by cocaine (Mihindou et al., 2011). Additionally, subsequent experience of similar interoceptive effects that mimic some aspects of cocaine's effects, such as stress, may also signal cocaine availability and trigger relapse (Mihindou et al., 2011).

1.1.1.2 Pavlovian associations

Pavlovian (also known as classical) conditioning is another form of associative learning that occurs in rodent self-administration models. Through repeated pairing, cocaine and its rewarding effects (unconditioned stimulus, US) become associated with the self-administration context (e.g., the operant chamber) and other contextual and discrete stimuli, such as the audiovisual cue that accompanies cocaine infusions (conditioned stimuli, CS) (**Figure 1B**) (Belin-Rauscent et al., 2016; Everitt, 2014). Pavlovian associative learning can occur passively, meaning it can occur in the absence of drug-seeking behavior (Perry et al., 2014). Even so, in rodent self-administration models, cocaine-associated cues signal drug availability, and therefore they can also promote behavior that is associated with the drug through a process termed conditioned reinforcement (Di Ciano and Everitt, 2004; Perry et al., 2014; Shahan, 2010). Exposure to drug-associated cues is a major catalyst for relapse in humans (Carter and Tiffany, 1999; Grant et al.,

1996; Milton and Everitt, 2012; Wang et al., 1999). Therefore, cue-induced reinstatement, the process by which exposure to cocaine-associated cues reinstates a previously extinguished drug-seeking behavior, was established in rodent self-administration to model the ability of cues to promote relapse under different conditions (Perry et al., 2014). Pavlovian drug-cue associations can be extinguished through the process of cue extinction, when the drug-paired cue is presented passively and repeatedly in the absence of the drug (Torregrossa et al., 2010). It is generally understood that extinction involves new learning about the lack of drug-cue association (Bouton, 2002). Cue extinction has been shown to reduce cue-induced drug seeking in rodent models of cocaine self-administration, which indicates that cue extinction impairs the ability of cues to promote cocaine-seeking behavior (Madsen et al., 2017; Murray et al., 2012; Perry et al., 2016; Rich et al., 2019; Torregrossa et al., 2013).

1.1.1.3 Goal-directed learning (response-outcome associations)

When cocaine is self-administered, instrumental (also known as operant) learning occurs alongside Pavlovian conditioning (Shiflett and Balleine, 2011). Initial learning of the instrumental response requires the formation of response-outcome associative memories that guide goal-directed behavior (De Wit and Dickinson, 2009; Smith and Laiks, 2017). An instrumental response or behavior (such as a lever press) becomes associated with the delivery of cocaine and its subsequent rewarding effects (**Figure 1C**) (Everitt and Robbins, 2013). Response-outcome associations that promote cocaine-seeking behavior can be extinguished through instrumental extinction, when the drug is withheld, and rodents learn that the behavior no longer results in drug delivery (Bouton, 2002).

1.1.1.4 Habit learning (stimulus-response associations)

In addition to goal-directed, response-outcome associative learning, stimulus-response associations that guide habitual behavior can also form (Smith and Laiks, 2017). After significant repetition of the same action in the presence of stimuli or cues, an association forms between the behavior and these stimuli (**Figure 1D**) (Smith and Laiks, 2017). Generally, Pavlovian drug-cue associations and their conditioned reinforcing properties are believed to facilitate the formation of stimulus-response associations (Di Ciano and Everitt, 2004). Under certain conditions, these stimuli can then promote the resulting habitual behavior in a way that is automatic and inflexible (O'Hare et al., 2018; Smith and Laiks, 2017).

1.1.2 Influence of cocaine memories on drug-seeking behavior

Multiple associative learning mechanisms can simultaneously contribute to drug-related behaviors, and the same behavior can also be produced by competing mechanisms (Perry et al., 2014; Root et al., 2009; Smith and Laiks, 2017; Troisi, 2013). Cues, through stimulus-response associations, can promote habitual drug-seeking behavior (**Figure 2**) (Smith and Laiks, 2017). However, drug-associated cues can themselves become reinforcing through their association with a reward, such that behaviors will be performed simply to obtain cue presentation, through a process known as conditioned reinforcement. (Perry et al., 2014; Shahan, 2010). Therefore, drug seeking may not only be initiated by stimulus-response or response-outcome associations but can also be influenced by drug-cue associations (Perry et al., 2014). Overlapping and dissociable effects of extinction learning also complicate separating the contribution of these associations (Goodman and Packard, 2019). Therefore, it can be difficult to elucidate the role of these different

associative memories on behavior, but extensive literature has sought to do so (Smith and Graybiel, 2016; Smith and Laiks, 2017; Wood and Neal, 2007).

In rodent models of cocaine self-administration, training subjects to make a new response that produces the cue alone may allow for isolation of the role of cocaine-cue associations in promoting behavior (Di Ciano and Everitt, 2004). Additionally, to determine whether a behavior is goal-directed and guided by response-outcome associations or habitual and guided by stimulus-response associations, experiments have manipulated the value of the outcome or the contingency of outcome delivery (Smith and Laiks, 2017). Because goal-directed behavior relies on the behavior's association with the outcome, devaluing the outcome through either specific satiety or by making the outcome aversive by inducing illness reduces goal-directed behavior but has no effect on habitual behavior (Smith and Graybiel, 2016; Smith and Laiks, 2017). Additionally, a reduction in responding after response-outcome contingency degradation, which involves noncontingent outcome presentations, can identify goal-directed behavior (Smith and Graybiel, 2016; Smith and Laiks, 2017). Normally, early on in training, behavior is goal-directed, and certain conditions later promote reliance on habitual behavior (Smith and Laiks, 2017). However, it has been suggested that response-outcome and stimulus-response associations may be learned at the same time early in training, even if stimulus-response associations do not initially guide behavior (Balleine and Dickinson, 1998; De Wit and Dickinson, 2009; Killcross and Coutureau, 2003).

Several limitations to these behavioral definitions of goal-directed and habitual behavior have been discussed (Watson and de Wit, 2018). Unfortunately, although a lack of effect of outcome devaluation or contingency degradation may suggest a behavior is habitual, it can also suggest impaired goal-directed learning (Watson and de Wit, 2018). Therefore, it can be difficult to identify habitual behavior through this method. Cocaine self-administration studies also usually

involve intravenous cocaine delivery, which presents a unique challenge to outcome devaluation and contingency degradation methods (Everitt, 2014; Everitt and Robbins, 2013; Ostlund and Balleine, 2008; Smith and Laiks, 2017). The extensive literature examining goal-directed and habitual behavior is also fraught with disagreements in definitions and semantics, which can make studying and defining these processes and interpreting their implications difficult (Houwer, 2019; Smith and Graybiel, 2016; Watson and de Wit, 2018).

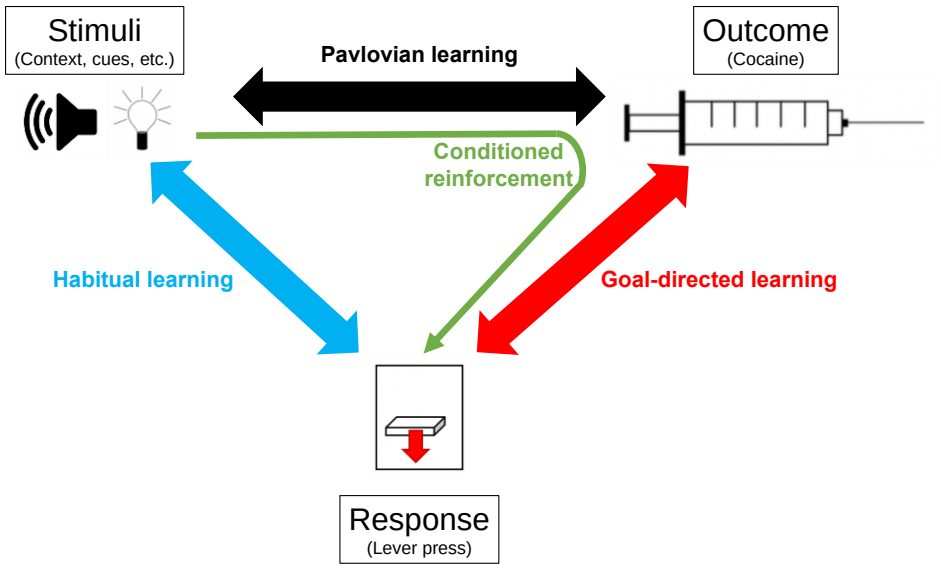

Figure 2: Influence of associative memories on drug seeking

In models of cocaine self-administration, the behavioral drug-seeking response, such as the lever press, can be initiated through goal-directed, response-outcome associations (red). The behavior can also be initiated by stimuli, either directly through habitual, stimulus-response associations (blue) or indirectly via the conditioned reinforcing effects of cocaine-cue associations (green).

1.1.3 Schedules of reinforcement

In rodent models of drug self-administration, the drug can be delivered in several ways, including orally or through vapor, but for most cocaine self-administration paradigms, cocaine is

delivered via infusion through an intravenous catheter (Gardner, 2000; Panlilio and Goldberg, 2007). If a drug has reinforcing properties, it increases the likelihood that the behavior that procured the drug will be performed again (Panlilio and Goldberg, 2007). In the simplest versions of self-administration experiments, when the drug is delivered via continuous reinforcement, every performance of the behavior (e.g. lever press) results in drug delivery (Panlilio and Goldberg, 2007). However, by manipulating the schedule of reinforcement, or the response contingency required to produce the reinforcer, experimenters can elucidate different information about drug-related memories and the behavior they promote (**Figure 3**). Here, we describe a few schedules of reinforcement relevant to the experiments described in this dissertation.

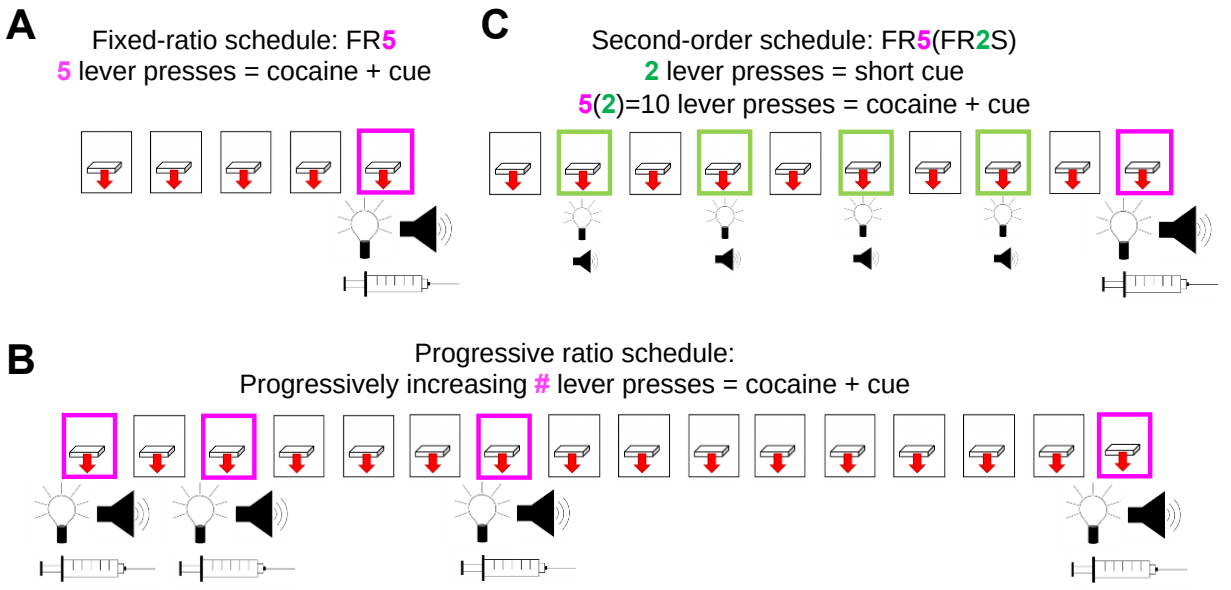

Figure 3: Schedules of reinforcement

Rodent cocaine self-administrations studies can utilize different schedules of reinforcement, or different contingencies for which cocaine is delivered. For a fixed-ratio 5 (FR5) schedule, cocaine and its associated cues are delivered after every 5th lever press (**A**). For a progressive ratio (PR) schedule, cocaine and its associated cues are delivered after the completion of a progressively increasing number of lever presses (**B**). For a second-order (SO) schedule, such as an FR5(FR2S) schedule, a short drug-associated cue is presented after every 2nd lever press, and after the 5th completion of this schedule (after the 10th total lever press), cocaine and its associated cues are delivered (**C**).

1.1.3.1 Fixed-ratio schedules

An overwhelming majority of rodent self-administration experiments utilize fixed-ratio one (FR1) schedules of reinforcement (Belin-Rauscent et al., 2016; Everitt, 2014). Under an FR schedule of reinforcement, a fixed number of lever presses is required to produce drug delivery (**Figure 3A**) (Belin-Rauscent et al., 2016). Therefore, an FR1 schedule describes continuous reinforcement, where each lever press is reinforced. Increasing the ratio, for example from 1 to 5, can model basic scenarios in which not every performance of a behavior is reinforced (Panlilio and Goldberg, 2007). Because of the strong relationship between the rate of lever pressing and the rate of cocaine delivery, FR schedules typically facilitate response-outcome learning and goal-directed behavior (Murray et al., 2012; Smith and Laiks, 2017). Rodents quickly learn to self-administer cocaine on FR schedules, making them valuable tools for evaluating the short-term effects of cocaine on the brain and behavior, as well as for identifying neural circuits involved in the reinforcing effects of cocaine (Belin-Rauscent et al., 2016). However, they fall short for in-depth analysis of the strength of reinforcers and do not reflect scenarios in which reinforcement delivery can be extensively delayed or variable or in which behavior is not necessarily goal-directed (Everitt, 2014).

1.1.3.2 Progressive ratio schedules

Under progressive ratio (PR) schedules of reinforcement, the number of behavioral responses required to procure the reinforcer increases progressively after each reinforcer delivery (**Figure 3B**) (Panlilio and Goldberg, 2007). Therefore, the subject must expend progressively more effort to continue to receive the reinforcer (Gardner, 2000). The breakpoint, or the ratio at which the subject fails to complete the schedule within a certain period of time, reflects the strength of the drug's reinforcement properties and is considered a measure of motivation for the drug

(Gardner, 2000; Panlilio and Goldberg, 2007). The breakpoint can be used to compare the reinforcement properties between different drugs or between experimental conditions (Gardner, 2000). Because the dose of the drug can affect the rate of lever-press behavior, breakpoints for multiple doses of drugs are typically evaluated (Johnson et al., 2022).

1.1.3.3 Second-order schedules

Second-order (SO) schedules of reinforcement were developed to produce prolonged cocaine-seeking dependent on the presentation of a cocaine-associated cue (Everitt, 2014). Under an SO schedule of reinforcement, a short cue that was previously associated with cocaine is presented after a set number of lever presses, and then cocaine infusions along with the longer audiovisual cue are delivered after either a fixed window of time or a fixed number of initial cue presentations (**Figure 3C**). These schedules of reinforcement were applied to cocaine self-administration studies to model the ability of cocaine-associated cues, through conditioned reinforcement, to promote prolonged drug-seeking behavior, even during drug-free periods (Belin-Rauscent et al., 2016; Di Ciano and Everitt, 2005). During responding on SO schedules of reinforcement, after the drug-cue association forms, cocaine-associated cues are not always accompanied by cocaine delivery, which reflects a common aspect of SUDs not often modeled by rodent self-administration studies (Di Ciano and Everitt, 2005). SO schedules promote stimulus-response learning and the rapid transition to habitual behavior, likely because of prolonged drug-seeking behavior without drug reinforcement, as well as increased stimulus-response pairings (Everitt, 2014).

1.1.4 Compulsivity and punishment resistance

A hallmark of SUDs is compulsive persistence in drug-seeking and drug use despite considerable negative outcomes for the individual (Belin-Rauscent et al., 2016). Therefore, several methods have been developed to model compulsive behavior in rodent self-administration using resistance to punishment as an indirect measure of compulsivity. Often, an aversive footshock delivered through the floor of an operant box is used to punish either cocaine seeking or self-administration, although an aversive conditioned stimulus can also be used (Jean-Richard-Dit-Bressel et al., 2018; Kawa et al., 2016; Vanderschuren and Everitt, 2004). In most cases, the punishment is delivered either when cocaine infusion occurs or when the rodent makes a drug-seeking lever press (Kawa et al., 2016; Pelloux et al., 2015).

When an aversive outcome, or punishment, is introduced, this involves new associative learning between the behavior, its associated stimuli, and the outcome (Smith and Laiks, 2017). Additionally, these models have allowed experimenters to evaluate how other drug-related associative memories may contribute to compulsive cocaine self-administration, but there is considerable disagreement in the field about how compulsive behavior arises. One predominant theory proposes that compulsive drug-seeking must arise from enhanced reliance on habitual behavior because it is insensitive to changes in outcome value (Everitt, 2014). Another theory suggests that compulsive behavior arises from inflexibility due to impaired decision-making or cognitive control of behavior (Verdejo-García et al., 2020; Wood and Neal, 2007). Yet another theory posits that an enhanced motivation for the drug over alternative choices results in persistent, maladaptive goal-directed drug seeking (Hogarth, 2020). In reality, there is evidence for each of these theories, and they all likely play some role in SUDs, which consist of several complex behaviors that may have different and competing motivations driven by distinct underlying

associations (Epstein, 2020; Lüscher et al., 2020; Vandaele and Ahmed, 2020; Vandaele and Janak, 2018).

1.1.5 Access models

In the majority of rodent self-administration studies, often referred to as short access (ShA), subjects have daily access to cocaine for a short amount of time, usually 1-2 hours (Allain and Samaha, 2019). Additionally, these studies usually provide continuous access (ContA) to cocaine, where rats can freely take cocaine throughout the session, and often last for 1-2 weeks. Because these experiments are relatively short, they are excellent for studying several aspects of drug use, such as evaluating the physical and neural effects of drugs, examining the learning involved in initial drug use, and identifying neural circuits involved in drug use. However, these methods fall short in their ability to model several important aspects of SUDs, including the long-term effects of drugs, escalation of drug intake over time, and the development of habitual, compulsive drug use (Ahmed and Koob, 1998; Belin-Rauscent et al., 2016).

In recent decades, several modifications to access methods have been made, including changes in session length and restrictions on cocaine availability (**Figure 4**) (Ahmed and Koob, 1998; Allain and Samaha, 2019; Belin-Rauscent et al., 2016). These modifications have sought to better model certain aspects of SUDs that may not be captured by short daily access for a short period of time (Allain and Samaha, 2019; Belin-Rauscent et al., 2016). Because drug access in humans is not usually restricted to a few hours a day, the long access model (LgA) was developed, where rodents are usually given 6 hours of drug access (Ahmed and Koob, 1998). Compared to ShA, LgA promotes escalation of cocaine intake, enhanced motivation for cocaine, and enhanced

cocaine-primed reinstatement (Ahmed and Koob, 1998; James et al., 2019; Knackstedt and Kalivas, 2007; Paterson and Markou, 2003).

However, evidence suggests that escalation of cocaine intake is not necessarily required for enhancement of these addiction-like behaviors (Allain et al., 2018). Regardless of session length, when provided with ContA, rats typically "load up" on cocaine in the beginning of the session to reach their desired brain cocaine concentration, and then steadily take more to maintain that concentration until the end of the session (Zimmer et al., 2012). However, evidence from clinical studies suggests that individuals with CUD or other stimulant use disorders tend to take drugs in an intermittent, binge-like pattern (Allain et al., 2015; Foltin et al., 1995; Leri et al., 2004a; Ward et al., 1997). To model this behavior in rats, intermittent access (IntA) methods were developed, in which short periods of cocaine availability are separated by longer periods of unavailability throughout the daily self-administration session (Zimmer et al., 2012). Restricted access facilitates binge-like behavior that leads to rising and falling brain cocaine concentrations that lead to different neural and behavioral effects of cocaine (Samaha et al., 2021; Zimmer et al., 2012). Compared to LgA which typically promotes tolerance to cocaine, IntA has been shown to promotes sensitization (Calipari et al., 2013; Kawa et al., 2019). IntA also enhances motivation for cocaine, cue-induced reinstatement, incubation of craving, and punishment-resistant cocaine self-administration (Algallal et al., 2020; Aragona et al., 2009; Calipari et al., 2015; James et al., 2019; Nicolas et al., 2019; Zimmer et al., 2012). Interestingly, it appears that it is the pattern and not the amount of cocaine intake that produces these effects, as one recent study showed similar effects of IntA whether sessions were 2 or 6 hours long (Allain and Samaha, 2019). Overall, the application of these different access models to the study of cocaine-related memories may provide new insights into how different patterns and extents of drug use may impact the ability of drug-

associated cues to promote drug-seeking, as well as the progression to habitual and compulsive drug self-administration.

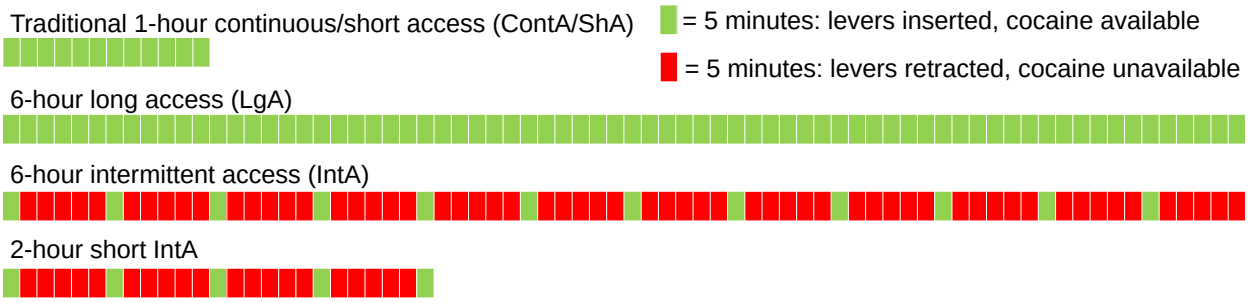

Figure 4: Access models

Different access models of cocaine self-administration allow rodents to self-administer for a set number of hours per day and also restrict cocaine access within those sessions. Green rectangles indicate 5-minute periods when levers are inserted in the operant box and cocaine is available, and red rectangles indicate 5-minute periods when levers are retracted and cocaine is unavailable. Continuous access (ContA) models do not restrict access, and rodents can self-administer cocaine freely for short (ShA, typically 1-2 hours) or long (LgA, typically 6-8 hours) periods each day. Intermittent access models (IntA) restrict cocaine availability to 5-minute periods that are typically separated by 25 minutes of unavailability. Some experiments have begun to use shortened IntA sessions (2-3 hours).

1.2 Cellular and molecular memory mechanisms

Rodent models of cocaine self-administration have allowed for the identification of several cellular and molecular processes that underlie the formation and maintenance of cocaine memories. Generally, memory traces are formed when a combination of neuronal input and activity triggers cellular events that ultimately result in changes in protein expression, including changes in the membrane expression of receptors, neuronal morphology, and the excitability of cells. All of these forms of neuroplasticity can lead to changes in the way the neuron responds to

inputs, thus allowing for the formation of a memory trace and the process of learning. Memory formation requires changes in how neurons communicate. Long-term potentiation (LTP) is a mechanism by which synapses are strengthened, and long-term depression (LTD) is a mechanism by which synapses are weakened (Nestler, 2002). These processes result in altered expression and localization of glutamate receptors and their subunits at the synapse, amongst other neurophysiological adaptations (Baez et al., 2018; Malinow and Malenka, 2002).

Although LTP and LTD can be regulated by modulatory neurotransmitters such as dopamine, these processes occur at glutamatergic excitatory synapses and rely on glutamate release from the presynaptic neuron along with temporally related post-synaptic depolarization (Gurden et al., 2000; Rich and Torregrossa, 2018). Depending on the timing of these events and calcium entry through NMDA receptors, LTP or LTD can occur, and result in altered spine density and changes in the AMPA:NMDA ratio at the synapse (Chen et al., 2007; Collin et al., 1997). LTP and LTD are induced by activation of particular kinases and phosphatases that regulate the phosphorylation and activation of signaling molecules (D'Alcantara et al., 2003). Typically, activation of protein kinases is associated with LTP (D'Alcantara et al., 2003; Li et al., 2012). Alternatively, activation of protein phosphatases is associated with LTD (D'Alcantara et al., 2003; Li et al., 2012). These proteins regulate the activity of downstream proteins, signaling molecules, and transcription factors by altering their phosphorylation state, resulting in changes in AMPA receptor insertion at the synaptic membrane and the expression and localization of other plasticity-related proteins (D'Alcantara et al., 2003; Hyman et al., 2006; Li et al., 2012; Rich and Torregrossa, 2018).

1.3 Neural substrates of associative memories and cocaine-seeking behavior

Because memory formation involves processing sensory information about contextual stimuli, detecting the rewarding effects of cocaine, directing attention, and producing reward-seeking behaviors, several brain regions interact to form cocaine-related memories (**Figure 5**). Though specific information about cocaine-related memories and the brain regions involved are limited in some cases, extensive literature has examined the role of many of these areas in learning related to other reinforcers, such as food or alcohol (Nestler, 2002). Drugs of abuse, including cocaine, increase dopamine concentrations at the synapse, which then influences memory formation processes throughout the reward circuitry, including the prefrontal cortex, striatum, and limbic system (Hyman et al., 2006). The role of dopaminergic projections from the midbrain in reward-related learning and memory has been extensively investigated (Hyman et al., 2006). The ventral tegmental area (VTA) and the substantia nigra (SN) are regions of the midbrain that send dopaminergic projections throughout the brain (Hyman et al., 2006). These regions and the dopamine they release have been shown to play major, but dissociable, roles in associative learning (Hyman et al., 2006; Saunders et al., 2018; Schultz et al., 1993).

Fairly distinct populations of dopaminergic neurons in the SN project to the dorsomedial striatum (DMS) and the dorsolateral striatum (DLS), two sub-regions of the striatum highly implicated in goal-directed and habitual learning and behavior (Belin et al., 2009; Lerner et al., 2015; Liljeholm and O'Doherty, 2012). Lesions to either the DLS or to dopaminergic projections to the DLS disrupt habit learning, whereas lesions to the DMS or DMS NMDA receptor blockade disrupt goal-directed learning in food-related tasks (Faure et al., 2005; Yin et al., 2005b, 2005a, 2004). Pharmacological dopamine antagonism in the DLS reduces cocaine-seeking behavior in rats trained on habit-promoting SO schedules of reinforcement, whereas DMS dopamine inhibition

has no effect (Murray et al., 2012). Alternatively, DMS dopamine antagonism, but not DLS dopamine antagonism, reduces FR-trained, goal-directed cocaine seeking (Murray et al., 2012). Therefore, the DMS and DLS are important for the learning of response-outcome and stimulus-response associations and the expression of goal-directed and habitual cocaine-seeking behavior, respectively.

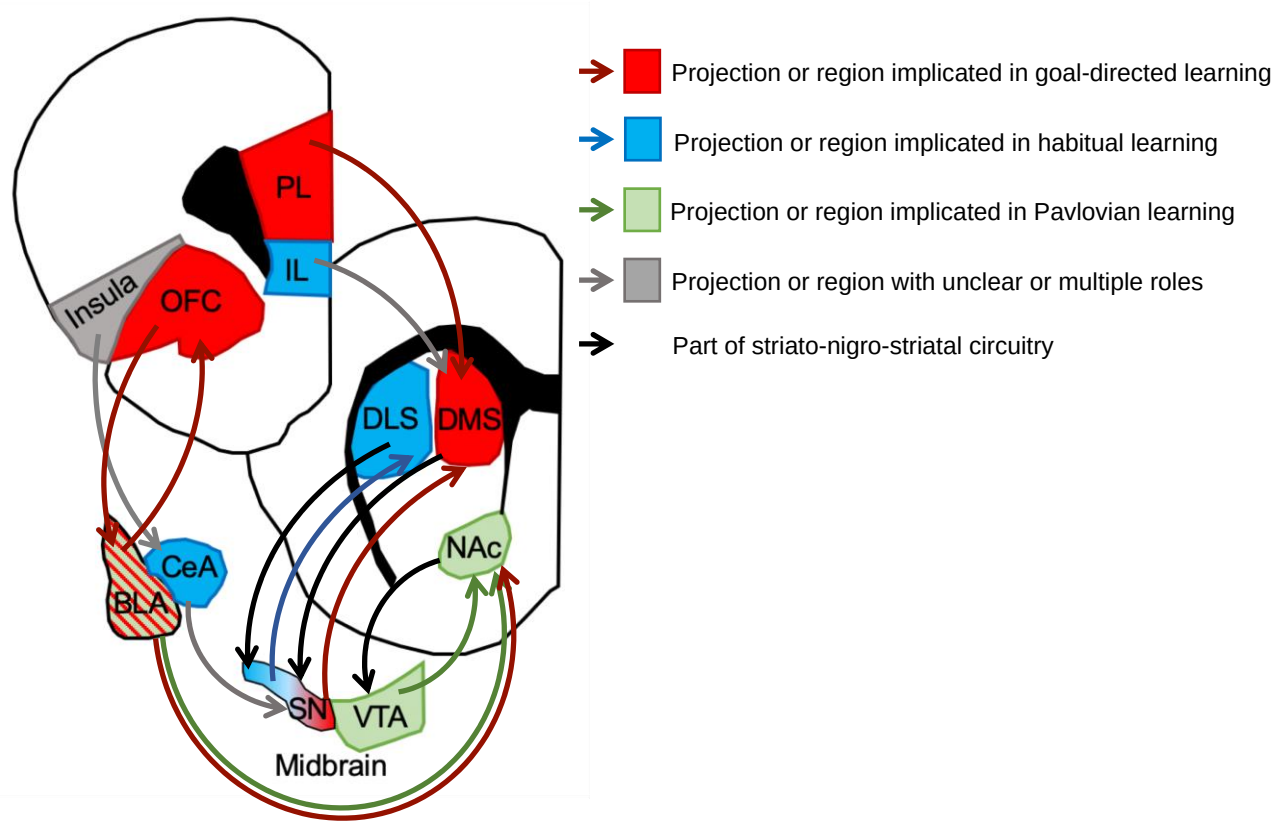

Figure 5: Associative memory circuitry

A diagram showing a subset of brain regions and their projections involved in different aspects of associative learning and related behavior. The dorsomedial striatum (DMS), a portion of the substantia nigra (SN), prelimbic cortex (PL), orbitofrontal cortex (OFC), and basolateral amygdala (BLA) have all been implicated in goal-directed learning and behavior. The dorsolateral striatum (DLS), a portion of the SN, the infralimbic cortex (IL), and central amygdala (CeA) have all been implicated in habitual learning and behavior. The nucleus accumbens (NAc), ventral tegmental area (VTA), and the BLA have been implicated in Pavlovian learning, and interactions between these and other regions play a role in behavior influenced by Pavlovian associations. Not included are several projections and

thalamic and other cortical regions that also interact with these regions. The insular cortex (insula), along with several other cortical regions, may play a role in compulsive behavior, but its role in goal-directed and habitual behavior is not known.

Another region of the striatum, the nucleus accumbens (NAc), receives dopaminergic input from the VTA. Dopamine activity in the NAc shell region is important for the consolidation of Pavlovian drug-cue associations, and the NAc core region is important for the ability of Pavlovian cues to promote goal-directed behavior (Di Chiara et al., 2004; Hall et al., 2001). Although the NAc's role in Pavlovian learning is clear, its role in goal-directed and habitual learning appears to be more indirect (Ding et al., 2013; Ito et al., 2004; Smith and Laiks, 2017; Wang et al., 2010). When Pavlovian associations between cocaine and environmental stimuli form in a paradigm where the drug is obtained by operant self-administration, these associations guide the acquisition of self-administration behavior (Everitt et al., 2008; Ito et al., 2004; Liljeholm and O'Doherty, 2012; Vanderschuren et al., 2005). Evidence suggests that while Pavlovian conditioning relies on the NAc, as drug-seeking progresses, Pavlovian associations guide the formation of goal-directed and eventually habitual instrumental response strategies aimed at obtaining the drug (Everitt et al., 2008; Ito et al., 2004; Liljeholm and O'Doherty, 2012; Vanderschuren et al., 2005). The facilitation of action-based learning may occur through the NAc's ability to influence dopamine release in the dorsal striatum via its projections to the midbrain and subsequent "spiraling" striato-nigro-striatal projections to progressively more dorsal and lateral regions of the striatum, which is important for habit formation (Belin and Everitt, 2008; Murray et al., 2015). Moreover, functional connectivity between the NAc shell and the DLS also facilitates cocaine self-administration, which indicates a role in these spiraling projections on cocaine-seeking behavior (Veeneman et al., 2015).

The amygdala and its interactions with the striatum play a role in several types of associative learning. The basolateral amygdala (BLA) encodes cocaine-cue associations, and

NMDA receptor activity in the BLA is necessary for the acquisition and consolidation of cocaine-cue associations (Carelli et al., 2003; M. Feltenstein and See, 2007; Sias et al., 2021). Additionally, BLA inhibition reduces goal-directed, but not habitual, cocaine self-administration, whereas inhibition of the central amygdala (CeA) has the opposite effect (Murray et al., 2015). The specific projections from the amygdala that mediate this dissociation in goal-directed and habitual behavior are unclear, but BLA projections to the NAc may play a role via the spiraling striato-nigro-striatal projections (Murray et al., 2015). The CeA, but not the BLA, is required for Pavlovian-instrumental transfer, a process by which a previously learned Pavlovian association promotes instrumental learning (Hall et al., 2001). The amygdala, particularly the CeA, also interacts with the midbrain; inhibitory projections from the CeA to the SN are required for Pavlovian-conditioned reward seeking (Steinberg et al., 2020). Inhibition of the CeA has been shown to promote cocaine seeking despite risk of footshock punishment, which implicates this region in compulsive behavior as well (Xue et al., 2012).

The amygdala also interacts with cortical regions in the formation and expression of associative memories. In general, the orbitofrontal cortex (OFC) and its connections to the BLA are important for goal-directed behavior as well as the ability of Pavlovian associations to guide goal-directed behavior (Arguello et al., 2017; Gremel and Costa, 2013; Li et al., 2022; Sias et al., 2021; Zimmermann et al., 2017). Interestingly, the projections from the medial and lateral portions of the OFC to the BLA play different roles in memory encoding and retrieval (Malvaez et al., 2019). The prelimbic cortex (PL) is also important for Pavlovian associations, and both the PL and infralimbic cortex (IL) play a role in regulating the expression of goal-directed and habitual behavior (Barker et al., 2014; Gourley and Taylor, 2016; Han et al., 2010; Zavala et al., 2003). Inhibition of the IL prevents the learning and expression of habitual behavior (Killcross and

Coutureau, 2003; Smith et al., 2012). Additionally, the PL and IL have been implicated in regulating compulsive behavior (Barker et al., 2013; Smith and Laiks, 2017). The insular cortex and its projections to the CeA also appear to have a role in reward seeking and potentially compulsive behavior, although this pathway is understudied for cocaine-specific memories and behavior (Campbell et al., 2019; Haaranen et al., 2020; Ponserre et al., 2020; Stern et al., 2019; Venniro et al., 2017). The striatum also receives direct projections from cortical regions (Smith and Laiks, 2017). The ventral OFC, PL, anterior cingulate, and premotor cortex project primarily to the DMS, whereas the DLS receives cortical input from the primary motor cortex and primary and secondary somatosensory cortices (Smith and Laiks, 2017). Projections from the PL to the DMS are required for goal-directed learning (Hart et al., 2018). Additionally, OFC-DMS projections are involved in the execution of goal-directed behavior (Li et al., 2022).

The thalamus, as a hub of sensory integration, plays a major role in relaying sensory information about drugs or other reinforcers to other brain regions, and therefore several thalamic nuclei are involved in associative learning. Projections from the medial geniculate nucleus (MGN) to the BLA are potentiated by cocaine-cue associative learning and depotentiated by Pavlovian cue extinction (Rich and Torregrossa, 2018). Additionally, the anterior thalamic nuclei have been implicated in Pavlovian learning in other operant tasks (Bradfield et al., 2013). Several thalamic nuclei send glutamatergic projections to the dorsal striatum that regulate dopamine release by modulating cholinergic interneuron activity (Alloway et al., 2017; Brown et al., 2010; Cover et al., 2019). Parafascicular nucleus projections to the DMS are involved in goal-directed behavior and modulating behavioral flexibility (Alloway et al., 2017; Brown et al., 2010). Thalamic projections to the cortex are also involved in goal-directed behavior (Alcaraz et al., 2018; Bradfield et al., 2013; La Terra et al., 2022; Stayte et al., 2021). In addition, the parafascicular nucleus and the

posteromedial nucleus relay sensory information to the DLS, and therefore have been suggested to be involved in habitual, stimulus-response learning, but their direct roles in habitual cocaine seeking have yet to be investigated (Alloway et al., 2017).

The complex interactions between these brain regions and their overlapping roles in different aspects of learning and memory make the identification of specific neural circuits very difficult. Still, advancing neuroscientific methods that allow for the isolation of specific projections, the visualization of neurotransmitter release, and the identification of distinct neuronal ensembles will help overcome some of these challenges. As our understanding of the neural circuitry involved in the learning and memories that contribute to SUDs grows, it becomes increasingly likely that we will be able to develop therapeutics that target these maladaptive memories (Belin-Rauscent et al., 2016; Milton and Everitt, 2012; Rich and Torregrossa, 2018; Taylor et al., 2009; Walsh et al., 2018).

1.4 Targeting maladaptive memories to treat SUDs

The idea that SUD treatment may require targeting maladaptive learning and memory has been discussed for many years (Díaz-Mataix et al., 2011; Milton and Everitt, 2012; Taylor et al., 2009; Walsh et al., 2018). Current treatment options for SUDs are limited, and their efficacy is often variable (Bentzley et al., 2021). There are currently no FDA-approved medications for CUD, so treatments are often psychosocial, such as cognitive behavioral therapy (CBT) or contingency management (Bentzley et al., 2021; Buchholz and Saxon, 2019). Although they can be effective for some individuals, large populations of patients are still in need of other treatment options

(Bentzley et al., 2021; Buchholz and Saxon, 2019). Therefore, combining medications with behavioral treatment may improve overall outcomes (Buchholz and Saxon, 2019).

1.4.1 Drug-associated cues and relapse

Several of the brain regions that play a role in cocaine memories in preclinical studies have been examined in individuals with CUD or SUDs (Jasinska et al., 2014). Particularly, the impact of drug-associated cues and the cravings they induce on brain activity have been extensively studied (Jasinska et al., 2014; Kantak and Nic Dhonnchadha, 2011). These studies typically use positron emission tomography (PET) or functional magnetic resonance imaging (fMRI) to measure changes in blood flow, glucose metabolism, or dopamine receptor availability in different regions of the brain as proxy measurements of neural activity or dopamine release (Jasinska et al., 2014). Brain activity in response to images or videos of drug-associated cues or words or scripts that describe drug use is compared to that evoked by neutral stimuli, often in both drug users and control subjects (Jasinska et al., 2014). These studies have shown increased cocaine cue-induced activity in the midbrain, NAc, dorsal striatum, amygdala, OFC, medial frontal cortex, dorsolateral prefrontal cortex, insula, anterior cingulate cortex, hippocampus, and thalamus (Childress et al., 1999; D'Amour-Horvat et al., 2022; Grant et al., 1996; Kilts et al., 2001; Maas et al., 1998; Prisciandaro et al., 2013a; Ray et al., 2015; Volkow et al., 2006; Wang et al., 1999; Wexler et al., 2001).

Moreover, the cue-induced activity of many of these brain regions has been associated with increased subjective reports of cocaine craving, including in the amygdala, thalamus, OFC, dorsolateral prefrontal cortex, insula, and anterior cingulate cortex (Bonson et al., 2002; Kilts et al., 2001; Maas et al., 1998; Ray et al., 2015; Wang et al., 1999). Additionally, studies have shown

increased dopamine release in the dorsal striatum in response to cocaine-associated cues, which was correlated with increased cocaine craving (Volkow et al., 2006; Wong et al., 2006). Interestingly, the onset of cue-induced craving in cocaine-dependent subjects resulted in increased activity in brain regions usually activated by sad videos, which suggests that cocaine craving also induces a negative affect (Wexler et al., 2001). Because cocaine-associated cues induce cravings that can lead to relapse, therapeutics that extinguish these Pavlovian cocaine-cue associations have been proposed as a strategy for reducing cue-induced craving and relapse (Conklin and Tiffany, 2002).

1.4.2 Cue exposure therapy

Extinction of Pavlovian cocaine-cue associations through repeated cue exposure in the absence of cocaine, a preclinical model of cue exposure therapy, leads to reduced cue-induced drug seeking in rodent models of cocaine self-administration (Madsen et al., 2017; Murray et al., 2012; Perry et al., 2016; Rich et al., 2019; Torregrossa et al., 2013). However, despite strong preclinical support for the success of Pavlovian cue extinction, cue exposure therapy has shown mixed results for reducing relapse to cocaine and other drug use (Conklin and Tiffany, 2002; Mellentin et al., 2017). In these experiments, participants with histories of drug use were exposed to drug-associated cues repeatedly, often in multiple sessions, in an attempt to replicate preclinical findings that these exposures can extinguish drug-cue associations (Conklin and Tiffany, 2002; Mellentin et al., 2017). Meta-analyses indicate that cue exposure treatments have only been modestly successful and point to potential areas of improvement (Conklin and Tiffany, 2002; Mellentin et al., 2017).

Multiple challenges have emerged in the application of Pavlovian extinction to clinical practice (Conklin and Tiffany, 2002). One prominent challenge is that cue extinction's effects appear to be context specific, limiting the efficacy of cue extinction outside the clinic (Bechard and Knackstedt, 2019; Parker et al., 2006; Torregrossa et al., 2010). Systemic and intra-NAc core partial NMDA receptor agonism with DCS after cue extinction reduces the context specificity of cue extinction for cocaine-associated cues in rodents, which indicates a possible avenue for overcoming the context specificity of cue extinction (Torregrossa et al., 2013, 2010). Still, the use of DCS to enhance the effects of cue exposure therapy has not yielded promising results, which suggests other or additional strategies may be necessary (Das and Kamboj, 2012; Prisciandaro et al., 2013b; Santa Ana et al., 2015). Because virtual reality exposure therapy has shown promising results for anxiety disorders, it is possible that methods using virtual reality could overcome these limitations by allowing for the simulation of non-clinical contexts (Powers and Emmelkamp, 2008).

1.4.3 Maladaptive habits and compulsive behavior

Along with the challenge of context specificity of cue exposure therapy, other factors may complicate the treatment of CUDs. Extensive cocaine use can result in weakened executive control, impaired working memory, and the development of habitual behavior (Belin-Rauscent et al., 2016; Gobin et al., 2019; Goldstein and Volkow, 2011; Mittenberg and Motta, 1993). Several limitations in the study of habitual behaviors in SUDs have been noted, but there is evidence that maladaptive habit learning and over-reliance on habitual control of behavior contribute to SUDs (Doñamayor et al., 2022; Houwer, 2019; Watson and de Wit, 2018). Individuals with an SUD outperformed controls in a stimulus-response behavioral task and also showed reduced behavioral

flexibility (McKim et al., 2016). Additionally, patients with alcohol use disorder (AUD) showed enhanced reliance on stimulus-response learning correlated with increased activity of the posterior putamen and decreased activity of the anterior putamen and ventromedial prefrontal cortex, brain regions implicated in habitual and goal-directed control, respectively (Sjoerds et al., 2013; Tricomi et al., 2009).

In individuals with CUD, there is also evidence for impaired goal-directed control of behavior in the learning of a new experimental task not related to drug use (Ersche et al., 2016). In a similar task, CUD patients were less sensitive to contingency degradation, and a longer history of cocaine use was associated with reduced sensitivity, which suggests increased reliance on habitual behavior (Ersche et al., 2021). Additionally, impaired glutamatergic signaling in the putamen was associated with reports of more automatic, stimulus-driven drug behaviors in patients with CUD, which may reflect impaired cortical control of habitual behaviors (Ersche et al., 2021). Future studies could focus on drug-related habits, as most of these experiments evaluated learning and behavior of a task unrelated to drug use.

An imbalance between goal-directed and habitual control has been proposed as the primary contributor to compulsive drug use, or the persistence of drug use despite negative consequences (Belin-Rauscent et al., 2016; Everitt, 2014; Lüscher et al., 2020; Vandaele and Janak, 2018). Although compulsive behavior is a prominent feature of SUDs, compared to the vast cocaine self-administration literature, few studies involve pairing negative consequences with cocaine. Still, there is evidence that cocaine seeking and self-administration can persist despite punishment, and prolonged or intermittent exposure to cocaine can enhance this perseverance of behavior (Deroche-Gamonet et al., 2004; James et al., 2019; Pelloux et al., 2013; Vanderschuren and Everitt, 2004). Additionally, inhibition of the DLS or CeA, two regions involved in habitual cocaine self-

administration, reduces punished cocaine-seeking behavior in rats, which further implicates habit circuitry in punishment-resistant behavior (Jonkman et al., 2012; Xue et al., 2012). Importantly, persistence of drug-seeking compared to drug-taking behavior may involve divergent neural circuitry (Lüscher et al., 2020). Furthermore, one limitation of many of these preclinical experiments is that they do not sufficiently model the negative consequences of drug use in humans, which is often delayed and long-term (Jean-Richard-Dit-Bressel et al., 2018; Vanderschuren et al., 2017).

It is still highly debated if habitual and compulsive behaviors significantly contribute to SUDs (Epstein, 2020; Heyman, 2013; Hogarth, 2020). It has been suggested that the ability of individuals with SUDs to eventually quit is evidence that they maintain control over their behavior, an argument against the role of compulsive behavior in addiction (Heyman, 2013). Although this theory disregards the fact that the inability to control drug use despite a desire to stop is part of the diagnostic criteria for SUDs, it does introduce interesting ideas about how human behavior generally involves flawed decision-making processes (Belin-Rauscent et al., 2016; Heyman, 2013). Additionally, it has been proposed that SUDs arise from an excess in goal-directed choice, driven by enhanced motivation for the drug over competing reinforcers, and not from habitual behavior driven by stimulus-response associations (Hogarth, 2020). Indeed, one preclinical study showed that the development of habitual behavior dependent on the DLS is not required for the enhancement of addiction-like behaviors such as escalation of drug intake and enhanced motivation for cocaine (Singer et al., 2018). However, supporters of this theory usually provide evidence for this enhanced motivation for goal-directed behavior without considering that the contribution of goal-directed choice, habits, and compulsive behaviors to SUDs are not mutually exclusive (Epstein, 2020; Lüscher et al., 2020). Because several types of associative memories are

formed during drug use and can contribute to drug-seeking behavior, they all likely play some role in SUDs, and these potential roles should all be investigated (Epstein, 2020; Vandaele and Janak, 2018).

Overall, much of the preclinical research targeting maladaptive cocaine-related memories involves experimenter-administered cocaine or short-term drug self-administration that facilitates goal-directed drug-seeking behavior, which cannot fully encompass the long-term effects of cocaine on memory functions and behavior that likely occur in humans with SUDs (Belin-Rauscent et al., 2016). Therefore, continued research should evaluate the formation and extinction of Pavlovian associations, as well as how these associations may guide behavior, in animal models that focus on under-studied aspects of addiction, such as extended or intermittent cocaine self-administration and habitual and compulsive drug use.

1.4.4 Age and sex considerations

Despite ample evidence for sex differences in drug use and susceptibility to developing SUDs in humans, most preclinical experiments examining cocaine-related memories have been conducted exclusively in adult, male rodents (Becker, 2016; Quigley et al., 2021). Therefore, little is known about how drug-related associative memories change throughout development and differ between sexes. Evidence suggests that adolescents are more sensitive to cocaine reward and less susceptible to extinction (Brenhouse and Andersen, 2008; Holtz and Carroll, 2015; Wong et al., 2013). Adolescent rats are quicker to acquire, take more, and have more inelastic economic demand for cocaine during self-administration than adults (Lynch, 2008; Wong et al., 2013). Additionally, adolescent rats are less sensitive to punishment when self-administering cocaine compared to adults (Holtz and Carroll, 2015). In both adolescents and adults, females acquire self-

administration more quickly and show a higher demand for cocaine under progressive ratios (Algallal et al., 2020; Lynch, 2008).

Age and sex differences in the rewarding effects of cocaine may result in altered formation of cocaine-related memories. Age and sex have a clear effect on formation of cocaine-context associations (Bobzean et al., 2010; Brenhouse and Andersen, 2008; Zakharova et al., 2009a, 2009b). In addition, exposure to cocaine in adolescent rats differentially impacts habit formation in adulthood in males and females (DePoy et al., 2016). Sex differences have also been established in cocaine-cue-induced neuronal activity and cue-induced reinstatement of cocaine-seeking, and some of these sex differences may be attributed at least in part to differential effects of gonadal hormones (Fuchs et al., 2005; Zhou et al., 2014). Overall, the interaction between age, sex, and the formation of cocaine-related associative memories is important to consider when these memories are targeted for treatment.

1.5 Dissertation aims

SUDs involve several complex behaviors, and it is important to understand how different learning and memory mechanisms contribute to these behaviors and promote relapse. Furthermore, uncovering the specific neural circuits involved in these learning and memory mechanisms and determining how they interact will allow for the development of future treatments that specifically target multiple aspects of drug-seeking behavior. Discrete and contextual stimuli, or cues, can drive drug-seeking behavior through their association with the drug of abuse (Everitt and Robbins, 2005; Gruber and McDonald, 2012; Shiflett and Balleine, 2011). Therefore, extinction of drug-cue associations through cue exposure therapy may reduce drug cravings and relapse. Despite

promising evidence that cue extinction reduces cue-induced drug seeking in animal models, several challenges remain in its successful clinical translation (Conklin and Tiffany, 2002; Madsen et al., 2017; Mellentin et al., 2017; Perry et al., 2016; Rich et al., 2019; Torregrossa et al., 2013; Troisi, 2013). One contributing factor to this lack of translation may be the development of habitual drug-seeking behavior, when the behavior is no longer driven by its association with the drug, but by the cues with which it is associated (Leong et al., 2016; Smith and Laiks, 2017). Preclinical evaluations of cue extinction have only used rodent cocaine self-administration models using FR schedules that promote goal-directed drug seeking (Madsen et al., 2017; Murray et al., 2012; Perry et al., 2016; Rich et al., 2019; Torregrossa et al., 2013). Additionally, cue extinction reduces drug seeking through its effects on the BLA, a structure that is not involved in well-established habitual drug seeking (Murray et al., 2015; Rich et al., 2019). Finally, the effects of cue extinction on compulsive behavior that persists in the face of punishment are also unknown.

Therefore, the overall goal of this dissertation is to determine how Pavlovian cue extinction differentially affects goal-directed, habitual, and punishment-resistant drug seeking and the neural circuits involved. We hypothesize that punishment-resistant and habitual cocaine-seeking behaviors and their underlying neural circuitry will be unaffected by Pavlovian cue extinction. We utilize FR and SO schedules of reinforcement to promote putatively goal-directed and habitual cocaine self-administration, respectively. Additionally, we utilize intermittent cocaine self-administration to facilitate punishment-resistant cocaine self-administration. Using a combination of behavioral, pharmacological, molecular, and fiber photometric techniques, we determine (a) if cue extinction differentially affects goal-directed, habitual, and punishment-resistant drug seeking, (b) how these different behavioral strategies impact protein expression and *in vivo* calcium and dopamine activity in the DMS and DLS, and (c) how calcium and dopamine activity in the DMS

and DLS are affected by cue extinction. The results from these experiments reveal important differences between the role of Pavlovian cocaine-cue associations in goal-directed and habitual drug-seeking behavior, indicate overlap between habitual and punishment-resistant behaviors, and suggest that future treatments for SUDs should use multiple approaches to target these distinct contributions to cue-induced craving and relapse.

2.0 Dorsolateral striatum dopamine-dependent cocaine seeking is resistant to Pavlovian cue extinction in male and female rats

This chapter is adapted from the following published manuscript:

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2.1 Introduction

Drugs of abuse influence learning and memory systems, and drug-related memories are thought to contribute to continued drug use and relapse (Milton and Everitt, 2012; Torregrossa et al., 2011). Drug use begins as a goal-directed action, driven by knowledge that a behavior will procure the drug (Belin-Rauscent et al., 2012). Under certain conditions, drug seeking can become a stimulus-response habit, when the behavior is driven by environmental stimuli (Leong et al., 2016; Murray et al., 2012; Zapata et al., 2010). The involvement of stimulus-response behaviors in substance use disorders (SUDs) has been widely debated, but complex human drug seeking likely involves both response-outcome and stimulus-response behaviors (Everitt and Robbins, 2005; Hogarth et al., 2019; Watson and de Wit, 2018; Woodhead and Robbins, 2017). Drug seeking initiated by response-outcome or stimulus-response associations can be distinguished behaviorally or through pharmacological manipulation of distinct anatomical structures (Corbit et al., 2014; DePoy et al., 2016; Murray et al., 2015; Murray et al., 2012; O'Hare et al., 2018). Response-outcome, also known as goal-directed behaviors rely on dopamine in the dorsomedial striatum (DMS), whereas habitual behaviors initiated by stimulus-response associations rely on

dopamine in the dorsolateral striatum (DLS) (Corbit et al., 2014; Faure et al., 2005; Hodebourg et al., 2019; Murray et al., 2015; Murray et al., 2012; Yin and Knowlton, 2004; Zapata et al., 2010). Goal-directed and habitual behaviors also rely on distinct amygdala nuclei, where inhibition of the basolateral amygdala (BLA) reduces DLS-independent cocaine seeking, while inhibition of the central amygdala (CeA) reduces DLS-dependent cocaine seeking (Murray et al., 2015).

Pavlovian associations between environmental cues and drug reinforcement promote drug-seeking behavior and involve synaptic plasticity in the BLA (Bender and Torregrossa, 2020; M. Feltenstein and See, 2007; Rich et al., 2019). Drug-associated cues can themselves reinforce drug-seeking behavior or non-drug-related behaviors through conditioned reinforcement (Di Ciano and Everitt, 2004). Likewise, exposure to drug-associated cues can induce craving and relapse in patients with SUDs (Carter and Tiffany, 1999; Grant et al., 1996; Wang et al., 1999). Cue exposure therapy (CET), which involves repeated unreinforced exposure to drug-associated cues, is a proposed behavioral treatment for SUDs (Conklin and Tiffany, 2002). Several preclinical studies support the efficacy of cue extinction, an animal model of CET (Kearns et al., 2012; Madsen et al., 2017; Perry et al., 2016; Rich et al., 2019, 2016; Rich and Torregrossa, 2018), but clinical applications have yielded mixed results (Conklin and Tiffany, 2002; Mellentin et al., 2017; Taylor et al., 2009). One reason for the lack of translation is likely due to the context dependency of extinction learning (Kantak and Nic Dhonnchadha, 2011; Rich et al., 2019; Torregrossa et al., 2010). Yet, another factor could be that human SUDs often involve inflexible, habitual behaviors that may be initiated by the cue even if the association between the cue and the drug have been extinguished (Belin-Rauscent et al., 2016; Everitt, 2014; Everitt et al., 2018; Sjoerds et al., 2013; Volkow et al., 2006).

Our lab has shown that the effects of cue extinction are mediated by depotentiation of BLA synapses (Rich et al., 2019). Combined with the knowledge that DLS-dependent, habitual behavior no longer relies on the BLA (Murray et al., 2015), this evidence led us to hypothesize that DLS-dependent drug seeking would be resistant to cue extinction that otherwise effectively reduces drug seeking. Here, we use fixed-ratio (FR) and second-order (SO) schedules of reinforcement to facilitate DLS dopamine-independent, or -dependent, putatively habitual cocaine self-administration, respectively, in male and female rats. We evaluated the effects of cue extinction on cue-induced drug seeking and compared the expression of proteins involved in glutamate-dependent plasticity in the dorsal striatum. Finally, we determined how the restoration of response-outcome control via DLS glutamate antagonism impacted the efficacy of cue extinction. Our results replicate findings that cue extinction reduces cue-induced drug seeking in FR-trained rats, and provide the first evidence that SO-trained rats, using DLS dopamine-dependent response strategies to self-administer cocaine, are resistant to cue extinction unless response-outcome goal-directed control is restored. These findings have important implications for the use of CET in SUD treatment.

2.2 Methods

2.2.1 Animals

Adult Sprague-Dawley rats (Envigo) weighed ~275 (male) or ~200 g (female) upon arrival (n=168; male n=120; female n=48). Animals were pair-housed in auto-ventilated racks with automated watering in a temperature- and humidity- controlled room maintained on a 12-hour

light-dark cycle and had *ad libitum* access to food and water. Rats were given ≥5 days to acclimate to the facility before surgical procedures, after which they were housed individually. Rats were food-restricted 24 hours before the start of training, and were maintained at ~90% of their free-feeding body weight. Behavioral experiments were run in the light cycle and began within ~3 hours of the same time of day. Procedures were conducted in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

2.2.2 Drugs

Cocaine hydrochloride (graciously provided by NIDA) was dissolved at 2 mg/ml in 0.9% sterile saline (Thermo Fisher) and filter-sterilized. *Cis*-flupenthixol hydrochloride (Cayman Chemical Company) was dissolved at 20 μg/μl in ddH_2O. NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline, Cayman Chemical Company) was dissolved at 0.3 and 1 μg/μl in 0.9% sterile saline.

2.2.3 Behavioral apparatus

Experiments were conducted in 24 standard operant conditioning chambers (MedAssociates) using MedPC software (MedAssociates). Each animal underwent all training and testing in the same chamber. Each chamber was equipped with bar floors, a house light, two cue lights above two levers, a tone generator, a head-entry magazine, and a syringe pump connected to a swiveled leash and housed in a sound-attenuating box with a fan for background noise. All boxes had 2 plexiglass walls, one wall containing the levers, magazine, and cue lights, and another

wall containing nose-poke apertures. Half of the boxes were equipped with 2 nose-poke apertures, while the other half were equipped with 5 nose-poke apertures with a removable opaque plexiglass cover.

2.2.4 Surgery

2.2.4.1 Anesthesia

Rats were fully anesthetized via intramuscular injections of ketamine (87.5-100 mg/kg, Henry Schein) and xylazine (5 mg/kg, Butler Schein) and were then given subcutaneous injections of the analgesic Rimadyl (5 mg/kg; Henry Schein) and 5 ml of Lactated Ringer's solution. Surgical sites were shaved and betadine (povidone iodine, 5%; Henry Schein) and 70% ethanol were applied to all incision sites as previously described (Rich et al., 2019).

2.2.4.2 Intravenous catheterization

All rats were implanted with a chronic indwelling intravenous catheter into the right jugular vein and fed subcutaneously to exit the midscapular region where a bent cannula (PlasticsOne) exited through a round incision, as previously described (Torregrossa and Kalivas, 2008). Catheters were capped to prevent blockage.

2.2.4.3 Intracranial cannulation

Immediately following jugular vein catheterization, rats used in experiments involving intra-DLS infusions were placed in a stereotaxic frame. A small injection of lidocaine (0.3-0.4 ml; Henry Schein) was used as a local anesthetic to the scalp. Two 22-gauge guide cannula (cut 6 mm below an 8 mm pedestal, PlasticsOne) were implanted bilaterally, aimed 1 mm dorsal to the

anterior DLS (in mm from Bregma, anterior and posterior (AP): +0.8; medial and lateral (ML): +/-3.0; dorsal and ventral (DV): -4.0)) and secured to the skull with 3 screws and OrthoJet dental cement (Lang Dental). Once dry, dummy cannulae (C313DC, PlasticsOne) the length of the guide cannulae were inserted to prevent obstruction.

2.2.4.4 Post-operative care

On the two days following surgery, rats were administered Rimadyl (5 mg/kg) subcutaneously. Catheter patency was maintained by daily infusion of 0.2-0.4 ml of a 0.99% sterile saline solution containing Gentamicin (3 mg/ml; Henry Schein), heparin (30 USP/ml; Henry Schein), and, for the week following surgery only, streptokinase (9.33 USP/ml; MP Biomedicals).

2.2.5 Behavioral procedures

2.2.5.1 Cocaine self-administration

Rats were trained to self-administer cocaine (1mg/kg/infusion) in 1-hour daily sessions. Each session began with the illumination of the house light, insertion of the active and inactive lever (counterbalanced between animals), and start of the fan. All cocaine infusions were paired with a 20-second audiovisual cue of a tone and the illumination of the cue light above the active lever, and initiated a 20-second time-out period when the house light was extinguished and levers retracted. Inactive lever presses were recorded, but had no programmed consequence. Each session was terminated after 1 hour or the delivery of 30 infusions.

Rats were trained on schedules designed to facilitate different behavioral strategies during self-administration using a protocol modified from experiments previously described (Murray et al., 2015; Murray et al., 2012). All rats were initially trained to self-administer cocaine on a fixed-

ratio 1 (FR1) schedule for 7 days, and then on an FR3 schedule for 3 days. **Figure 7A** illustrates how rats were then divided into groups that were either trained to maintain response-outcome drug seeking (FR-trained) or to develop DLS dopamine-dependent behavior through second-order (SO) schedule training (SO-trained). Following the three FR3 training sessions, the FR-trained rats were maintained on an FR3 schedule for an additional 5 days and were then switched to an FR5 schedule for 5 more days to increase responses required for each infusion. The SO-trained rats were switched to an SO schedule for a total of 10 days, with 5 days on an FR5(FR2S) followed by 5 days on an FR7(FR2S) schedule. Rats were matched for responding before splitting into FR-trained and SO-trained groups. Based on our own pilot experiments and evidence that just two weeks of training on an FR10(FR4S) SO schedule facilitates DLS dopamine-dependent cocaine seeking, we chose this level and time-course of training to maximize acquisition and catheter patency while minimizing experiment length and differences in responding between FR- and SO-trained rats (Murray et al., 2015).

Under an FR1 schedule, each active lever press resulted in a cocaine infusion paired with the audiovisual cue and the initiation of the time-out period. Under an FR3 and FR5 schedule, every third or fifth lever press, respectively, resulted in an infusion, the cue, and the time-out period. Under an FR5(FR2S) SO schedule, every two lever presses (indicated by FR2S) resulted in a short 1-second presentation of the audiovisual cue, and the 5th completion of this schedule (indicated by FR5), or a total of 10 lever presses, resulted in cocaine delivery, the audiovisual cue, and time-out period. Lever presses during the 1-second cue presentations were recorded, but did not contribute toward the completion of the schedule. Under an FR7(FR2S) schedule, every two lever presses resulted in a short 1-second cue presentation, and the cue, cocaine, and time-out occurred after the seventh completion of that schedule (14 total lever presses).

2.2.5.2 Drug-seeking tests

For the experiment involving intra-DLS infusion of *cis*-flupenthixol, separate groups of rats underwent a 15-minute drug-seeking test immediately before self-administration on day 9 (during FR training) or day 13 (during SO training) (**Figure 6A**). Rats were given bilateral intra-DLS infusions of vehicle (ddH_2O) or *cis*-flupenthixol (10 μg) 5 minutes prior to testing, a timepoint for which this dose of *cis*-flupenthixol has previously been shown to reduce habitual drug seeking (Murray et al., 2015; Murray et al., 2012). Rats then began the 15-minute drug-seeking test, during which cues and time-outs were presented contingently as previously described on the current training schedule (FR3 for day 9; FR5(FR2S) for day 13), but cocaine was withheld. Lever presses were recorded, and a 1-hour self-administration session immediately followed to prevent extinction learning.

2.2.5.3 Pavlovian cue extinction

On the day immediately following the final day of self-administration, rats underwent Pavlovian cue extinction or a control procedure, during a 1-hour session when 0 or 120 20-second audiovisual conditioned stimuli (CSs), separated by 10 seconds, were presented non-contingently in the same context as self-administration, with levers retracted.

2.2.5.4 Cue-induced drug-seeking test

One day following cue extinction, rats underwent a 1-hour cue-induced drug-seeking test, during which cues were presented contingently on the rat's previous training schedule, but no cocaine was delivered. In one experiment (**Figure 9A**), rats received intra-DLS infusions of either vehicle (0.9% bacteriostatic saline) or NBQX (0.3 or 1 μg/μl) 5 minutes prior to the start of the session. Although this cue-induced drug-seeking test is typically referred to as cue-induced

reinstatement, these rats did not undergo typical instrumental extinction to avoid its potential degradation of stimulus-response associations important for the maintenance of DLS-dependent behaviors. Previous findings suggest that cue extinction's behavioral and biological effects are not dependent on instrumental extinction (Rich et al., 2019), but in order to uphold precise terminology, we will refer to this test as a cue-induced drug-seeking test because of the omission of instrumental extinction. Rats used for the drug-seeking tests after *cis*-flupenthixol or vehicle were also used to examine the effects of a lower dose of NBQX (0.3 μg/μl). Because there were no differences between vehicle rats between experiments, data were collapsed across cohorts for analysis.

2.2.5.5 Re-training and self-administration without cue

On the day following the cue-induced drug-seeking test, a subset of rats used in the NBQX or vehicle experiment returned to self-administration for one day of re-training on an FR7(FR2S) schedule. In order to examine if the discrete audiovisual cue was important for the maintenance of established, DLS dopamine-dependent cocaine self-administration, these rats then underwent a self-administration session where the discrete audiovisual cue was removed, but the same number of lever presses resulted in a cocaine infusion. In this session, 14 lever presses (FR14, no cues) resulted in a cocaine infusion and a 20-second time-out period, during which the house light was extinguished and the levers were retracted, but no discrete audiovisual cue was presented.

2.2.5.6 Conditioned reinforcement

The same rats that were re-trained and used for self-administration without cues were then used to examine the reinforcing properties of the discrete audiovisual cue. Rats were returned to the self-administration context and underwent conditioned reinforcement training. During these

sessions, the house light was illuminated, but levers were retracted. Head entries into the active nose-poke aperture (counterbalanced) resulted in presentation of discrete cues on an FR7(FR2S) schedule, with no cocaine infusions. Cues were presented for 1 second after every two nose-pokes and for 20 seconds upon the completion of the second order schedule on the same cue light above the previously active lever. In operant conditioning chambers containing 5 nose-poke apertures, the left-most and right-most apertures were used as the active and inactive nose pokes. Active nose pokes were recorded, and inactive nose pokes were recorded after the first active nose poke because prior to the first active nose poke the rats have no knowledge of the contingencies in place on either aperture, which avoids the possibility that a rat could spend the majority of the session poking the inactive aperture without discovering that the active aperture would produce the cue, which would then skew the results. Rats were matched for responding and underwent a second cue extinction procedure on the following day as described above (counterbalancing for previous cue extinction group), and on the following day underwent conditioned reinforcement testing, which did not differ from the training session.

2.2.6 Intra-DLS infusions

For experiments involving intra-DLS infusions, drug or vehicle was infused through a 28-guage internal cannula extending 1 mm below the guide cannula into the DLS using a 10 µl Hamilton syringe connected to a syringe pump (Harvard Apparatus). *Cis*-flupenthixol was infused at 0.33 µl/min for 90 seconds. NBQX was infused at 0.3 µl/min for 60 seconds. Internal cannulae were left in place for 1 minute following infusion.

2.2.7 Western blot analysis

2.2.7.1 Sample preparation

Rats used in the 0 vs. 120 cue extinction experiment were killed by decapitation immediately after the cue-induced drug seeking test. Brains were flash-frozen in isopentane on dry ice and kept at -80 °C. The anterior DLS (AP +2 mm to +0.8 mm) and posterior DMS (AP +1 mm to -0.4 mm) were dissected over dry ice from coronal sections made ~1 mm thick on a stainless steel brain matrix (Braintree Scientific). We chose to focus our analysis on the anterior portion of the DLS and the posterior portion of the DMS based on previous publications suggesting this distinction is important (Furlong et al., 2018; Yin et al., 2005b). Tissue was fractionated into membrane- and non-membrane-bound components as previously described (Bañuelos et al., 2014). Protein concentrations for each sample were determined using a Thermo Scientific Pierce Micro BCA Protein Assay (Thermo Fischer Scientific) as previously described (Kirschmann et al., 2017). For each sample, 20 μg of protein was diluted in 30 μl containing 8 μl of sample buffer (a 9:1 mixture of 4x Laemmli protein sample buffer [Bio-Rad] to 2-Mercaptoethanol [Millipore Sigma]).

2.2.7.2 Immunoblotting

Membrane fractions were resolved on ice by SDS-PAGE on 4-12% Tris-glycine gels (Invitrogen) and electrophoretically transferred to a PVDF membrane (Immuno-Blot PVDF Membrane, Bio-Rad). Membranes were blocked for 1 hour at room temperature by incubation in 5% bovine serum albumin (Thermo Scientific Pierce) and then incubated overnight at 4 °C with specific primary antibodies (Millipore) against the following proteins: NMDA receptor subunits GluN2A (1:1000, 04-901) and GluN2B (1:1000, 05-920), AMPA receptor subunits GluA1

(1:1000, MAB2263) and GluA2/3 (1:500, 07-598), vesicular glutamate transporter VGluT1 (1:1000, ABN1647), and β-actin (1:1000, Cell Signaling Technology, 3700S). Antigen binding was visualized by incubating membranes for 1 hour at room temperature in secondary fluorescent antibodies (IRDye 800 CW anti-rabbit, 1:5000, LI-COR Odyssey, 926-32211; IRDye 680 CW anti-mouse, 1:5000, LI-COR Odyssey, 926-68070). All antibodies were diluted in blocking solution (1:1 LI-COR Odyssey blocking buffer to 1x PBST). Protein expression was quantified using LI-COR Odyssey imaging and ImageStudio software. Each sample was normalized to its own β-actin expression, and expression for the SO-trained group was normalized to average levels of the FR-trained group within each gel.

2.2.8 Histology

Rats with cannula were killed by CO_2 followed by decapitation, and brains were removed and immersed in 10% buffered formalin phosphate (Fisher Chemical) for ~24 hours. Brains were then moved to a cryoprotectant solution (30% sucrose) for ≥2 days before they were frozen and sectioned on a cryostat (Leica CM1950) at 50 μm. Every third section was immediately mounted on a slide, and cannula tracts were visualized and their location noted. Histological misses were characterized by placement more than 2.2 mm anterior to Bregma, less than 0.6 mm anterior to Bregma, dorsal to the striatum, or medial or lateral to the DLS.

2.2.9 Exclusion criteria

Rats were excluded from analysis due to death or illness after surgery (n=7), histological misses in drug-treated rats (n=8), failure to acquire cocaine self-administration (n=10) (>4

infusions on the final day of self-administration, note that the low infusion number is due to the high response requirement), or loss of catheter patency (n=33) (determined by a 0.1 ml intravenous infusion of 10 mg/ml sodium brevital at the end of self-administration training). Note that the high rate of loss due to catheter patency was due to the development of a new catheter system in the lab at the time. Attrition rates are not commonly that high, and were improved by the addition of streptokinase (9.33 U/ml) to the post-operative flushing solution.

2.2.10 Quantification and statistical analysis

Behavioral data were collected using MedPC software. For 2 rats, self-administration data for day 17 were unavailable due to a power outage, so data for the missing day were entered by averaging behavioral responses on day 16 and 18. When possible, experimenters were blinded to rats' treatment conditions. All statistical analyses were performed using GraphPadPrism and SPSS Statistics software. When split into groups, animals were matched for responding. For 15-minute drug-seeking tests, the ratio of responding during test to previous responding was calculated by dividing the active lever presses during test by the average number of active lever presses per 15 minutes in the previous self-administration session: active lever presses during test / (active lever presses on previous day / 4). For 1-hour cue-induced drug-seeking tests, the ratio of responding was calculated by dividing the number of active lever presses during test by the number of active lever presses in the previous self-administration session: active lever presses during test / active lever presses on previous day. We use the ratio of responding, where a ratio of 1 indicates no change in responding during the cue-induced drug-seeking test compared to the previous day of self-administration to accurately visualize, with y axes of the same scale, the effects of cue extinction on animals responding on different schedules of reinforcement that produce different

rates of responding. We additionally show active lever presses, but note that plotting raw active lever presses requires the use of y axes of different scales and occludes valuable information about how rats using different response strategies differentially respond under extinction conditions compared to during self-administration. Despite this, we show that ratio of responding and active lever presses reveal similar results, but ultimately draw our major conclusions from data showing the ratio of responding.

For all statistical analyses, significance was set at $p<0.05$. All data were determined to be normally distributed using the Shapiro-Wilk test, and Bartlett's test was used to determine that there were no significant differences in the estimated variance between groups. Infusions were analyzed by using either a two-way or three-way rmANOVA, using time as one factor and either lever, sex, training schedule, drug treatment, treatment day, or future extinction groups as the other factor(s) as indicated. Protein expression, ratio of responding, active lever presses, inactive lever presses, active nose pokes, or inactive nose pokes were analyzed using either a student's t-test (with paired measures when indicated) or a two-way ANOVA as indicated. When an interaction was detected by two-way ANOVA analysis, significant effects were further analyzed by Sidak's post-hoc multiple comparisons analysis. Student's t-tests were used to make planned comparisons between FR-trained and SO-trained rats.

2.3 Results

2.3.1 Cocaine seeking relies on DLS dopamine after training on SO, but not FR, schedules of reinforcement

To evaluate the effects of cue extinction on DLS dopamine-dependent cocaine seeking, we sought to adapt previously established methods utilizing SO schedules to facilitate the formation of DLS dopamine-dependent, putatively habitual behavior (Murray et al., 2015; Murray et al., 2012). Under SO schedules, short presentations of the drug-paired cue sustain responding over longer periods when the drug is not available. It has been proposed that SO schedules may better model the human experience of encountering drug-associated cues both in the presence and absence of drug reinforcement (Belin-Rauscent et al., 2016). Our first goal was to verify that we could replicate prior studies indicating that cocaine self-administration reinforced on an FR schedule would not require dopamine signaling in the DLS, while responding under an SO schedule would be dependent on DLS dopamine. Because these previous experiments used male rats, we used only male rats for this initial investigation.

Rats learned to self-administer cocaine (1 mg/kg/infusion) for 20 days, and their full self-administration data is included in **Figure 9B-C**, as these rats were also used for those experiments. Here, we show their self-administration data for the first 15 days, which is relevant for these experiments. Vertical gray lines on graphs indicate changes in self-administration reinforcement schedule outlined in **Figure 6A**. On days 9 or 13 respectively after either 8 (FR-group) or 12 (SO-group) days of self-administration, rats were given intra-DLS infusions of vehicle or *cis*-flupenthixol (10 μg) before a 15-minute drug-seeking test, when cocaine was unavailable (**Figure 6A**). The drug seeking test was immediately followed by the appropriate self-administration

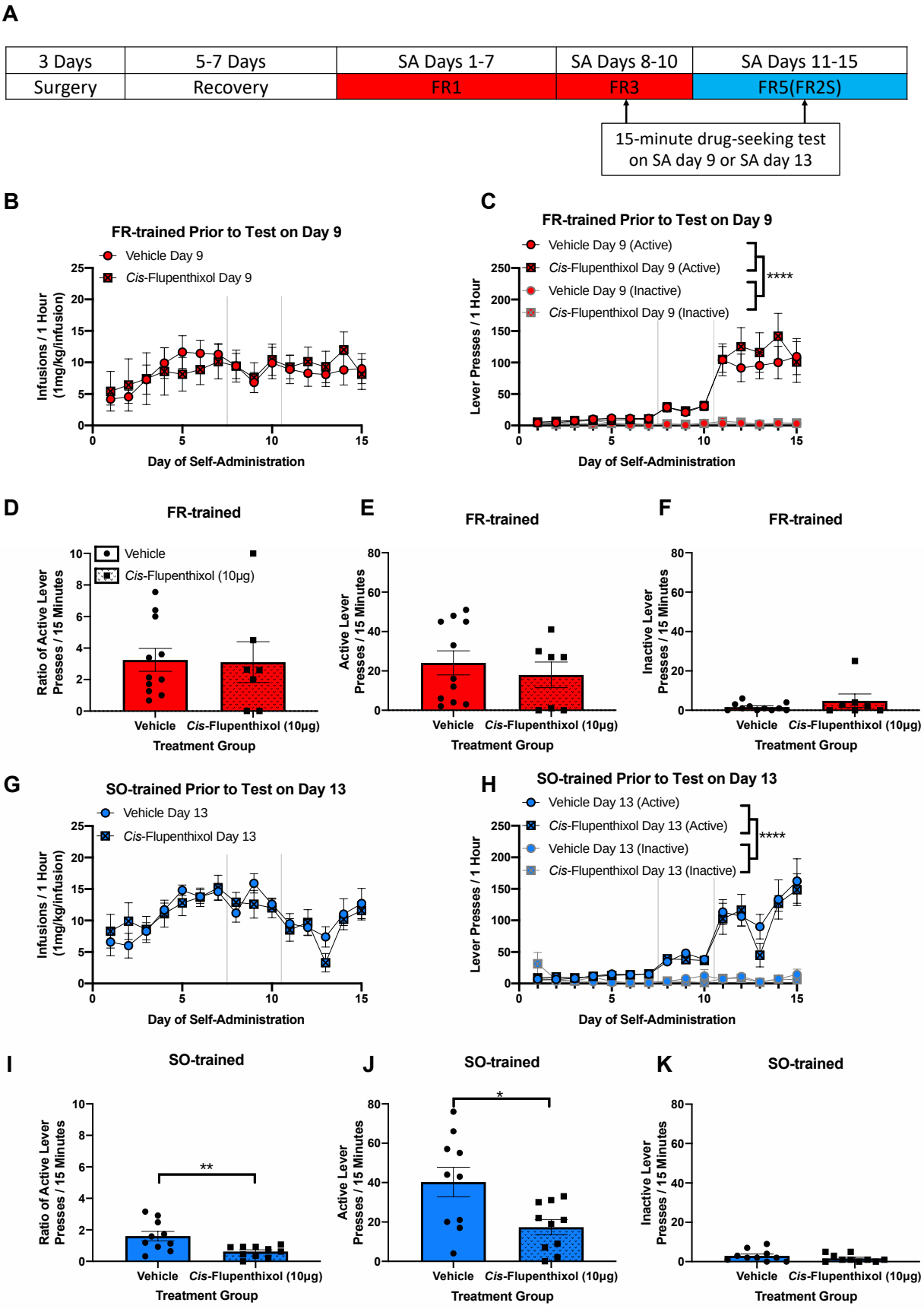
A
3 Days
5-7 Days
SA Days 1-7
SA Days 8-10
SA Days 11-15
Surgery
Recovery
FR1
FR3
FR5(FR2S)
15-minute drug-seeking test on SA day 9 or SA day 13
B
FR-trained Prior to Test on Day 9
Vehicle Day 9
Cis-Flupenthixol Day 9
Infusions / 1 Hour (1mg/kg/infusion)
Day of Self-Administration
C
FR-trained Prior to Test on Day 9
Vehicle Day 9 (Active)
Cis-Flupenthixol Day 9 (Active)
Vehicle Day 9 (Inactive)
Cis-Flupenthixol Day 9 (Inactive)
****
Lever Presses / 1 Hour
Day of Self-Administration
D
FR-trained
Vehicle
Cis-Flupenthixol (10µg)
Ratio of Active Lever Presses / 15 Minutes
Vehicle
Cis-Flupenthixol (10µg)
Treatment Group
E
FR-trained
Active Lever Presses / 15 Minutes
Vehicle
Cis-Flupenthixol (10µg)
Treatment Group
F
FR-trained
Inactive Lever Presses / 15 Minutes
Vehicle
Cis-Flupenthixol (10µg)
Treatment Group
G
SO-trained Prior to Test on Day 13
Vehicle Day 13
Cis-Flupenthixol Day 13
Infusions / 1 Hour (1mg/kg/infusion)
Day of Self-Administration
H
SO-trained Prior to Test on Day 13
Vehicle Day 13 (Active)
Cis-Flupenthixol Day 13 (Active)
Vehicle Day 13 (Inactive)
Cis-Flupenthixol Day 13 (Inactive)
****
Lever Presses / 1 Hour
Day of Self-Administration
I
SO-trained
**
Ratio of Active Lever Presses / 15 Minutes
Vehicle
Cis-Flupenthixol (10µg)
Treatment Group
J
SO-trained
*
Active Lever Presses / 15 Minutes
Vehicle
Cis-Flupenthixol (10µg)
Treatment Group
K
SO-trained
Inactive Lever Presses / 15 Minutes
Vehicle
Cis-Flupenthixol (10µg)
Treatment Group

Figure 6: Second-order schedule training facilitates the development of DLS dopamine-dependent, putatively habitual cocaine-seeking behavior

Experimental timeline for self-administration (SA) and drug-seeking tests (**A**). Rats (n=38, male) were trained to self-administer cocaine (1 mg/kg/infusion) for 15 days (**B-C**; **G-H**), and the reliance of drug-seeking on DLS dopamine was analyzed by intra-DLS infusion of the nonspecific dopamine antagonist *cis*-flupenthixol (10 μg) in a 15-minute drug-seeking test, with no cocaine available, on either SA day 9, after FR training (**B-F**), or SA day 13, after SO training (**G-K**). There was no effect of treatment group on infusions (**B, G**) or active lever presses (**C, H**) during self-administration. On SA day 9, after FR training, DLS dopamine antagonism did not affect the ratio of active lever presses during test to the previous day of SA (**D**), active lever presses (**E**), or inactive lever presses (**F**) during a 15-minute drug-seeking test. On SA day 13, after SO training, DLS dopamine antagonism reduced the ratio of active lever presses during test to the previous day of SA (**G**) and active lever presses (**H**) compared to vehicle controls, but did not affect inactive lever presses (**I**). Graphs show group means ± SEM and individual data points. Vertical gray bars indicate changes in reinforcement schedule (**B-C**). *p<0.05. **p<0.01. ****p<0.0001.

session when cocaine was available. In the first group of rats, which underwent only FR training prior to DLS infusion of vehicle or *cis*-flupenthixol before the 9th day of self-administration, analysis of daily self-administration behavior revealed no main effect of treatment group on infusions ($F_{(1,16)}$=0.001817, p=0.9665) (**Figure 6B**) (two-way rmANOVA). Active lever presses per session increased as the schedule of reinforcement required more presses per infusion, and inactive lever presses remained low. There was a main effect of training day ($F_{(14, 224)}$=25.97, p<0.0001), lever ($F_{(1,16)}$=33.81, p<0.0001), and a training day × lever interaction ($F_{(14, 224)}$=24.75, p<0.0001) (**Figure 6C**) (three-way rmANOVA). After only FR training, during the 15-minute drug-seeking test, *cis*-flupenthixol had no effect on ratio of responding (p=0.9186, η^2=0.0006736) (**Figure 6D**), active lever presses (p=0.5210, η^2=0.02621) (**Figure 6E**), or inactive lever presses (p=0.2630, η^2=0.07761) (**Figure 6F**) (independent samples *t*-tests), which indicates that lever pressing did not rely on DLS dopamine and suggests the maintenance of response-outcome behavior. Note that a subset of animals in each group expressed a low number of active lever

presses during this 15-minute test, which likely reflects individual differences in acquisition of the FR3 contingency, but these individual differences do not impact the results.

A separate group of rats was infused with *cis*-flupenthixol or vehicle on the 13th day of training, when responding had previously been reinforced on an FR5(FR2S) SO schedule. Analysis of daily self-administration behavior revealed no main effect of treatment group on infusions ($F_{(1,18)}$=0.05192, p=0.9665) (**Figure 6G**) (two-way rmANOVA). Active lever presses per session increased as the schedule of reinforcement required more presses per infusion, and inactive lever presses remained low. There was a main effect of training day ($F_{(14, 252)}$=28.57, p<0.0001), lever ($F_{(1,18)}$=74.54, p<0.0001), and a training day × lever interaction ($F_{(14, 252)}$=29.09, p<0.0001) (**Figure 6H**) (three-way rmANOVA). After SO training, during the 15-minute drug-seeking test on day 13, DLS dopamine antagonism led to a reduced ratio of responding (p=0.0071, η^2=0.3390) (**Figure 6I**) and active lever presses (p=0.0144, η^2=0.2894) (**Figure 6J**) compared to vehicle controls, but had no effect on inactive lever presses (p=0.2593, η^2=0.07010) (**Figure 6K**) (independent samples *t*-tests), which suggests that SO training facilitated the formation of DLS dopamine-dependent behavior, similar to prior results observed in other labs. Because rats were used for later experiments, DLS cannula placements for rats in this experiment are included in **Figure 9F**.

2.3.2 Cocaine seeking is resistant to cue extinction in SO-trained rats

Having established that self-administration on an SO schedule facilitates DLS dopamine-dependent cocaine seeking, we sought to compare the effect of cue extinction on cue-induced drug seeking in rats trained on either FR or SO schedules of reinforcement. In this experiment we used both males and females and all rats were trained for 20 days, with the last five days being an FR5

schedule for the FR group or an FR7(FR2S) schedule for the SO group. Rats were then divided into those that underwent cue extinction (120 CS presentations), or a control no cue extinction group (0 CS presentations), and then were given a 1-hour cue-induced drug seeking test (**Figure 7A**). During self-administration training, main effects of sex and training schedule were found, where females self-administered more cocaine than males ($F_{(1,33)}$=5.928, p=0.0205), and FR-trained rats self-administered more cocaine than SO-trained rats ($F_{(1,33)}$=4.308, p=0.0458) (three-way rmANOVA) (**Figure 7B**). When evaluated based on future extinction group and training schedule collapsed across sex, FR-trained rats again self-administered more cocaine than SO-trained rats ($F_{(1,33)}$=4.650, p=0.0384), but there were no differences between rats in to-be extinction vs. to-be control groups ($F_{(1,33)}$=0.3307, p=0.5691) (three-way rmANOVA) (**Figure 7C**). Active lever presses per session increased as the schedule of reinforcement required more presses per infusion, and inactive lever presses remained low, demonstrated by a main effect of training day ($F_{(19,1330)}$=64.7, p<0.0001), lever ($F_{(1,70)}$=170.5, p<0.0001), and a training day × lever interaction ($F_{(19,1330)}$=58.39, p<0.0001) (three-way rmANOVA) (**Figure 7D**). There was a main effect of schedule ($F_{(1,70)}$=31.49, p<0.0001) and a schedule × lever interaction ($F_{(1,70)}$=29.57, p<0.0001) (three-way rmANOVA) (**Figure 7D**).

In a cue-induced drug-seeking test after cue extinction, there was a main effect of training schedule ($F_{(1,33)}$=6.0196, p=0.0196, partial η^2=0.154), a main effect of cue extinction ($F_{(1,33)}$=4.267, p=0.0468, partial η^2=0.115), and no significant interaction ($F_{(1,33)}$=1.751, p=0.1948, partial η^2=0.050) on the ratio of active lever presses during test to active lever presses on the final day of self-administration (two-way ANOVA) (**Figure 7E**). For raw active lever presses, there was a main effect of training schedule ($F_{(8.582,)}$=8.582, p=0.0061, partial η^2=0.206), but no main effect of cue extinction ($F_{(1,33)}$=2.424, p=0.1290, partial η^2=0.068) or interaction ($F_{(1,33)}$=0.01412, p=0.9061,

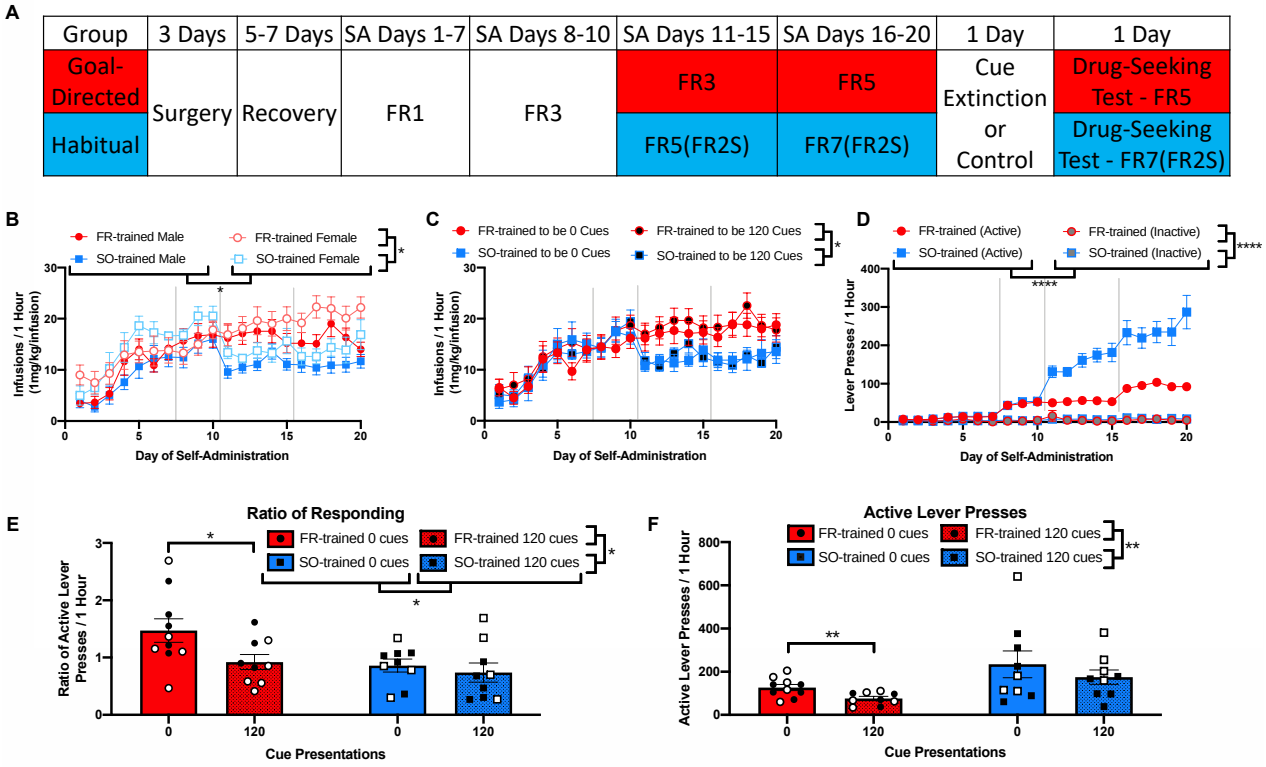

Figure 7: DLS dopamine-dependent cocaine-seeking behavior is resistant to cue extinction

Experimental timeline (**A**). Rats (n=37; 19 male, 18 female) were trained to self-administer cocaine on either FR or SO schedules of reinforcement (**B-D**). They underwent cue extinction (120 CS) or a control (0 CS) procedure followed by a cue-induced drug-seeking test (**E-F**). Females self-administered more cocaine than males (**B**) and FR-trained rats self-administered more cocaine than SO-trained rats (**B-C**), but there were no differences between rats to be assigned to different cue extinction groups (**C**). As rats learned to self-administer cocaine, their active lever presses increased, while inactive lever presses remained low, and SO-trained rats pressed the active lever more than FR-trained rats (**D**). In a cue-induced drug-seeking test, there was a main effect of cue extinction and training schedule, but no interaction, on ratio of responding (**E**), and a main effect of training schedule, but no main effect of cue extinction or interaction, on active lever presses (**F**). Planned comparisons revealed that previous cue extinction (120 CS) resulted in reduced ratio of responding (**E**) and active lever presses (**F**) in FR-trained, but not SO-trained, rats relative to a 0-cue control group. Graphs show group means ± SEM and individual data points. Open symbols indicate females, and closed symbols indicate males. Vertical gray bars indicate changes in reinforcement schedule (**B-D**). *p<0.05. **p<0.01. ****p<0.0001.

partial η^2=0.000) (two-way ANOVA) (**Figure 7F**). Planned comparisons between 0 CS controls and 120 CS extinction groups indicated an effect of cue extinction on ratio of responding in FR-trained (p=0.0426, η^2=0.2203), but not SO-trained rats (p=0.5601, η^2=0.02166) (**Figure 7E**) (independent samples *t*-tests). Planned comparisons showed that cue extinction also reduced raw active lever presses in FR-trained (p=0.0098, η^2=0.2203) but not SO-trained (p=0.4120, η^2=0.2203) (**Figure 7F**) rats (independent samples *t*-tests). These results suggest that cue extinction does not impact responding in SO-trained rats, but reduces responding in FR-trained rats. Although these planned comparisons do not correct for multiple comparisons, these findings have been replicated in multiple experiments. These experiments were not powered to detect sex differences in the effects of cue extinction, but no obvious differences between males and females were observed, as indicated by the individual, differentially shaded data points in the bars.

2.3.3 FR and SO training result in differential expression of plasticity-related proteins in the dorsal striatum

Next, we wanted to determine if there were molecular indices of altered plasticity in the DMS or DLS of SO-trained rats relative to FR-trained rats. We analyzed tissue (**Figure 8**) from the male and female rats used in the previous cue extinction experiment (n=37, **Figure 7**) by western blot analysis to compare the membrane-bound expression of NMDA receptor subunits GluN2A and GluN2B, AMPA receptor subunits GluA1 and GluA2/3, and vesicular glutamate transporter VGluT1. We chose these proteins because they have previously been reported to change in response to a methamphetamine administration procedure that facilitates habit learning (Furlong et al., 2018). In the DMS, there were no differences in the expression of GluN2A (p=0.5006, η^2=0.01526), GluN2B (p=0.6412, η^2=0.006870), GluA1 (p=0.4212, η^2=0.03260), or

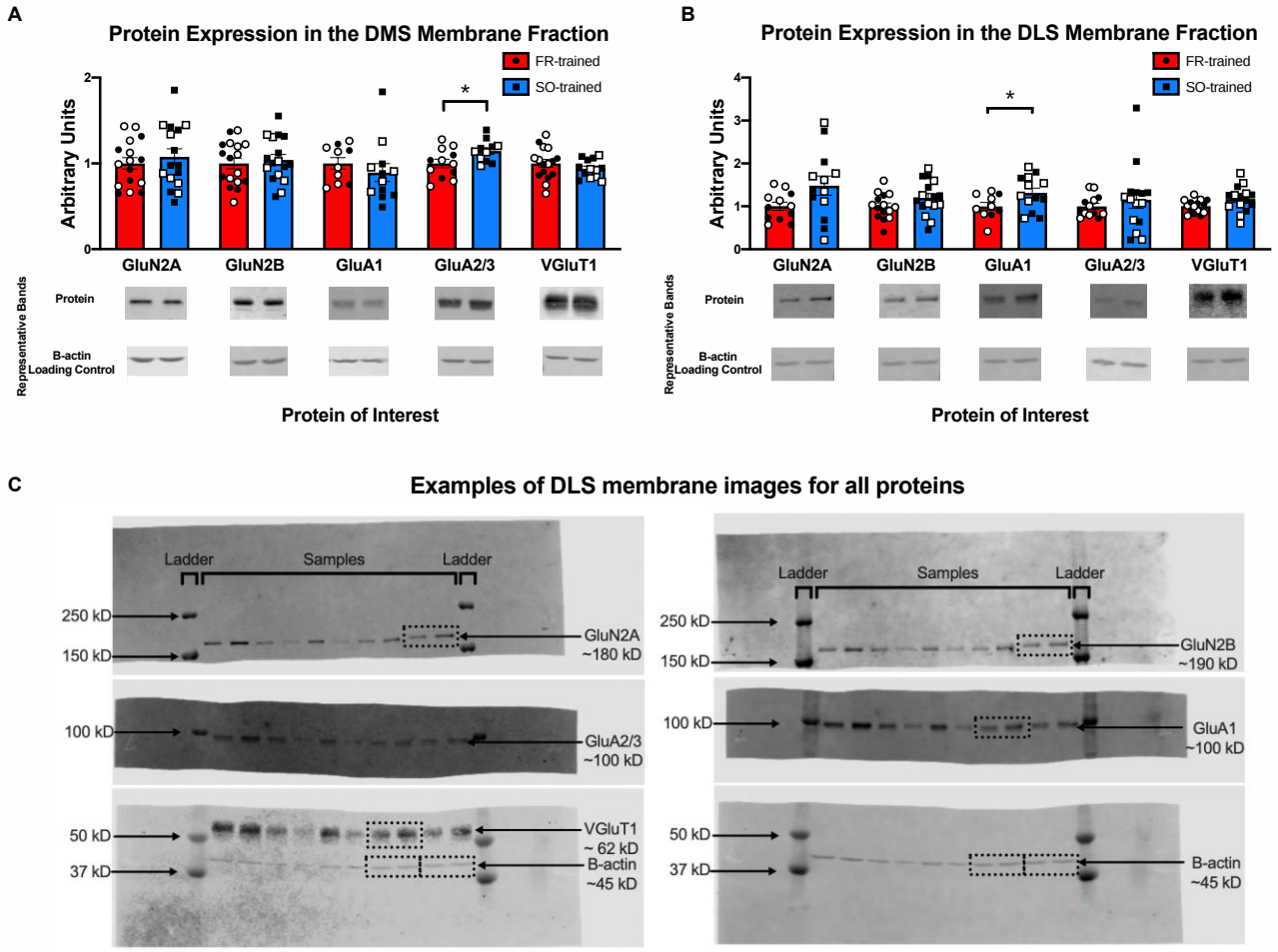

Figure 8: FR and SO training result in differential expression of plasticity-related proteins in the dorsal striatum

Western blot analysis was used to quantify the membrane-bound expression of glutamate plasticity-related proteins in the DMS and DLS in rats trained to self-administer cocaine on FR vs. SO schedules or reinforcement. In the DMS, SO-trained rats had increased expression of GluA2/3 in the membrane compared to FR-trained rats, but there were no differences in GluN2A, GluN2B, GluA1, or VGluT1 (**A**). In the DLS, SO-trained rats had significantly increased GluA1 in the membrane, but there were no differences in GluN2A, GluN2B, GluA2/3, or VGluT1 (**B**). Note that p values were not corrected for multiple comparisons. Representative bands indicate the protein of interest above the β-actin loading control for one animal in each group (**A, B**). Each protein was detected at its expected molecular weight, indicated on examples of DLS membranes with samples alongside a ladder (**C**). Dotted boxes indicate pairs of samples used as representative bands in panel B. Graphs show group means ± SEM and individual data points. Open symbols indicate females, and closed symbols indicate males. *p<0.05.

VGluT1 ($p=0.2879$, $\eta^2=0.0673$), but SO-trained rats had increased expression of GluA2/3 ($p=0.0343$ $\eta^2=0.1961$) compared to the FR-trained group (independent sample *t*-tests, not corrected for multiple comparisons) (**Figure 8A**). In the DLS, there were no significant differences in the expression of GluN2A ($p=0.0653$, $\eta^2=0.1401$), GluN2B ($p=0.1343$, $\eta^2=0.07563$), GluA2/3 ($p=0.5045$, $\eta^2=0.01723$), or VGluT1 ($p=0.0561$, $\eta^2=0.1242$), but GluA1 ($p=0.0496$, $\eta^2=0.3249$) was significantly increased in SO-trained rats (independent sample *t*-tests, not corrected for multiple comparisons) (**Figure 8B**). These results indicate that training on different reinforcement schedules may be sufficient to lead to differential expression of glutamate receptor subunits in the membrane in the dorsal striatum. There was no correlation between total cocaine exposure and protein expression for any protein in either region, and there were no differences between males and females in either region (data not shown). Each protein was detected at its expected molecular weight, as indicated by representative images of gels from samples from the DLS alongside a ladder (**Figure 8C**). Proteins with overlapping molecular weights were detected using different secondary antibodies and were imaged on the same membrane at different wavelengths.

2.3.4 Restoration of response-outcome control reveals an effect of cue extinction

We next wanted to determine if inhibiting glutamatergic signaling in the DLS would be sufficient to reveal an effect of cue extinction learning in rats trained on the habit-promoting SO schedule. We hypothesized that DLS-dependent stimulus-response associations formed during self-administration on an SO schedule resulted in the initiation and persistence of lever pressing during cue-induced drug seeking tests despite cue extinction learning; but, in the absence of DLS signaling, response-outcome responding would be restored and lever pressing would decrease after cue extinction learning. Rats completed the full 20 days of self-administration training, underwent

cue extinction (0 or 120 cues), and then their cue-induced cocaine seeking was tested after intra-DLS infusion of vehicle or the AMPA antagonist NBQX (**Figure 9A**). NBQX in the DLS has been shown previously to restore response-outcome behavior in rats that orally self-administered alcohol (Corbit et al., 2014b). Rats used in the *cis*-flupenthixol experiment (n=23, male) were initially used to evaluate the effects of vehicle or a lower dose of NBQX (0.3 μg/μl) because we were concerned that NBQX might suppress overall responding. When no overall suppression was observed, additional rats (n=35; 21 male, 14 female) were used to evaluate the effects of vehicle or a higher dose of NBQX (1 μg/μl). Because there were no differences between rats in the vehicle groups, results were collapsed for analyses. There were no differences in infusions throughout training between sexes ($F_{(1,56)}$=0.5050, p=0.4803) or between future drug treatment groups ($F_{(2,56)}$=0.01573, p=0.9844) (two-way rmANOVA) (**Figure 9B-C**). During cue-induced drug-seeking after cue extinction, after vehicle or NBQX was infused in the DLS, there was a main effect of cue extinction ($F_{(1,50)}$=7.255, p=0.0096, partial η^2=0.127), no main effect of drug treatment ($F_{(2,50)}$=0.8011, p=0.4545, partial η^2=0.031), and an extinction × drug treatment interaction ($F_{(2,50)}$=3.456, p=0.0393, partial η^2=0.121) on ratio of responding (two-way ANOVA) (**Figure 9D**). There was a main effect of cue extinction on active lever presses ($F_{(1,50)}$=7.178, p=0.0100, partial η^2=0.126), but no main effect of drug treatment ($F_{(2,50)}$=0.7278, p=0.4880, partial η^2=0.028) or interaction ($F_{(2,50)}$=2.057, p=0.1386, partial η^2=0.076) (two-way ANOVA) (**Figure 9E**). Further post-hoc analyses (Sidak's multiple comparisons) on ratio of responding revealed no differences between the control and extinction rats in rats treated with vehicle (p=0.9539, d=0.503) or the low-dose of NBQX (p>0.999, d=0.013), but in rats treated with the high-dose of NBQX, there was a significant difference in ratio of responding between the cue extinction and 0-CS

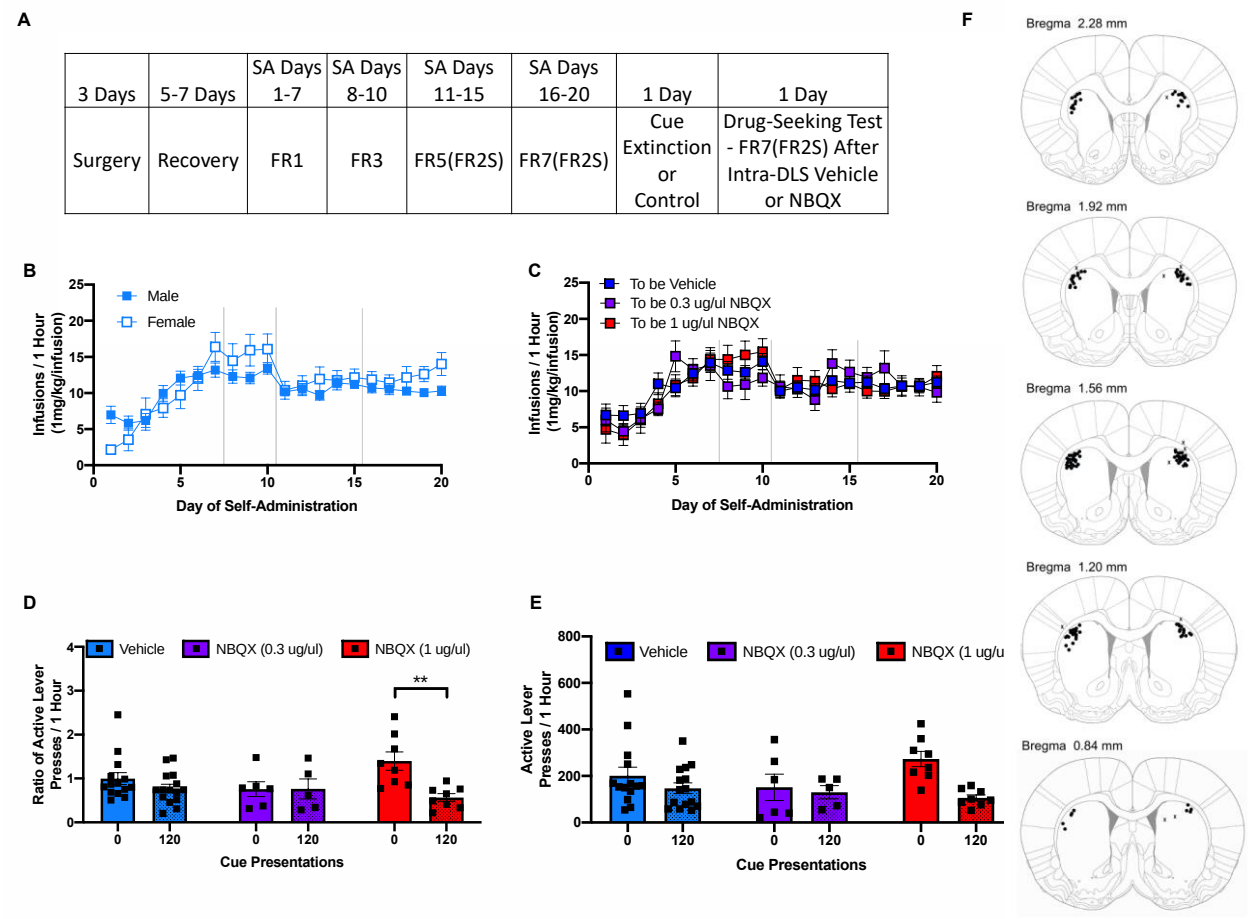

Figure 9: AMPA receptor antagonism during cue-induced drug seeking in previously SO-trained rats reveals an effect of cue extinction

Experimental timeline for self-administration, cue extinction, and cue-induced drug-seeking tests (**A**). Rats underwent 20 days of training to facilitate DLS dopamine-dependent cocaine self-administration, and there was no main effect of sex (**B**) or future drug treatment group (**C**) on infusions throughout training. After cue extinction, rats were given intra-DLS infusions of vehicle, a low dose (0.3 μg/μl), or a high dose (1 μg/μl) of the AMPA antagonist NBQX prior to a 1-hour cue-induced drug-seeking test. During the drug-seeking test, there was a main effect of cue extinction, no main effect of drug treatment, and an extinction × drug treatment interaction on ratio of responding (**D**). Post-hoc analyses revealed no differences between the 0-cue control and extinction rats in the vehicle groups or the low-dose NBQX groups, but rats in the high-dose NBQX group that underwent cue extinction had a reduced ratio of responding compared to 0-cue controls also given a high dose (**D**). For active lever presses, there was a main effect of cue extinction, but no main effect of drug treatment or interaction (**E**). Placement of DLS cannula was histologically

evaluated for all rats used in *cis*-flupenthixol and NBQX experiments (total n=73), and each on-target placement is indicated by a black dot, while off-target misses are indicated by black Xs (**F**). Graphs show group means ± SEM and individual data points. Open symbols indicate females, and closed symbols indicate males. Vertical gray bars indicate changes in reinforcement schedule (**B-C**). **p<0.01.

control groups (p=0.0078, d=1.839) (**Figure 9D**). Note that there was no difference between the 120-vehicle group and the 120-high dose NBQX group (p=0.9948, d=0.668) (**Figure 9D**). These results suggest that when response-outcome control is restored by a sufficient level of AMPA antagonism in the DLS, the effect of cue extinction is uncovered.

2.3.5 The conditioned reinforcing properties of the drug-paired cue are sensitive to cue extinction in SO-trained rats

Cue extinction's lack of effect on cocaine seeking in SO-trained rats brings into question both the necessity of the cue to maintain DLS dopamine-dependent responding and the reinforcing properties of the cue. Therefore, a subset of rats from the previous experiment (n=15; 4 male, 11 female) were used to address these questions. Rats returned to self-administration for two days, but on the second day, audiovisual cues were removed from the session (**Figure 10A**). There were no differences in active (p=0.7469, η^2=0.007678) or inactive (p=0.3268, η^2=0.06883) lever presses between self-administration days with and without cues (paired *t*-tests) (**Figure 10B-D**), which suggests the drug-paired audiovisual cue is not required to maintain DLS dopamine-dependent self-administration. To assess whether or not the cue maintained conditioned reinforcing properties after SO self-administration, rats were trained for 1 day on an acquisition of a new response task, when rats had the opportunity to nose-poke for the cue alone, in the absence of drug reinforcement, on the same SO schedule (**Figure 10A**). During conditioned reinforcement training, rats made

A

30-33 Days	1 Day	1 Day	1 Day	1 Day	1 Day
Surgery, SA training, cue extinction or control, and drug-seeking test	Return to SA – FR7(FR2S)	SA without cues – FR14 (no cues)	Conditioned Reinforcement Training FR7(FR2S)	Cue Extinction or Control	Conditioned Reinforcement Testing FR7(FR2S)

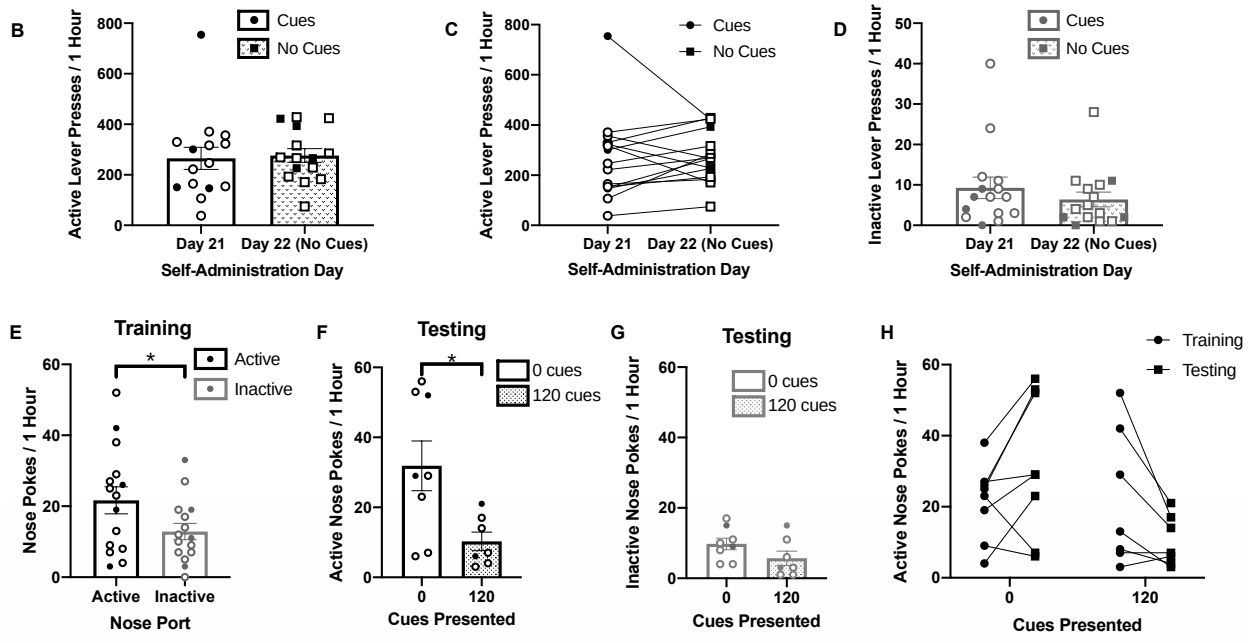

Figure 10: The conditioned reinforcing properties of the drug-paired cue are sensitive to cue extinction in SO-trained rats

Experimental timeline (**A**). A subset of rats from the previous experiment returned to SA for 2 days, with cues removed on the second day. They then underwent conditioned reinforcement training, when they learned to nose-poke for cues on an FR7(FR2S) schedule, and then underwent cue extinction and conditioned reinforcement testing (**A**). Removal of the audiovisual cue during cocaine SA had no effect on active (**B**-**C**) or inactive lever presses (**D**). During conditioned reinforcement training, rats made more active than inactive nose pokes (**E**). After cue extinction, during conditioned reinforcement testing, extinction rats made fewer active nose pokes than control rats (**F**) but their inactive nose pokes were not affected (**G**). When comparing active nose-pokes during training to testing, there was a significant day × extinction interaction (**H**). Graphs show group means ± SEM and individual data points. Open symbols indicate females, and closed symbols indicate males. *p<0.05.

more active than inactive nose-pokes ($p=0.0121$, $\eta^2=0.3723$) (paired *t*-test), which suggests the cue retained its conditioned reinforcing properties that were revealed because the rats were asked

to perform a new goal-directed action for cue presentation (**Figure 10E**). Rats then underwent cue extinction (120 CS) or control (0 CS) procedures and were re-tested for conditioned reinforcement. Rats that underwent cue extinction made fewer active nose pokes during test than control rats ($p=0.0183$, $\eta^2=0.3586$), but there were no differences between groups in inactive nose pokes ($p=0.1428$, $\eta^2=0.1576$) (unpaired *t*-test) (**Figure 10F-G**). There was a day × extinction interaction, where rats in the 0-cue control group tended to increase responding on the second day of conditioned reinforcement testing, while rats in the cue extinction group primarily decreased their active nose pokes ($F_{(1,13)}=8.912$, $p=0.0105$) (2-way ANOVA) (**Figure 10H**). These results suggest that the reinforcing properties of the drug-paired cue can be extinguished in a new response-outcome task in rats that were previously trained on SO schedules to self-administer cocaine.

2.4 Discussion

Overall, we find that cue extinction reduces cue-induced drug seeking when rats are trained on an FR schedule that promotes response-outcome behavior, as previously published (Rich et al., 2019, 2016; Torregrossa et al., 2013). However, we find that cue extinction is ineffective in rats trained on SO schedules that promote DLS-dependent behavior, despite comparable length of training and cocaine intake. Although a lack of significant interaction between training schedule and cue extinction prevents the conclusion that cue extinction is more effective in FR-trained than SO-trained rats, cue extinction's efficacy has been well demonstrated in FR-trained rats in several previous publications (Madsen et al., 2017; Rich et al., 2019, 2016; Torregrossa et al., 2013). Thus, we provide new evidence that putatively habitual drug seeking is resistant to cue extinction. We also show that training rats on these different schedules of reinforcement leads to differential

expression of membrane-bound glutamate receptors in the dorsal striatum, though this effect may have been more pronounced were we able to examine expression in a cell-type specific manner. Additionally, we show that blocking AMPA receptors in the DLS or requiring rats to produce a novel nose-poke response for cocaine cues leads to an apparent restoration of response-outcome behavior and reveals the suppressive effect of cue extinction.

Pavlovian cocaine-cue associations are dependent on the BLA, and we have previously shown that the effects of cue extinction are mediated by depotentiation of BLA synapses (Rich et al., 2019). Prior research indicates that the CeA, not the BLA, is necessary for DLS dopamine-dependent, habitual cocaine seeking (Murray et al., 2015). Our findings are consistent with these prior studies and suggest that cue extinction's lack of effect on DLS-dependent cocaine seeking is due to reduced reliance of cocaine-seeking behavior on the BLA. An alternative interpretation for the lack of effect of cue extinction after SO training includes the "partial reinforcement effect," which is known to occlude extinction learning (Chan and Harris, 2019). While this is a possibility, it does not completely explain our findings because DLS inhibition revealed an effect of cue extinction, which suggests that extinction is not prevented, but its effects are masked by DLS-dependent neural signaling. Furthermore, the efficacy of cue extinction on the conditioned-reinforcement task refutes the theory that responding on an SO schedule alone leads to resistance to cue extinction, which suggests that the neural circuits underlying behavioral control, and not the particular schedule of reinforcement, is what masks the effects of cue extinction. Previous work from our lab has shown a positive correlation between the number of cocaine-cue pairings and the strength of thalamo-BLA synapses, which suggests that fewer cocaine-cue pairings could result in increased susceptibility to cue extinction (Rich et al., 2019). Interestingly, we found that SO-trained rats were resistant to cue extinction despite taking significantly less cocaine, and therefore

receiving fewer cocaine-cue pairings, than FR-trained rats. Therefore, this finding provides evidence that it is the reduced reliance on this circuitry, and not a failure of cue extinction to impact BLA synaptic strength, which results in unaltered responding in SO-trained rats. Although unlikely, it is possible that the results of the present study reveal differences in the underlying Pavlovian processes of cue learning that are unrelated to habit circuitry, but these findings remain relevant to understanding how drug-cue associations guide behavior, especially because SO schedules may better model how drug-associated cues can be encountered in the absence of subsequent drug procurement.

Here, we used concentrations of the AMPAR antagonist NBQX in the DLS that restored response-outcome control in rats that orally self-administered alcohol (Corbit et al., 2014b). It has been established that dopamine antagonism in the DLS can reduce overall rates of responding, whereas AMPA receptor antagonism restores response-outcome control without reducing general responding (Corbit et al., 2014; Murray et al., 2015; Murray et al., 2012). The mechanisms by which dopamine or AMPA antagonism in the DLS differentially affect behavioral output are unclear, but are likely due to differential effects of manipulating midbrain dopaminergic inputs versus cortical and subcortical glutamatergic inputs.

Although other schedules of reinforcement, such as random ratio and random interval schedules, can also produce different patterns of behavioral control over drug seeking, we used SO and FR schedules for a few reasons. SO schedules may better model how humans with SUDs encounter drug-associated cues (Belin-Rauscent et al., 2016). Additionally, we sought to remain consistent with the methods used in the experiments that motivated our hypothesis, and previous experiments determined that while SO training facilitates DLS dopamine-dependent cocaine self-administration, an FR schedule maintains DLS dopamine-independence of behavior (Belin-

Rauscent et al., 2016; Murray et al., 2015; Murray et al., 2012; Rich et al., 2019). In agreement with previous studies, we found that SO schedule training facilitates DLS dopamine antagonist-sensitive cocaine seeking, which suggests our training methods also facilitated habit formation. Interestingly, we found that just a few days of SO training rendered responding sensitive to DLS dopamine antagonism, which suggests the neural circuitry involved in drug seeking may shift more rapidly than expected. How this shift occurs is still unclear, but likely involves the dramatic increase in lever presses that are sustained by the intermediate cues presented on an SO schedule. Future experiments utilizing *in vivo* imaging in the striatum and its input regions throughout this shift would provide further insight into the time-course and mechanism. One way these experiments deviated from previous research was by increasing the response requirement for FR-trained rats to FR5 to increase their overall responding (Murray et al., 2012). Although FR-trained rats still maintained fewer active lever presses than SO-trained rats, this adjustment rules out the possibility that cue extinction only affects low levels of responding. One limitation to these experiments is the use of a pharmacological manipulation to define habitual behavior, but this was necessitated because there is no well-established behavioral method, such as devaluation or contingency degradation, for defining habitual IV drug self-administration. Given the results of the present experiments, which suggest a role of Pavlovian associations in guiding behavior relying on goal-directed but not habitual response circuitry, we propose that future research should examine the application of sensitivity to the effects of cue extinction as a viable behavioral assay to distinguish between goal-directed and habitual response strategies, which would be easier to implement in IV drug self-administration models.

In order to further characterize rats in different training groups, we utilized western blot analysis to compare the expression of glutamate receptor subunit and vesicular transporter proteins

in the membrane in the DMS and DLS as markers of glutamatergic plasticity. We designed this experiment based on evidence that methamphetamine exposure biases toward habitual response strategies with an associated downregulation of glutamate proteins in the DMS and an upregulation in the DLS (Furlong et al., 2018). Our experiments differ in that all rats self-administered cocaine, so there was no drug-naïve group, and thus training schedule is the distinction between groups. In SO-trained rats, we detected increased membrane-bound GluA2/3 in the DMS and GluA1 in the DLS compared to FR-trained rats. The GluA1 subunit of the AMPA receptor has been implicated in long-term potentiation (Boehm et al., 2006; Shi et al., 2001). Upregulated GluA1 in the DLS in SO-trained rats suggests increased glutamatergic plasticity and further supports the conclusion that SO training facilitates the formation of DLS dopamine-dependent, habitual behavior. Because there is no drug-naïve group to compare to, interpretation of upregulated GluA2/3 in the DMS of SO-trained rats is more difficult. It is possible that this is an effect of significantly reduced membrane insertion of GluA2/3 in FR-trained rats coinciding with a nonsignificant increase in GluA1, which overall suggests an increased reliance on the plasticity-related GluA1 subunit compared to GluA2/3 in FR-trained rats, which would support the hypothesis of increased reliance on the DMS.

In these studies, we expanded on previous findings with the use of both sexes. Although these experiments were not powered to detect sex differences, we show similar effects of cue extinction in male and female rats. In the experiment utilizing both FR and SO schedules, females self-administered significantly more cocaine than males, which agrees with the literature (Jackson et al., 2006; Swalve et al., 2016). The sex difference in cocaine infusions was not significant in the NBQX experiment. There were also no sex differences in the membrane-bound expression of plasticity-related proteins. Although these data suggest similar effects of cue extinction in males

and females, future studies could further examine potential sex differences, as sex and sex hormones may impact cue-mediated drug seeking and habit formation (Barker et al., 2010; Fuchs et al., 2005; Schoenberg et al., 2019).

In addition to presenting raw lever press data, we chose to present data as a ratio of active lever presses during the cue-induced drug-seeking test to active lever presses on the final day of self-administration. Importantly, this involves comparing responding for the cocaine-paired cue when cocaine is not available to responding during normal self-administration, which are related, but not equivalent, measures. The ratio, or the relationship between responding under these different conditions, reveals interesting caveats to our findings that are otherwise occluded by the raw lever press data. In FR-trained rats, cue extinction appears to suppress increased responding that occurs in the 0-CS control group. Increased responding in the control group is likely due to intact potentiation of BLA synapses that drive an increase in cue-induced drug seeking in the absence of cocaine (Rich et al., 2019). Interestingly, SO-trained rats in the control treatment group do not increase their responding in the absence of cocaine, supporting the hypothesis that drug-cue associations, encoded by BLA synaptic strength, do not impact DLS dopamine-dependent responding. SO-trained rats continue to respond at the same rate independent of whether or not they underwent cue extinction or are receiving cocaine infusions, providing further evidence that their behavior is not driven by response-outcome associations. Therefore, restoration of response-outcome control with NBQX reveals an effect of cue extinction, but restoring response-outcome control after cue extinction does not significantly reduce responding below that of rats utilizing DLS-dependent response strategies that also underwent cue extinction.

Restoration of response-outcome control produces bidirectional effects on ratio of responding of SO-trained rats depending on their extinction history, indicated by a significant

interaction between extinction experience and NBQX treatment, and the restored effect of cue extinction appears to primarily rely on an increase in responding in the 0-CS control group. Therefore, it is important to note that although restoring response-outcome control reveals an effect of cue extinction, and this gives insight into the synaptic and circuit mechanisms underlying DLS dopamine-dependent behavior's resistance to cue extinction, it does not significantly reduce responding in the absence of cue extinction. Restoration of response-outcome control is the most likely explanation for NBQX's bidirectional effects on cue-induced drug seeking because a previous study showed that the same dose of NBQX infused in the DLS restored response-outcome control of oral alcohol seeking, which was established using outcome devaluation to behaviorally distinguish between response-outcome and habit-like behavior (Corbit et al., 2014b). Nevertheless, it is possible that NBQX could impact motivation for the drug or cue or impair retrieval of the cocaine-cue association. However, if NBQX could somehow impact retrieval of the cocaine-cue association or motivation for cocaine, we would expect impaired retrieval or motivation to result in a decrease in cue-induced drug seeking, not the increase observed in the 0-cue control group. These findings have implications for the use of CET in SUD treatment. Although they show that DLS dopamine-dependent behavior is resistant to cue extinction, they also suggest that restoring response-outcome control alone could be harmful because, without other manipulations, it may increase cue-induced drug seeking and risk of relapse. The combination of restoring response-outcome control with other therapeutic methods including CET may be more effective and should be further studied.

A major obstacle in the use of CET to treat SUDs is context dependency (Kantak and Nic Dhonnchadha, 2011; Rich et al., 2019; Torregrossa et al., 2010). Even if this challenge is overcome, our findings suggesting that habitual behaviors are resistant to cue extinction, which

may be important for the design and interpretation of clinical CET studies. There is ample evidence for a dissociation in the circuitry involved in DLS-independent and -dependent behaviors (Murray et al., 2015; Murray et al., 2012; Steinberg et al., 2020). Future experiments are required to determine how DLS dopamine-dependent response circuitry is strengthened, how it processes environmental stimuli to initiate behavior, and what role drug-associated cues play when they are reinforcing but not required for responding. Additionally, our findings reveal an interesting dissociation between the reinforcing properties of cues and how they guide behavior depending on response strategy, demonstrated by the susceptibility of conditioned reinforcement to cue extinction in SO-trained rats. These results further underscore the complex dynamics that may motivate drug seeking depending on environmental conditions and past experience.

3.0 Changes in DMS activity mediate expression of goal-directed vs. habit-like cue-induced cocaine seeking

3.1 Introduction

A major obstacle in the treatment of substance use disorders (SUDs) is maladaptive learning and memory, which can promote drug cravings, drug use, and relapse (Carter and Tiffany, 1999; Grant et al., 1996; MacNiven et al., 2018; Milton and Everitt, 2012; Wang et al., 1999). Several types of associative learning can contribute to persistent drug use, and drug exposure can also enhance learning and the strength of these memories (Bender and Torregrossa, 2020; Everitt and Robbins, 2005; Furlong et al., 2018; Nelson and Killcross, 2006; Nordquist et al., 2007; Olausson et al., 2007; Torregrossa et al., 2011). Response-outcome learning occurs when a desired outcome, such as a drug of abuse, becomes associated with a behavioral response, or action, that produces the drug effect (Everitt and Robbins, 2013; Ostlund and Balleine, 2008; Smith and Laiks, 2017). These associations can then promote goal-directed drug-seeking behavior (Gruber and McDonald, 2012). As learning continues and an action repeatedly leads to the same outcome, stimulus-response associations begin to form where the environmental stimuli present during response-outcome learning (e.g, contexts or discrete cues) become sufficient to drive the behavioral response independent of the value of the outcome, which is defined as habitual behavior (Everitt and Robbins, 2013; Ostlund and Balleine, 2008; Smith and Laiks, 2017). Therefore, over time these stimuli alone can promote putatively habitual drug-seeking behaviors (Leong et al., 2016; Smith and Laiks, 2017). Finally, in addition to action-related learning, Pavlovian associations also form between environmental stimuli and the effects of a drug, which can also

promote motivated behavior (Everitt and Robbins, 2005; Gruber and McDonald, 2012; Shiflett and Balleine, 2011). The presentation of drug-associated stimuli, or cues, has been shown to enhance subjective levels of drug craving in individuals with SUDs, promote relapse, and activate implicated brain regions, including the nucleus accumbens, dorsal striatum, and regions of the cortex (Carter and Tiffany, 1999; Garavan et al., 2000; Kosten et al., 2006; Maas et al., 1998; MacNiven et al., 2018; Prisciandaro et al., 2013a; Volkow et al., 2006; Wang et al., 1999; Wexler et al., 2001).

Therefore, the extinction of Pavlovian drug-cue associations has been proposed as a potential therapeutic target in the treatment of SUDs (Conklin and Tiffany, 2002; Milton, 2013; Torregrossa and Taylor, 2013). Cue exposure therapy, the repeated presentation of cues in the absence of the associated outcome, has been shown to be an effective behavioral treatment for others psychiatric disorders that involve maladaptive Pavlovian associations, such as phobias and post-traumatic stress disorder (Powers et al., 2010; Powers and Emmelkamp, 2008). Additionally, cue extinction, a preclinical model of cue exposure therapy, reduces cue-induced cocaine seeking in rodent cocaine self-administration models (Madsen et al., 2017; Perry et al., 2016; Rich et al., 2019; Torregrossa et al., 2013). However, the clinical application of cue exposure therapy to SUDs has yielded modest results (Conklin and Tiffany, 2002; Mellentin et al., 2017). There are likely several reasons for this difficulty in translation, including context dependency (Kantak and Nic Dhonnchadha, 2011; Rich and Torregrossa, 2018; Torregrossa et al., 2010). Additionally, our lab has shown that Pavlovian cue extinction reduces goal-directed cocaine seeking, but has no effect on habitual cocaine seeking unless goal-directed control is restored (Bender and Torregrossa, 2021). Therefore, a lack of effect of cue extinction on habitual components of drug seeking may also be a contributing factor to this difficulty in translation.

Extensive literature has implicated distinct neural circuits in goal-directed and habitual behavior (Corbit et al., 2012; Knowlton and Patterson, 2018; Shiflett and Balleine, 2011). Dopaminergic inputs from the substantia nigra to the dorsomedial striatum (DMS) and dorsolateral striatum (DLS) are important for the initiation of goal-directed and habitual behavior, respectively (Barker et al., 2015; Belin and Everitt, 2008; Faure et al., 2005; Murray et al., 2012). Additionally, other direct and indirect inputs to the dorsal striatum, including those from the cortex, thalamus, and amygdala, may be important for toggling between reliance on goal-directed and habitual behavior (Cover et al., 2019; Gremel and Costa, 2013; Kato et al., 2018; Killcross and Coutureau, 2003; Lingawi and Balleine, 2012; Murray et al., 2015). Dopamine release in the DMS and DLS during operant reward seeking can differ between regions depending on the operant task and extent of training (Brown et al., 2011; Ito et al., 2002; Klanker et al., 2017; Shnitko and Robinson, 2015; Willuhn et al., 2012). Several studies using *in vivo* electrophysiology to compare DMS and DLS activity during operant reward-seeking behavior have also shown distinct patterns of neural activity in these regions and indicate these patterns change as habitual behavior develops (Fanelli et al., 2013; Kimchi et al., 2009; Vandaele et al., 2019; Vandaele and Janak, 2022). However, the specific contributions of dopaminergic and other inputs to the dorsal striatum's response to drug-associated cues and how they might be impacted by Pavlovian cue extinction remain unclear.

In the present study, we employed fixed-ratio (FR) and second-order (SO) schedules of reinforcement, which have been shown to facilitate either goal-directed, DMS dopamine-dependent or habitual, DLS dopamine-dependent cocaine seeking, respectively (Bender and Torregrossa, 2021; Murray et al., 2015, 2012). We utilized fiber photometry to examine dorsal striatal calcium and dopamine activity during drug seeking throughout the establishment of goal-directed and habitual cocaine self-administration and evaluated the effects of cue extinction on

activity in these regions. We found distinct signatures of calcium and dopamine activity in the dorsal striatum in rats trained on FR or SO reinforcement schedules to promote goal-directed or habitual cocaine seeking, respectively. Additionally, we showed that cue extinction impacted DMS, but not DLS, calcium and dopamine activity, which suggests that cue extinction does not impact the neural circuitry promoting habitual behavior.

3.2 Methods

3.2.1 Animals

Adult Sprague-Dawley rats (Envigo) were 8-9 weeks old upon arrival (n=26; male n=14; female n=12). Animals were housed in auto-ventilated racks with automated watering in a temperature- and humidity- controlled room maintained on a 12-hour light-dark cycle. Rats were given ≥4 days to acclimate to the facility before surgical procedures and were pair-housed until catheter implantation. Rats had *ad libitum* access to food and water until 24 hours before the start of training, when they were food restricted to maintain ~90% of their free-feeding body weight. Behavioral experiments were run in the light cycle and began within ~3 hours of the same time each day. Procedures were conducted in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

3.2.2 Viral vectors

The fluorescent dopamine indicator dLight1.2 (AAV5-hSyn-dLight1.2) (Addgene, titer ≥ 4×10^{12} vg/mL) and calcium indicator jRCaMP1b (AAV1.Syn.NES-jRCaMP1b.WPRE.SV40) (Addgene, titer ≥ 1×10^{13} vg/mL) were mixed in a 1:1 ratio and vortexed immediately prior to intracranial infusion surgeries.

3.2.3 Drugs

Cocaine hydrochloride (graciously provided by NIDA) was dissolved at 2 mg/ml in 0.9% sterile saline (Thermo Fisher) and filter-sterilized.

3.2.4 Behavioral apparatus

Experiments were conducted in 4 standard operant conditioning chambers using MedPC software (Med Associates). Each animal underwent all training and testing in the same chamber. Each chamber was equipped with bar floors and a syringe pump connected to a swiveled leash. All chambers had 3 plexiglass walls and one wall containing two levers with cue lights above them, a head-entry magazine, a houselight, and a tone generator. Chambers were housed in a sound-attenuating box with a fan for background noise.

3.2.5 Surgery

3.2.5.1 Anesthesia

Rats were fully anesthetized with ketamine (100 mg/kg, Henry Schein) and xylazine (5 mg/kg, Butler Schein) and prepared for surgery as previously described (Bender and Torregrossa, 2021; Rich et al., 2019).

3.2.5.2 Viral infusion

Viral infusion surgery took place at least 4 weeks prior to photometry recordings to allow for virus expression. Rats were placed in a stereotaxic frame and lidocaine (0.3 ml, Butler Schein) was injected subcutaneously above the skull as previously described (Rich et al., 2019). A 26-gauge injection cannula connected to a Hamilton Syringe and pump was used to inject 1 μl of virus mixture at a rate of 0.1 μl/s unilaterally into the anterior DLS (in mm from bregma, anterior and posterior (AP): +0.8; medial and lateral (ML): ± 2.8; dorsal and ventral (DV): -5.0) and, in the opposite hemisphere, the posterior DMS (AP: -0.2 mm; ML: ± 2.2 mm; DV: -4.7 mm). The hemisphere receiving each injection was counterbalanced.

3.2.5.3 Intravenous catheterization and optic fiber implantation

In a second surgery up to a week before rats began self-administration, rats were implanted with a chronic indwelling intravenous catheter into the right jugular vein as previously described (Bender and Torregrossa, 2021; Rich et al., 2019; Torregrossa and Kalivas, 2008). Rats were then placed in a stereotaxic frame and lidocaine was injected subcutaneously above the skull. Fiber optic cannulae (Thorlabs, 2.5 mm ferrule, 400 μm core, 5 mm long) were lowered into the DLS

(AP: +0.8 mm; ML: ± 3.0 mm; DV: -4.5 mm) and the DMS (AP: -0.2 mm; ML: ± 2.0 mm; DV: -4.0 mm) and were secured as previously described (Rich et al., 2019).

3.2.5.4 Post-operative care

Rats were administered analgesic and catheters were flushed to maintain patency as previously described (Bender and Torregrossa, 2021).

3.2.6 Behavioral procedures

3.2.6.1 Pre-learning stimuli exposure

A subset of rats (n=5) underwent photometry recordings during a series of stimuli presentations prior to operant behavioral training to determine if these stimuli, when novel, would impact dorsal striatal dopamine or calcium activity. In 2 daily 15-minute sessions, rats were exposed to 20-seconds of a stimulus every 30 seconds. Stimuli consisted of houselight, tone, cue light, lever insertion, and simultaneous tone + cue light. Each stimulus was presented 4 times in each session.

3.2.6.2 Cocaine self-administration

Rats were trained to self-administer cocaine (1mg/kg/infusion) in 1-hour daily sessions for 20 days as previously described (Bender and Torregrossa, 2021). Briefly, cocaine infusions were paired with a 20-second audiovisual cue accompanied by a 20-second time-out when levers were retracted and the houselight was extinguished, and inactive lever presses were recorded but had no consequences. Rats were initially trained to self-administer cocaine on a fixed-ratio 1 (FR1) schedule for 7 days (acquisition) then on an FR3 schedule for 3 days (early training). Rats were

then split into FR-trained (goal-directed) and SO-trained (habit-like) groups. FR-trained rats were maintained on an FR3 schedule for 5 days (middle training) and then trained on an FR5 schedule for 5 days (late training). SO-trained rats were trained for 5 days on an FR5(FR2S) schedule (middle training) followed by 5 days on an FR7(FR2S) schedule (late training). Details for SO schedule training were as previously described (Bender and Torregrossa, 2021), briefly, every second lever press resulted in a 1-second presentation of the audiovisual cue (FR2S), and every fifth (FR5) or seventh (FR7) completion of the FR2S cycle resulted in cocaine infusion and timeout paired with the 20-second audiovisual cue. Levers were not retracted during 1-second cues during SO training.

3.2.6.3 Daily cue-induced drug-seeking tests

During fiber photometry recordings, rats underwent a 15-minute drug-seeking test immediately before self-administration on days 9-20. To prevent cocaine from influencing extracellular dopamine, no cocaine was administered during these tests, but audiovisual cues and timeouts occurred on the same reinforcement schedule each rat self-administered under on the previous day. Standard self-administration sessions immediately followed each of these 15-minute test sessions.

3.2.6.4 Pavlovian cue extinction

On the day immediately following the final day of self-administration, with levers retracted, rats were non-contingently exposed to 120 20-second audiovisual cues separated by 10 seconds during photometry recordings.

3.2.6.5 Cue induced drug-seeking test

On the day following cue extinction, rats underwent a 1-hour drug-seeking test during fiber photometry recordings. Cocaine was withheld and cues and timeouts occurred on the rat's previous reinforcement schedule. The cue-induced drug-seeking test was 1 hour long because, in our hands, rats can take longer to resume cue-induced drug seeking after cue extinction, which was supported by our finding that that multiple rats (n=4) did not complete their reinforcement schedule within the first 15 minutes of this drug-seeking test.

3.2.7 Fiber photometry

3.2.7.1 Recordings

Photometry recordings were collected using a multi-wavelength photometry system (Plexon) and a branched low-autofluorescence fiber-optic patch cord (Doric: 2 branches, 400 μm core, 440 μm cladding, 0.37 NA). Laser output for each excitation wavelength (560 nm, 465 nm, and 410 nm) was set that laser intensity at the cable tip was 20-30 μW. Laser was passed through the patch cord for 30 minutes prior to daily photometry recordings to minimize autofluorescence of the cable during recordings. Recordings occurred using 3-phase cycling of 415, 465, and 560 nm LEDs. Fluorescence data were collected at 30 frames per second using Plexon software, and behavioral events were aligned to photometry fluorescence using TTL timestamp outputs from MedPC software. Rats were habituated to the optic cable setup in the operant chamber for at least 1 day prior to photometry recordings.

3.2.7.2 Processing and analysis

Data were processed and analyzed using custom MATLAB (Mathworks, Natick, MA, USA) scripts, which are available upon request. Fluorescent signals were forward and reverse low-pass filtered at 30 Hz. Traces were visualized and motion artifacts were removed. The isosbestic control trace was fitted to fluorescent signal traces at 465 nm (dLight) and 560 nm (RCaMP) using a least squares polynomial fit of degree 1. ΔF/F was calculated by subtracting the fitted isosbestic signal from the fluorescent signal and then dividing by the fitted isosbestic signal. Z-scores were calculated across the entire fluorescent signal trace by subtracting the mean ΔF/F value from each data point and dividing by the standard deviation. Traces around behavioral events (including 3 seconds before and 30 seconds after) were separated and averaged for each animal for each recording day, and SEM was calculated for the average trace. To account for shifts of baseline in cue extinction sessions, event traces were normalized by subtracting the average fluorescence during the 3-second period before cue onset from each event's trace.

Because the majority of the responses we observed occurred within 1 second of behavioral events, the area under the curve (AUC) of the z-score and peak z-score amplitude in the 1 second post-event were calculated for all events. For each phase of training, each animal's daily average z-score AUC and peak amplitude were averaged to obtain a single value per animal. For pre-training recordings during stimuli exposure, traces from each event type were averaged for each region for each animal, and average AUC per second and peak z-score were calculated during the 3-second baseline period and during the 10-second stimulus exposure. For results, "cue-reinforced active lever presses" refer to lever presses that resulted in cue presentation and timeout (20-second audiovisual cue, houselight off, and lever retraction). "Unreinforced active lever presses" refer to

active lever presses that did not result in any cue presentation, and for which there were no active lever presses (and therefore no cue presentations) in the 3 seconds before or 5 seconds after.

3.2.8 Vaginal cytology

Estrous cycle phase was determined daily by cellular morphology as previously described (Bender and Torregrossa, 2023; Parrish et al., 2019).

3.2.9 Histology

Rats were anesthetized and perfused and brains were removed, sliced, mounted, and coverslipped as previously described (Rich et al., 2019). Every fourth section containing the dorsal striatum was mounted and imaged at 10X magnification using an Olympus BX61VS epifluorescent slide-scanning microscope to verify fiber placement location and virus expression at the base of the fiber.

3.2.10 Exclusion criteria

Rats were excluded from all analysis due to death or illness after surgery (n=5), loss of catheter patency (n=1) (determined by a 0.1 ml intravenous infusion of 10 mg/ml sodium brevital, Covetrus), or bilateral histological misses (n=4). For rats with a unilateral histological miss (n=6), data for the region with the histological miss were excluded. One rat was excluded from the final cue-induced drug-seeking test due to loss of headcap. Rats were excluded from analysis during cue-induced drug-seeking tests if they failed to make enough lever presses to reach a long cue

during all phases of training (n=2). Rats were excluded from comparing activity during the late training to activity during drug seeking after cue extinction if they failed to obtain a long cue or make an isolated unreinforced lever press during either test (n=3).

3.2.11 Quantification and statistical analysis

Behavioral data were collected using MedPC software. All statistical analyses were performed using GraphPad Prism. For the 1-hour cue-induced drug-seeking test after cue extinction, the ratio of responding was calculated by dividing the number of active lever presses during test by the number of active lever presses in the final self-administration session to normalize changes in responding across rats with different magnitudes of lever pressing behavior due to their training schedule.

For all statistical analyses, significance was set at $p<0.05$. All data were determined to be normally distributed using the Shapiro-Wilk test, and Bartlett's test was used to determine that there were no significant differences in the estimated variance between groups. For analyses using repeated-measures ANOVAs, a Geisser-Greenhouse correction was used to account for potential lack of sphericity. Criteria for outlier data points was set at >2 standard deviations from the mean, and outlier points were excluded along with their paired data.

Infusions were analyzed by two-way rmANOVA, using time and training schedule as factors. Lever presses during self-administration were analyzed by three-way rmANOVA with time, training schedule, and lever as factors. Ratio of active lever presses during the post-cue extinction cue-induced drug-seeking test was analyzed with an unpaired student's t-test. Calcium and dopamine peak z-score amplitude and AUCs during cue-induced drug-seeking tests were analyzed by two-way or three-way ANOVA with future training schedule, training schedule, sex,

cue reinforcement, cue length, phase of training, or cue extinction as factors as indicated. Calcium and dopamine peak z-score amplitude and AUC during early training were analyzed to determine if there was an effect of estrous phase on signals by mixed-effects analysis with estrous phase and cue reinforcement as factors because one rat was never in estrus during those three days of testing. Calcium and dopamine peak z-score amplitude and AUCs during cue extinction were analyzed by one-way rmANOVA. For correlation analyses, Pearson's correlation coefficients were calculated with average active lever presses as the independent variable and peak z-score amplitude or AUC as the dependent variable. Calcium and dopamine peak z-score amplitude and AUCs after pre-learning stimuli exposure were analyzed by two-way ANOVA with stimulus type and stimulus presentation as factors. When a significant effect was detected by one-way ANOVA or an interaction was detected by two-way or three-way ANOVA analysis, significant effects were further analyzed by Tukey's or Sidak's post-hoc multiple comparisons analysis, respectively. Throughout our analyses, we report the peak z-score amplitude as our primary measure of calcium and dopamine responses. Results from analyses of AUC data were overall similar and are therefore not presented in the main text, but there were some minor differences, so AUC results are presented in the appendix.

3.3 Results

3.3.1 FR- and SO-trained rats do not differ in daily cocaine self-administration or cue-induced drug-seeking after cue extinction

The dopamine fluorescent sensor dLight and calcium sensor RCaMP were expressed contralaterally in the aDLS and pDMS in male and female rats (n=26), and optic fibers and jugular vein catheters were implanted (**Figure 11A**). After all experiments, virus expression at the base of the fiber (**Figure 11B**) and fiber placement (**Figure 11C**) were confirmed by fluorescent microscopy. A total of 10 rats were excluded due to death after surgery, loss of catheter patency, or bilateral fiber misplacement or misplaced virus expression. Remaining rats (n=16; 9 males and 7 females) were trained to self-administer cocaine (1 mg/kg/inf) for 20 days before they underwent cue extinction and a subsequent cue-induced drug-seeking test (**Figure 11D**). During daily self-administration, there was a main effect of training day on number of infusions ($F_{(4.776,66.86)}$=5.759, p=0.0002), but no main effect of training schedule ($F_{(1,14)}$=0.002179, p=0.9634) or interaction ($F_{(19,266)}$=1.486, p=0.0899) (2-way rmANOVA) (**Figure 11E**). For lever presses, there was a 3-way training day × schedule × lever interaction ($F_{(19,266)}$=9.801, p<0.0001) (3-way rmANOVA) (**Figure 11F**). These data indicate that both groups received increased cocaine infusions and made more active lever presses as training progressed, and that the increase in active lever presses was more pronounced in SO-trained rats, which is expected because these rats had to increase their lever presses to receive the same number of infusions. During the cue-induced drug-seeking test after cue extinction, there was no difference in the ratio of active lever presses during test to the final day of self-administration between FR-trained and SO-trained rats (p=0.3229, η^2=0.08873) (unpaired *t*-test) (**Figure 11G**).

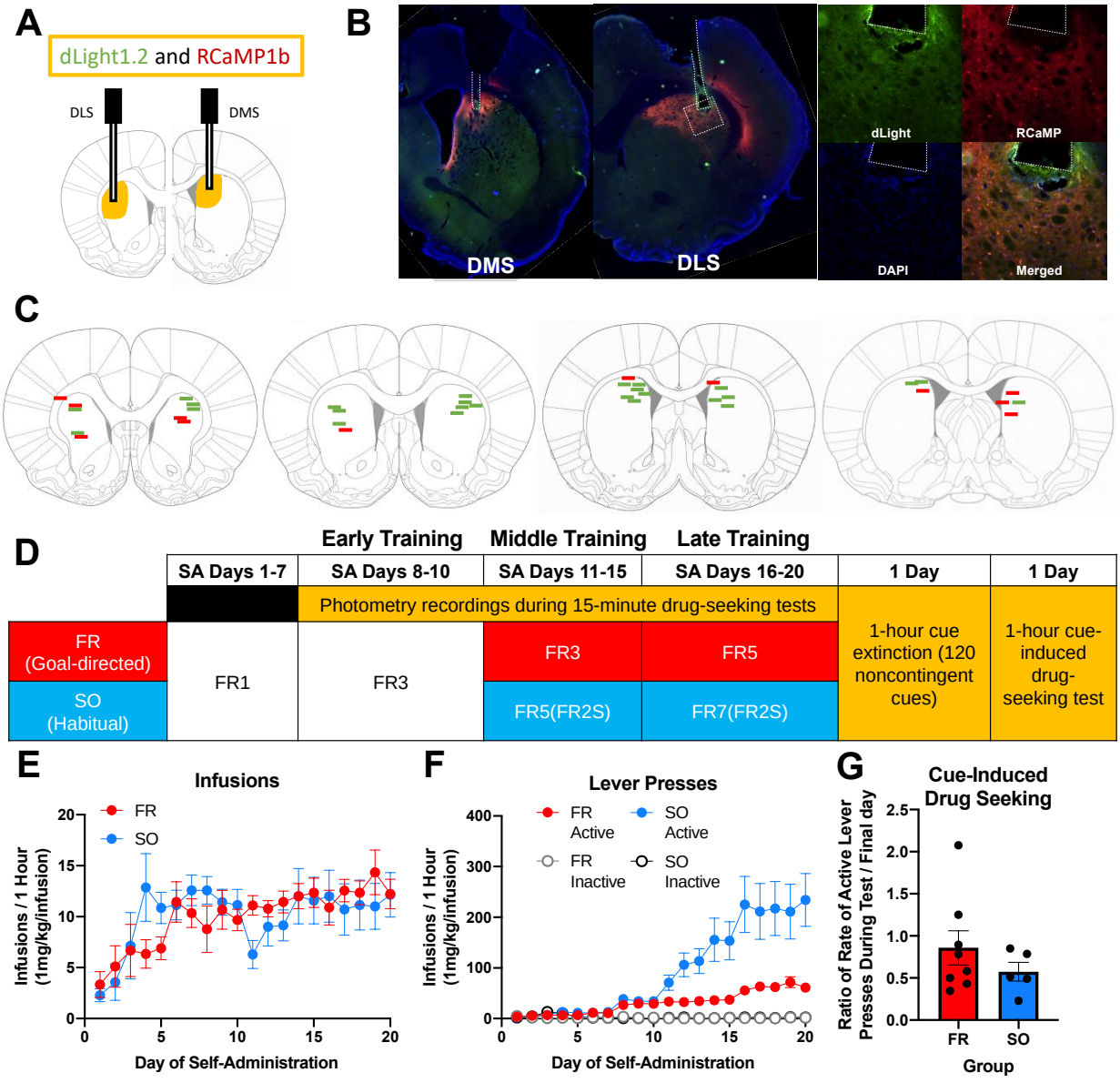

Figure 11: FR- and SO-trained rats do not differ in daily cocaine self-administration or cue-induced drug-seeking after cue extinction

Schematic for fiber placement and virus expression in the DLS and DMS (**A**). Representative images of fiber placement and virus expression in the DMS and DLS (left) and at higher magnification in the DLS (right) with fluorescent channels shown individually and merged, and fiber locations are outlined in dotted white lines (**B**). For all rats, fiber placement and virus expression were evaluated via fluorescent microscopy, and green bars represent fibers appropriately placed with confirmed virus expression at the base of the fiber, while red bars indicate fibers excluded from analysis due to either fiber misplacement and/or lack of virus expression (**C**). Rats were trained to self-administer

cocaine for 20 days on different schedules of reinforcement (FR or SO) before undergoing cue extinction and a subsequent cue-induced drug-seeking test, and after acquisition, photometry recordings occurred in 15-minute drug-seeking tests that preceded daily self-administration, during cue extinction, and during the subsequent cue-induced drug-seeking test (**D**). During self-administration, there was a main effect of training day on the number of daily cocaine infusions, where infusions increased for both groups as training progressed (**E**). For lever presses during daily self-administration, there was a 3-way training day × training schedule × lever interaction (**F**). There was no difference between groups in the ratio of active lever presses during the post-cue extinction cue-induced drug-seeking test compared to the final day of self-administration (**G**). Graphs show group means ± SEM and individual data points where possible.

3.3.2 After acquisition, dorsal striatal calcium responses are greater for cue-reinforced than unreinforced active lever presses

After 7 days of cocaine self-administration on an FR1 schedule, all rats self-administered on an FR3 schedule for 3 days. Following the first day of FR3 training, fiber photometry recordings took place in daily 15-minute drug-seeking tests prior to self-administration. The first 3 days of recording to examine dorsal striatal responses during "early training" occurred after acquisition but before rats were split into FR- and SO-trained groups. Because we wanted to determine if there were any differences between rats split into future groups, we first compared dorsal striatal responses between rats that would later be separated into FR- or SO-trained groups during this early training phase. On the schedules of reinforcement used in these studies, some active lever presses resulted in cue presentation upon the completion of the reinforcement schedule, but others that occurred before schedule completion had no consequences. Because we were particularly interested in the role of the drug-paired cue, we compared calcium and dopamine responses in the dorsal striatum between future training group for cue-reinforced versus unreinforced active lever

presses using 2-way ANOVAs. Therefore, any differences were due to differences in the response to cue presentation after lever press and were not a motion artifact of performing the lever press. Because we used both male and female rats, we first determined that there was no effect of sex or estrous phase on dorsal striatal responses to lever pressing (Appendix, **Figure 20**). Therefore, males and females were combined for analyses throughout.

During early training prior to splitting animals into groups, there was a main effect of cue reinforcement ($F_{(1,8)}$=12.98, p=0.0070) on calcium peak z-score amplitude in the DLS in the 1 second after lever press, but no effect of future training schedule ($F_{(1,8)}$=4.263, p=0.0728) or interaction ($F_{(1,8)}$=2.214, p=0.1751) (**Figure 12A**). Similarly, for calcium peak amplitude in the DMS, there was a main effect of cue reinforcement ($F_{(1,9)}$=9.035, p=0.0148), but no effect of future training schedule ($F_{(1,9)}$=0.006144, p=0.9392) or interaction ($F_{(1,9)}$=0.1558, p=0.7022) (**Figure 12B**). For dopamine peak amplitude in the DLS, there were no main effects of cue reinforcement ($F_{(1,9)}$=0.06979, p=0.7976) or future training schedule ($F_{(1,9)}$=1.907, p=0.2006) or interaction ($F_{(1,9)}$=0.2988, p=0.5979) (**Figure 12C**). Similarly, for dopamine peak amplitude in the DLS, there were no main effects of cue reinforcement ($F_{(1,9)}$=0.06176, p=0.8093), future training schedule ($F_{(1,9)}$=0.0007846, p=0.9783) or interaction ($F_{(1,9)}$=0.02546, p=0.8767) (**Figure 12D**). Overall, these data suggest that after acquisition of cocaine self-administration and learning Pavlovian drug-cue associations, calcium activity in the DMS and DLS is greater in response to cue-reinforced lever presses compared to lever presses that did not result in cue presentation. Interestingly, cue reinforcement after lever press did not impact dopamine activity in either region during this early training phase. Throughout, we present peak amplitude data in the main figures, but we also calculated AUC data, which can be found in the appendix (**Figures 20-25**). During early training, results from AUC data were similar to peak data, and we also found that there were

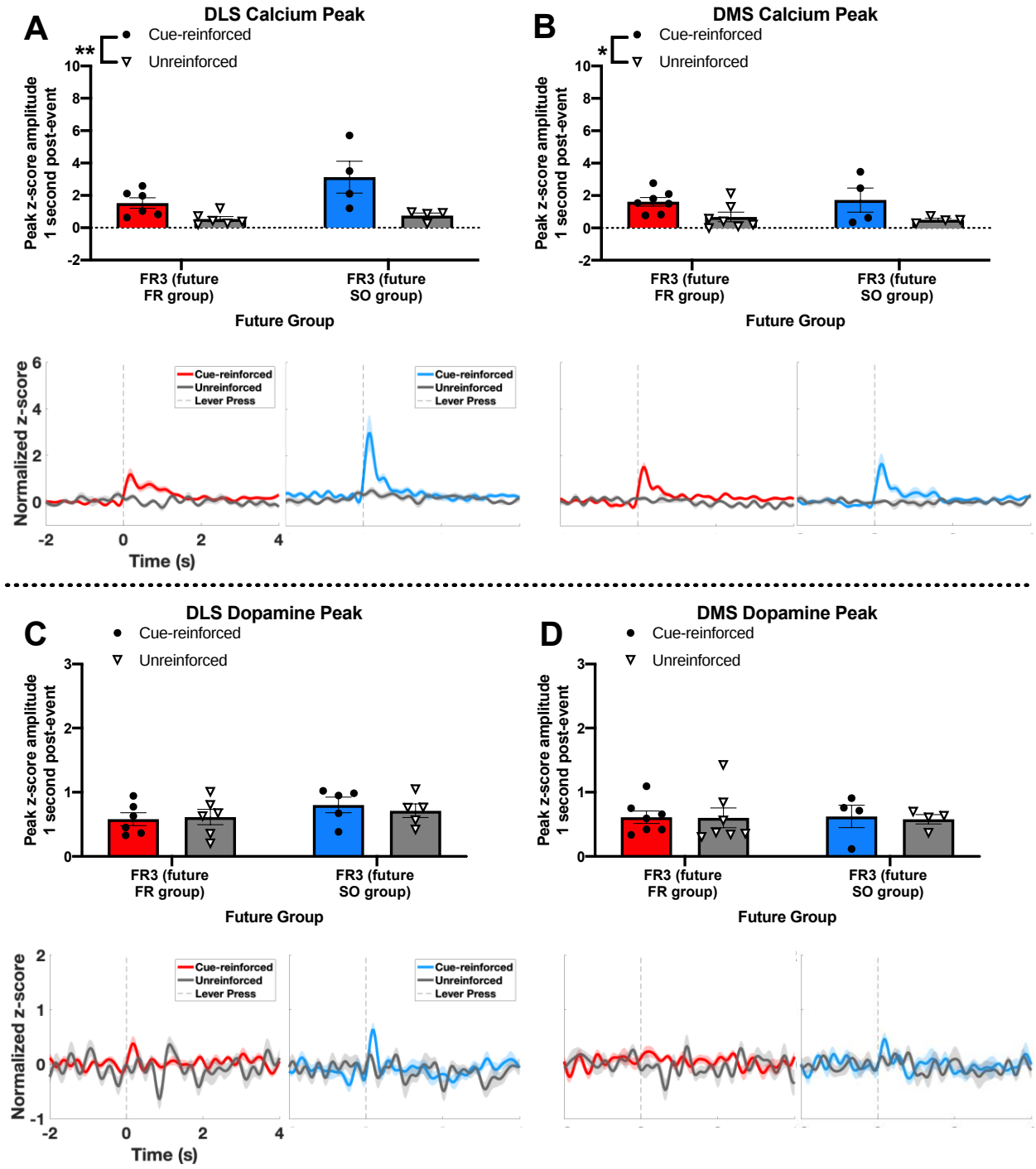

Figure 12: After acquisition, dorsal striatal calcium responses are greater for cue-reinforced than unreinforced active lever presses

After acquisition and prior to splitting rats into FR- and SO-trained groups, fiber photometry recordings occurred during 15-minute drug-seeking tests prior to daily self-administration, during which some active lever presses had no

consequence (unreinforced) and an active lever press that completed the FR3 schedule resulted in cue presentation (cue-reinforced) and timeout (levers retracted, houselight extinguished). For DLS (**A**) DMS (**B**) calcium, there was a main effect of cue reinforcement on peak z-score amplitude in the 1 second after lever press, but there was no effect of future training schedule or interaction. For DLS (**C**) and DMS (**D**) dopamine peak z-score amplitude, there was no effect of cue reinforcement, future training group, or interaction. Graphs show group means ± SEM and individual data points. Traces show overall average trace for each event for each future group aligned to behavioral events with SEM shown with shading and dashed vertical lines indicating time of lever press. *p<0.05; **p<0.01.

no significant correlations between the average number of active lever presses during drug-seeking tests and the average calcium or dopamine peak amplitude or AUC for cue-reinforced lever presses (Appendix, **Figure 21**), which suggests that different rates of lever pressing do not impact dorsal striatal responses to cues.

3.3.3 FR-trained rats, but not SO-trained rats, show greater DMS calcium activity after cue-reinforced compared to unreinforced lever presses

After 10 days of self-administration, rats were split into FR-trained and SO-trained groups and trained on different schedules of reinforcement accordingly. The next 10 days were split into 5 days of middle training and 5 days of late training. During SO-schedule training, short 1-second cues are presented in addition to 20-second cues presented upon schedule completion, and for some measures SO-trained rats had different responses to short cues compared to long cues (Appendix, **Figure 20**). Therefore, only long cues were compared between FR- and SO-trained rats. To determine if SO-trained rats had different dorsal striatal responses to lever presses than FR-trained rats, we compared cue-reinforced to unreinforced lever presses between groups for each phase of training (middle or late) using 3-way ANOVAs. For DLS calcium peak amplitude, there was a main effect of cue reinforcement ($F_{(1,9)}$=31.21, p=0.0003), but no main effects of

training schedule ($F_{(1,9)}$=4.259, p=0.0691) or phase of training ($F_{(1,9)}$=3.205, p=0.1633), and there were no cue reinforcement × training schedule ($F_{(1,9)}$=0.00748, p=0.9318), cue reinforcement × phase of training ($F_{(1,9)}$=0.6451, p=0.4426), phase of training × training schedule ($F_{(1,9)}$=3.954, p=0.0780), or 3-way interactions ($F_{(1,9)}$=0.6999, p=0.4245) (**Figure 13A**). For DMS calcium peak amplitude, there was a main effect of cue reinforcement ($F_{(1,9)}$=9.569, p=0.0129) and a cue reinforcement × training schedule interaction ($F_{(1,9)}$=5.370, p=0.0457), but no main effect of training schedule ($F_{(1,9)}$=1.870, p=0.2047) or phase of training ($F_{(1,9)}$=1.130, p=0.3155), and no cue reinforcement × phase of training ($F_{(1,9)}$=0.003176, p=0.9563), phase of training × training schedule ($F_{(1,9)}$=4.718, p=0.0579), or 3-way interaction ($F_{(1,9)}$=0.01580, p=0.9027) (**Figure 13B**). These data suggest that while both FR-trained and SO-trained rats have greater calcium responses in the DLS to cue-reinforced than unreinforced lever presses, this difference is only present in the DMS for FR-trained, but no SO-trained rats. In other words, SO training leads to a loss of cue-induced calcium-indicated activity selectively in the DMS.

3.3.4 SO-trained rats, but not FR-trained rats, show greater DLS dopamine responses to cue-reinforced compared to unreinforced lever presses

For DLS dopamine peak amplitude during middle and late training, there was a main effect of cue reinforcement ($F_{(1,9)}$=11.42, p=0.0081) and a cue reinforcement × training schedule interaction ($F_{(1,9)}$=5.494, p=0.0437), but no main effect of training schedule ($F_{(1,9)}$=0.001535, p=0.9696) or phase of training ($F_{(1,9)}$=0.06291, p=0.8076), and no cue reinforcement × phase of training ($F_{(1,9)}$=0.3683, p=0.5589), phase of training × training schedule ($F_{(1,9)}$=0.8370, p=0.3841), or 3-way interaction ($F_{(1,9)}$=1.951, p=0.1960) (**Figure 13C**). There were no main effects of cue

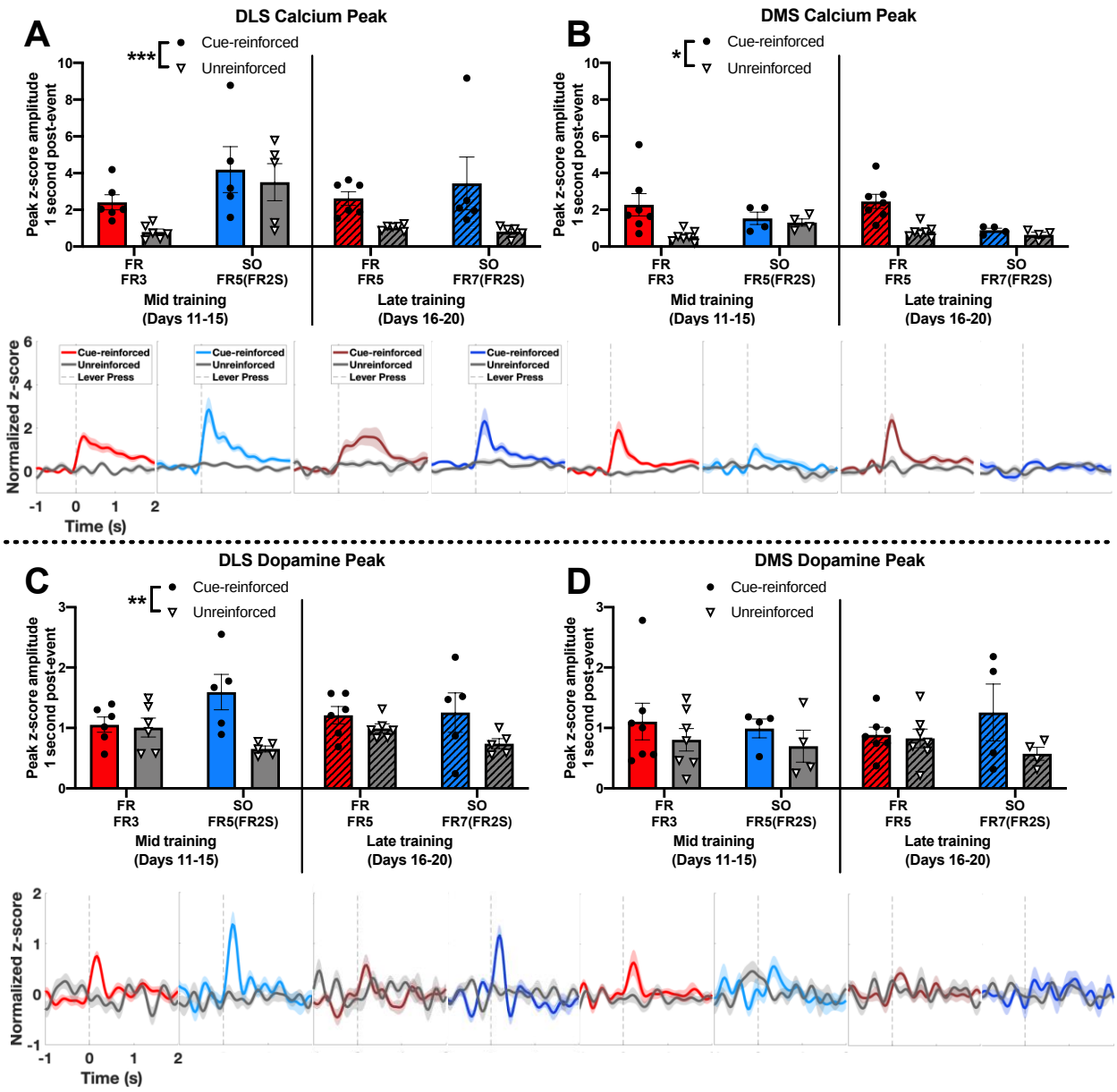

Figure 13: FR- and SO-trained rats have different patterns of dorsal striatal calcium and dopamine activity during drug seeking

Rats were separated into FR-trained and SO-trained groups for the remaining 10 days of self-administration and trained on different schedules of reinforcement accordingly for the middle and late phases of training. Dorsal striatal calcium and dopamine responses to cue-reinforced and unreinforced lever presses were compared for each training schedule and phase of training. There was a main effect of cue reinforcement on DLS calcium peak amplitude, but no main effects of training schedule or phase of training or interactions (**A**). For DMS calcium peak amplitude, there was a main effect of cue reinforcement and a cue reinforcement × training schedule interaction, but no other main effects

or interactions (**B**). There was a main effect of cue reinforcement and a cue reinforcement × training schedule interaction for DLS dopamine peak amplitude (**C**), but there were no main effects or interactions for DMS dopamine peak amplitude (**D**). Graphs show group means ± SEM and individual data points. Traces show overall average trace for each event for each group aligned to behavioral events with SEM shown with shading and dashed vertical lines indicating time of lever press. *p<0.05; **p<0.01; ***p<0.001.

reinforcement ($F_{(1,9)}$=0.2947, p=0.1202), training schedule ($F_{(1,9)}$=0.01592, p=0.9024), or phase of training ($F_{(1,9)}$=0.008296, p=0.9294) on DMS dopamine peak amplitude, and there were no cue reinforcement × training schedule ($F_{(1,9)}$=6271, p=0.4488), cue reinforcement × phase of training ($F_{(1,9)}$=0.1141, p=0.7433), phase of training × training schedule ($F_{(1,9)}$=0.2727, p=0.6141), or 3-way interactions ($F_{(1,9)}$=2.060, p=0.1851) (**Figure 13D**). These data suggest that SO-trained rats, but not FR-trained rats, had increased DLS dopamine activity after cue-reinforced lever presses, but neither group had greater DMS dopamine in response to cue-reinforced compared to unreinforced lever presses. Data for AUC of calcium and dopamine responses during middle and late training are presented in the appendix (**Figure 22**).

3.3.5 Dorsal striatal calcium responses to noncontingent cues decrease throughout cue extinction

After 20 days of self-administration, rats underwent cue extinction, during which levers were retracted and 120 audiovisual cues were passively presented every 30 seconds over one hour while photometry recordings occurred. During cue extinction, there were no differences in calcium or dopamine responses between FR-trained and SO-trained rats, so groups were combined. Sessions were divided into 15-minute, 30-cue bins and analyzed with a one-way rmANOVA. There was no main effect of bin on DLS calcium peak amplitude ($F_{(1.496,14.96)}$=3.855, p=0.0549) (**Figure 14A**). For DMS calcium peak amplitude, there was a main effect of bin on peak amplitude

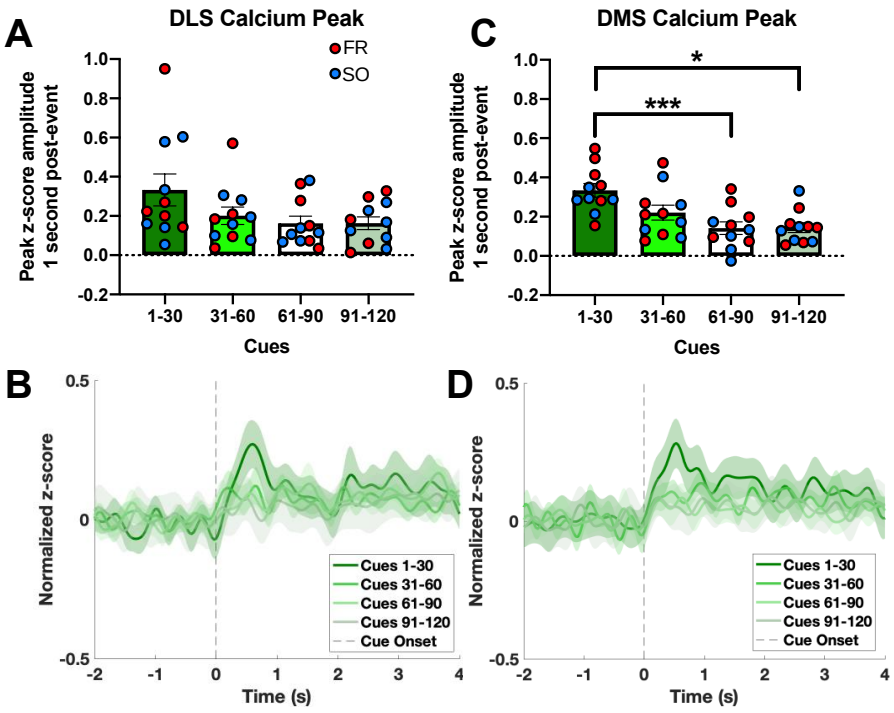

Figure 14: Dorsal striatal calcium responses to noncontingent cues decrease throughout cue extinction

Dorsal striatal calcium and dopamine activity were recorded during cue extinction, when 120 audiovisual cues were passively presented every 30 seconds over one hour. Cues were separated into 15-minute, 30-cue bins, and data from FR-trained and SO-trained rats were combined for analysis. There was no main effect of bin on DLS calcium peak amplitude (**A, B**). For DMS calcium peak amplitude, there was a main effect of bin, and post-hoc analyses showed that the calcium amplitude for cues 1-30 was greater than for that of cues 61-90 and cues 91-120 (**C, D**). Graphs show group means ± SEM and individual data points, with FR-trained rats indicated by red symbols and SO-trained rats indicated by blue symbols. Traces show overall average trace for each bin aligned to cue onset with SEM shown with shading and dashed vertical lines indicating time of cue onset. *p<0.05; ***p<0.001.

($F_{(2.096,20.96)}$=10.16, p=0.0007), and post-hoc analyses indicated that DMS calcium peak amplitude for cues 1-30 was significantly greater than for cues 61-90 (p=0.0007, q=8.378) and for cues 91-120 (p=0.0113, q=5.655) (Tukey's multiple comparisons) (**Figure 14B**). Results were similar for calcium AUC data (Appendix, **Figure 23**). These results suggest that throughout cue extinction, dorsal striatal calcium peak amplitude responses to noncontingent cues decreased. Note that this reduction was only significant for DMS calcium signal, but there was a nonsignificant trend toward

a reduction in the DLS, so this reduction may not be specific to the DMS. Interestingly, there was no significant effect of repeated passive drug-paired cue exposure on dopamine responses to cues (Appendix, **Figure 23**).

3.3.6 Cue extinction results in reduced DMS calcium peak amplitude during drug seeking selectively in FR-trained rats

Given that we have previously found that SO-trained rats are resistant to the effects of cue extinction in modulating their cocaine-seeking behavior, and that this is reliant on activity in the DLS (Bender and Torregrossa, 2021), we wanted to determine if cue extinction differentially affected dorsal striatal activity in a drug seeking test after cue extinction. Rats underwent a 1-hour cue-induced drug-seeking test during which photometry recordings occurred. Dorsal striatal calcium and dopamine peak amplitudes after cue-reinforced or unreinforced lever presses during this post-cue extinction drug-seeking test (post-ext) were compared to the late phase of training (pre-ext) using 3-way ANOVAs. There was a main effect of cue reinforcement ($F_{(1,7)}$=8.302, p=0.0236) on DLS calcium peak amplitude, but no main effects of training schedule ($F_{(1,7)}$=3.393, p=0.2080) or cue extinction ($F_{(1,7)}$=0.4329, p=0.5316) or cue reinforcement × training schedule ($F_{(1,7)}$=1.480, p=0.2632), cue reinforcement × cue extinction ($F_{(1,7)}$=0.06984, p=0.7992), training schedule × cue extinction ($F_{(1,7)}$=1.385, p=0.2778), or 3-way interactions ($F_{(1,7)}$=0.3617, p=0.5665) (**Figure 15A**). These data suggest that cue extinction did not impact DLS calcium activity for either FR- or SO-trained rats. For DMS calcium peak amplitude, there was a main effect of cue reinforcement ($F_{(1,7)}$=11.42, p=0.0118) and a main effect of cue extinction ($F_{(1,7)}$=6.514, p=0.0380), as well as significant cue reinforcement × training schedule ($F_{(1,7)}$=6.362, p=0.0397) and training schedule × cue extinction interactions ($F_{(1,7)}$=7.953, p=0.0258) (**Figure 15B**). There

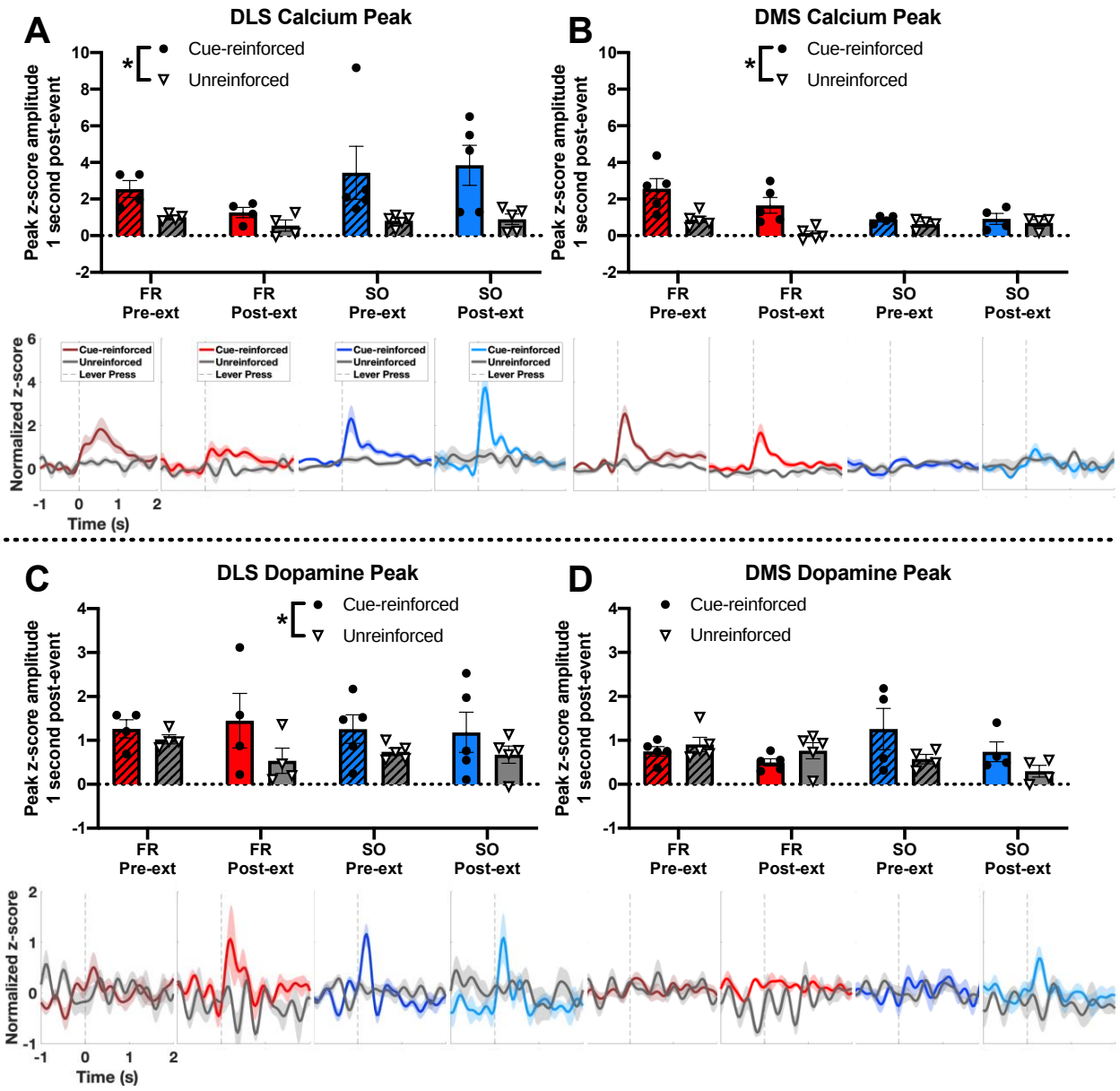

Figure 15: Cue extinction results in changes in DMS, but not DLS, calcium and dopamine activity during drug seeking

To examine the effects of cue extinction on dorsal striatal calcium and dopamine activity, peak amplitudes during the post-cue extinction drug-seeking test (post-ext) were compared to the late phase of training (pre-ext). There was a main effect of cue reinforcement on DLS calcium peak amplitude, but no effects of cue extinction or training schedule or interactions (**A**). For DMS calcium peak amplitude, there was a main effect of cue reinforcement, a main effect of cue extinction, and significant cue reinforcement × training schedule and training schedule × cue extinction interactions, but no other interactions (**B**). There was a main effect of cue reinforcement on DLS dopamine peak

amplitude, with no other main effects or interactions (**C**). Finally, there was a main effect of cue extinction on DMS dopamine peak amplitude, but no other effects or interactions (**D**). Graphs show group means ± SEM and individual data points. Traces show overall average trace for each event for each group aligned to behavioral events with SEM shown with shading and dashed vertical lines indicating time of lever press. *p<0.05.

was no main effect of training schedule ($F_{(1,7)}$=2.980, p=0.1279) on DMS dopamine peak amplitude, and there was no cue reinforcement × cue extinction ($F_{(1,7)}$=0.1234, p=0.7358) or 3-way interaction ($F_{(1,7)}$=0.04091, p=0.8455) (**Figure 15B**). These results suggest that cue extinction resulted in a reduction in DMS calcium peak amplitude for FR-trained rats, while SO-trained rats were unaffected, which may be due to their already minimal DMS calcium response. Effects of cue extinction on calcium AUC can be found in the appendix (**Figure 24**).

3.3.7 Cue extinction results in reduced DMS dopamine peak amplitudes during drug seeking in both groups

For DLS dopamine peak amplitude, there was a main effect of cue reinforcement ($F_{(1,7)}$=6.724, p=0.0358), but no effect of training schedule ($F_{(1,7)}$=0.1056, p=0.7547) or cue extinction ($F_{(1,7)}$=0.2119, p=0.6592), and there were no cue reinforcement × training schedule ($F_{(1,7)}$=0.02193, p=0.8865), cue reinforcement × cue extinction ($F_{(1,7)}$=2.090, p=0.1915), training schedule × cue extinction ($F_{(1,7)}$=0.02783, p=0.8722), or 3-way interactions ($F_{(1,7)}$=2.151, p=0.1859) (**Figure 15C**). There was a main effect of cue extinction ($F_{(1,7)}$=8.889, p=0.0205) on DMS dopamine peak amplitude, but there was no main effect of cue reinforcement ($F_{(1,7)}$=1.106, p=0.3280) or training schedule ($F_{(1,7)}$=0.004638, p=0.9476), and there were no cue reinforcement × training schedule ($F_{(1,7)}$=5.388, p=0.0533), cue reinforcement × cue extinction ($F_{(1,7)}$=0.6134, p=0.4592), training schedule × cue extinction ($F_{(1,7)}$=1.005, p=0.3495), or 3-way interactions

($F_{(1,7)}$=0.07993, p=0.7856) (**Figure 15D**). These results suggest that cue extinction resulted in an overall reduction in DMS dopamine peak amplitude after any lever press, regardless of cue reinforcement, in both groups, while DLS dopamine responses were not affected. Effects of cue extinction on dopamine AUC can be found in the appendix (**Figure 24**).

3.4 Discussion

In the present study, we show distinct patterns of calcium and dopamine activity during drug seeking in the dorsal striatum in rats trained on different schedules of reinforcement, where SO training results in an enhanced DLS dopamine response and a reduced DMS calcium response to cue-reinforced lever presses compared to FR training. Additionally, we show evidence that cue extinction impacts DMS, but not DLS, calcium and dopamine activity during later drug-seeking, which suggests that extinction of the Pavlovian cocaine-cue association impacts the DMS circuitry important for goal-directed drug seeking, but does not impact the DLS circuitry important for habitual drug seeking (Murray et al., 2015, 2012). We have previously shown that cue extinction does not affect cue-induced drug seeking in rats trained on SO schedules of reinforcement to promote DLS dopamine-dependent behavior unless goal-directed behavior is restored (Bender and Torregrossa, 2021). In the present study, cue extinction's lack of effect on the DLS provides a biological explanation for why cue extinction does not affect habitual drug seeking. These findings add to existing literature that indicates divergent roles of the DMS and DLS in goal-directed and habitual drug seeking and expand our understanding of how cocaine-cue associations facilitate dorsal striatal activity.

In these experiments, we chose to use fiber photometry, which allows for the *in vivo* comparison of bulk changes in fluorescent output of fluorescent indicators in the regions of interest. Fluorescent dopamine sensors provide the advantage of enhanced temporal resolution compared to other methods of monitoring dopamine release *in vivo*, including microdialysis and fast-scanning cyclic voltammetry (FSCV) (Patriarchi et al., 2019, 2018; Wang et al., 2021). Using fiber photometry also allowed us to simultaneously monitor dopamine release (via dLight) and intracellular calcium (via RCaMP) in the same rats (Li et al., 2019). Although *in vivo* electrophysiology has enhanced temporal sensitivity compared to fiber photometry, fiber photometry is more stable for long-term comparison across days, which was important for the present study (Li et al., 2019). Additionally, monitoring intracellular calcium may provide distinct information compared to *in vivo* electrophysiology. It was initially proposed that intracellular calcium activity reported by calcium sensors like GCaMP and RCaMP are proxy indicators of neural activity, or cell firing (Siciliano and Tye, 2019). However, recent evidence suggests that at least in the dorsal striatum, where neurons have extensive dendritic arborization, changes in the fluorescent calcium signal reported by fiber photometry are more indicative of changes in non-somatic calcium and do not necessarily reflect the same results as *in vivo* electrophysiology (Legaria et al., 2022). Importantly, this evidence suggests that the changes in calcium fluorescence we report here may be interpreted as the summation of excitatory and inhibitory input into dorsal striatal neurons (Legaria et al., 2022).

We initially recorded dorsal striatal dopamine and calcium activity during cue-induced drug seeking after acquisition. Notably, we did not show any significant differences in dopamine or calcium peak amplitudes between rats that would later be separated into FR- and SO-trained groups, which suggests that later differences in calcium and dopamine responses were the result

of effects of training on different schedules of reinforcement and were not due to baseline differences in sensor expression. Additionally, our comparison between cue-reinforced and unreinforced active lever presses controls for motion artifacts that could be produced by the lever press action and isolates the contribution of cocaine-paired cue presentation on dorsal striatal activity (Mejaes et al., 2022). Throughout the main text, we chose to show results for the peak z-score amplitude in the 1 second after the behavioral event, and results for area under the curve (AUC) during this time window are reported in the appendix. Both of these methods have been used to evaluate differences in fluorescent output measured by fiber photometry (Bruno et al., 2021; Sherathiya et al., 2021). Overall, we show similar results for both measures, with some key differences discussed in the appendix. Future studies should examine how different information provided by these measures could impact interpretation of results.

After just one week of cocaine self-administration, there was an enhanced calcium response to cue-reinforced lever presses in both the DMS and DLS. Because these recordings took place after limited self-administration training at a timepoint when behavior would presumably be dependent on the DMS (Corbit et al., 2012; Murray et al., 2012), the DLS calcium response to reinforced lever presses was somewhat surprising. There is some uncertainty about when the stimulus-response associations that later guide habitual behavior are learned. Typically, habitual behavior can be differentiated from goal-directed behavior through its lack of sensitivity to outcome devaluation and outcome contingency degradation (Smith and Laiks, 2017). There is evidence that even after minimal operant training, DMS inhibition, DMS lesions, or exposure to stimulants can render behavior insensitive to outcome devaluation or contingency degradation (Corbit et al., 2014a, 2012; Furlong et al., 2018; Nelson and Killcross, 2006; Yin et al., 2005b). Insensitivity to these paradigms could suggest reliance on habitual behavior, but in some cases it

could also be attributed to impaired execution of goal-directed behavior (Watson and de Wit, 2018). Our results showing increased calcium activity after cue-reinforced lever presses in the DLS at this early timepoint, when behavior is presumably DMS-dependent and goal-directed, support the theory that stimulus-response learning occurs during early training, even though the behavioral response may still be goal-directed.

Interestingly, we did not show increased DMS dopamine release in response to cue-reinforced lever presses compared to unreinforced lever presses in either group at any phase of training. Because previous experiments have shown that after similar training, DMS dopamine antagonism reduces drug-seeking behavior, this finding was particularly surprising in the early training phase, as well as during the middle and late phase in FR-trained rats (Murray et al., 2012). The lack of effect is not likely due to limitations of the dopamine sensor dLight, as we did detect a DMS dopamine response to novel stimuli prior to training, in accordance with a previous study showing that midbrain dopamine neurons, including those that project to the dorsal striatum, respond to novel stimuli (Morrens et al., 2020). It is well-established that dopamine release in the nucleus accumbens core occurs upon the presentation of a reward-predictive or reward-associated cue (Flagel et al., 2011; Shnitko and Robinson, 2015). However, the impact of reward-associated cues on DMS dopamine release is less clear. Because the DMS is particularly important for goal-directed drug seeking, which is promoted by the association between the lever press behavior and reward, DMS dopamine release may not be directly tied to cue presentation, but to reward delivery. Indeed, in mice that were trained to self-administer sucrose, calcium activity in dopamine neuron terminals in the DMS did show increased responses to nosepokes that were reinforced by sucrose delivery (Seiler et al., 2022). Therefore, the data in the present study may not show a DMS dopamine response because we recorded DMS dopamine under extinction conditions, when lever

presses were reinforced by the cue alone and not cocaine, due to technical limitations and to prevent cocaine exposure from impacting DMS dopamine. However, in rats trained to self-administer sucrose after a discriminative stimulus was presented, presentation of the discriminative stimulus resulted in increased DMS, but not DLS, dopamine release as measured by FSCV (Brown et al., 2011). In this case, the stimulus does not signal reward delivery, but the imminent availability of a lever that, when pressed, results in sucrose delivery (Brown et al., 2011). Therefore, future experiments should investigate the conditions for which DMS dopamine release occurs for reward-predictive or reward-associated cues. Interestingly, results from the present study did suggest DMS dopamine overall during drug seeking was reduced after cue extinction, even though this reduction was not specific to cue-reinforced lever presses.

On the other hand, our results indicate that SO, but not FR training, does enhance DLS dopamine release after cue-reinforced lever presses. These results agree with those of another study using in vivo microdialysis to measure dopamine release in the DLS in rats trained to self-administer cocaine on second-order schedules of reinforcement, which also showed enhanced DLS dopamine release during cue-induced drug seeking (Ito et al., 2002). Our findings complement these by providing enhanced temporal resolution and showing that this increased DLS dopamine release is specific to cue-reinforced lever presses. In another study, rats were trained to self-administer alcohol or sucrose on a variable-interval schedule, which usually promotes habitual behavior, and striatal dopamine release was measured with FSCV during operant responding for reward-associated cues (Shnitko and Robinson, 2015; Smith and Laiks, 2017). They observed enhanced dopamine release after cue-reinforced compared to unreinforced lever presses in the DLS, but not in the DMS (Shnitko and Robinson, 2015). Our results extend these findings by indicating that SO-training to self-administer cocaine facilitates a similar pattern of dorsal striatal

dopamine release, which suggests this enhanced DLS dopamine response to cue-reinforced lever presses is consistent across multiple reinforcers (cocaine, ethanol, and sucrose) and is also generalized between multiple schedules of reinforcement known to facilitate habitual behavior. Although FR-trained rats in the present study did not develop an enhanced DLS dopamine response to cue-reinforced lever presses, one previous study did report enhanced dopamine release in the DLS, measured by FSCV, after just 2-3 weeks of similar cocaine self-administration, but rats also received cocaine along with the cocaine-associated cue when they made an active nose poke (Willuhn et al., 2012). Therefore, reward delivery may also be required to promote this DLS dopamine response in FR-trained rats, and future experiments should determine what experimental parameters are required to facilitate a DLS dopamine response to cue-reinforced drug seeking.

With regard to calcium responses, we found increases in the DMS to cue-reinforced lever presses through the late phases of training in FR-trained, but not SO-trained rats. Importantly, rats that would later be SO-trained did show increased DMS calcium response during the early phase of training, which suggests that SO-training led to a loss of the DMS calcium response to cues. Because calcium activity primarily reflects dendritic calcium, the reduction in calcium activity likely reflects reduced excitatory or enhanced inhibitory input into DMS neurons after SO training (Legaria et al., 2022). Future studies should determine the contribution of different DMS inputs to the DMS calcium response to cue-reinforced behavior. Indeed, there is evidence that mPFC projections to the DMS are disengaged as a motor skill is refined, so it is possible that a reduction in excitatory mPFC input could occur in SO-trained rats (Kupferschmidt et al., 2017). Additionally, the orbitofrontal cortex (OFC) is important for goal-directed behavior and modulates the DMS via both direct projections and indirectly through the other cortical areas and the amygdala, and could also be involved in this decrease in DMS calcium response in SO-trained rats

(Gremel and Costa, 2013; Zimmermann et al., 2017). In addition to projections from the cortex and the amygdala, the DMS also receives thalamic inputs, which should also be investigated (Alloway et al., 2017). Future studies could also evaluate the contribution of different neuronal sub-types, as direct- and indirect-pathway medium spiny neurons and the more sparse local interneurons may have different roles in these behaviors (Garr and Delamater, 2020; Holly et al., 2019; O'Hare et al., 2017; Yin et al., 2009).

Next, we evaluated changes in dorsal striatal calcium and dopamine responses to the non-contingent presentation of cocaine-paired cues in a Pavlovian cue extinction paradigm. We found that DMS and DLS calcium responses to passive cue presentations were of a magnitude lower than those observed when the cue was presented after a lever press during drug seeking tests. Moreover, the amplitude of the responses to the cue significantly decreased throughout the extinction session in the DMS, and, although not statistically significant, a similar trend occurred in the DLS. One limitation to these findings is that photobleaching, a phenomenon by which fluorescence intensity is lost as a function of light exposure, could have contributed to this reduction, and this possibility should be further investigated (Siciliano and Tye, 2019). However, the use of an isosbestic control channel, which we employ in the present study, can aid in the correction of changes in fluorescence due to photobleaching, and we also corrected for any potential shifts in baselines during these recordings (Bruno et al., 2021). Additionally, the reduction we observed began early in the session (within the first 45 minutes), when few effects of photobleaching have been reported (Saunders et al., 2018). Additionally, we did not observe increased dorsal striatal dopamine in response to noncontingent cues during cue extinction, and there was no reduction throughout the session. These findings agree with those of another study showing that noncontingent cue presentations do not result in increased DLS dopamine release, as measured by *in vivo* microdialysis, after SO

training to self-administer cocaine (Ito et al., 2002). Taken together, these results suggest that DLS dopamine release during habitual drug seeking is dependent upon both the lever press action as well as contingent cue presentation, further supporting the role of DLS dopamine in connecting cues (stimuli) with the lever press behavior.

Finally, we conducted an additional drug seeking test after cue extinction and compared dorsal striatal calcium activity to activity during the late phase of training, prior to cue extinction. In doing this comparison, we found that FR-trained rats had reduced DMS calcium responses to lever presses after cue extinction, but there was no effect in SO-trained rats or in either group in the DLS. These data suggest that the learned lack of association between cocaine and the drug-associated cue that occurs during cue extinction results in reduced DMS calcium activity during subsequent drug seeking, but only in animals trained on a schedule that promotes goal-directed behavior. Inputs to the DMS that contribute to this reduction in DMS calcium activity after cue extinction would be an interesting topic of future research. Our lab has previously shown that cue extinction results in reduced synaptic strength of thalamo-amygdala synapses, and optogenetic depotentiation of these synapses mimics the effects of cue extinction (Rich et al., 2019). Therefore, it is possible that cue extinction affects DMS calcium activity by reducing input to the DMS either directly from the BLA or through the BLA's interaction with the OFC, which could be the topic of future investigations (Gremel and Costa, 2013; Zimmermann et al., 2017).

We have previously shown that cue extinction reduces cue-induced drug seeking in FR-trained, but not SO-trained rats (Bender and Torregrossa, 2021). In our previous study, the effect of cue extinction in FR-trained rats was apparent when animals the underwent cue extinction were compared to those that did not. Therefore, the lack of difference in response ratio after cue extinction in FR- compared to SO-trained rats in the current study is consistent with our previous

findings (Bender and Torregrossa, 2021). Interestingly, cue extinction affected DMS calcium activity in FR-trained rats and DMS dopamine activity in both groups, which suggests that cue extinction may inhibit circuitry that facilitates goal-directed action towards seeking cocaine when cocaine is expected but not provided.

Overall, the present study expands upon previous literature examining the differential roles of the DMS and DLS in goal-directed and habitual behavior, respectively. We also present novel results showing that cue extinction reduces calcium and dopamine activity in the DMS during later drug seeking but has no effects on the DLS, which indicates that extinction of the cocaine-cue association impacts the circuitry involved in goal-directed, but not habitual cocaine seeking. Future experiments should examine specific projections to the DMS and how they are impacted by SO-schedule training and cue extinction. Together, these results provide novel insights into how cue extinction may reduce drug seeking that is goal-directed but not affect habitual drug seeking and indicate that future treatments for SUDs may need to address these aspects of drug-seeking behavior using different methods.

4.0 Intermittent cocaine self-administration has sex-specific effects on addiction-like behaviors in rats

This chapter is adapted from the following published manuscript:

Bender, B.N., Torregrossa, M.M., 2023. Intermittent cocaine self-administration has sex-specific effects on addiction-like behaviors in rats. *Neuropharmacology*. 230:109490. doi: 10.1016/j.neuropharm.2023.109490

4.1 Introduction

The progression from recreational drug use to substance use disorder (SUD) involves escalated drug intake, increased motivation for the drug, maladaptive learning and memory, and persistence in drug seeking and taking despite negative consequences (Belin-Rauscent et al., 2016; Bender and Torregrossa, 2020; Edwards and Koob, 2013; Koob and Le Moal, 2008; Smith and Laiks, 2017). Rodent cocaine self-administration models are used to evaluate these and many other characteristics of SUDs and the circuits involved in order to inform understanding of SUDs and develop novel treatments (Panlilio and Goldberg, 2007). Evidence suggests that cocaine use may consist of short periods of drug binge-like consumption separated by periods without consumption (Allain et al., 2015; Foltin et al., 1995; Leri et al., 2004b; Samaha et al., 2021; Ward et al., 1997). Recently, intermittent access (IntA) cocaine self-administration has been implemented to more accurately model this intermittent pattern of cocaine intake. Whereas rodents self-administering under traditional continuous access (ContA, either short [ShA] or long access [LgA]) models tend to maintain high, sustained brain cocaine concentrations throughout sessions, IntA models produce

the pattern of spiking and falling brain-cocaine concentration that may occur during human cocaine binges (Allain et al., 2015; Bentzley et al., 2014; Samaha et al., 2021).

The spiking brain-cocaine concentration patterns characteristic of IntA have different pharmacological and behavioral effects than the continuous elevated cocaine levels sustained in ContA models. IntA increases cocaine-induced dopamine release and uptake and promotes sensitization of the dopamine transporter (DAT) to cocaine in the nucleus accumbens, whereas LgA promotes tolerance (Calipari et al., 2015, 2013; Kawa et al., 2019). Several studies have shown that IntA increases motivation for cocaine (Algallal et al., 2020; Calipari et al., 2015; James et al., 2019; Zimmer et al., 2012), and this enhanced motivation is correlated with sensitization of cocaine-induced dopamine release in the nucleus accumbens (NAc) (Kawa et al., 2019). Because dopamine in the NAc is also important for cocaine-cue associations, it is unsurprising that IntA also augments cue-induced reinstatement and incubation of cocaine craving (Aragona et al., 2009; James et al., 2019; Kawa et al., 2019; Nicolas et al., 2019), but the ability to extinguish cocaine-cue associations after IntA has not been examined. Punished cocaine taking, an important aspect of compulsive behavior in SUDs, is also enhanced by IntA (Belin-Rauscent et al., 2016; James et al., 2019; Lüscher et al., 2020). Although it has been theorized that compulsive drug seeking arises from an over-reliance on habitual behavior compared to goal-directed drug seeking, the effects of IntA on habit formation or reliance on dopamine in the dorsolateral striatum (DLS), a structure important for habit initiation, have not been evaluated (Everitt, 2014; Ostlund and Balleine, 2008). One study showed that compared to LgA, IntA increased cocaine-induced *c-fos* expression in the dorsal striatum, including the DLS, but not the nucleus accumbens, which suggests IntA may impact habit circuitry, but its behavioral effects on habits are unknown (Minogianis and Samaha, 2020).

Additionally, sex differences in the effect of IntA on addiction-like behaviors are understudied, though existing studies show that IntA produces greater enhancement of psychomotor sensitization, locomotor sensitization, and greater and more rapid enhancement of motivation for cocaine in females (Algallal et al., 2020; Carr et al., 2020; Kawa and Robinson, 2019). The ability of IntA to enhance punishment-resistant cocaine self-administration has only been evaluated in males and has not been examined in females (James et al., 2019). Furthermore, a recent study showed that dopamine sensitization, which appears to be central to IntA's effects on addiction-like behavior, differentially affects the rate of habit formation in males and females (Schoenberg et al., 2022), which suggests that IntA could have differential effects on the rate at which DLS dopamine-dependent, habit-like cocaine seeking is established between sexes.

Overall, IntA has been shown to promote many addiction-like behaviors characteristic of SUDs and provides a model for analyzing how the spiking brain cocaine concentrations that induce sensitization may promote the progression from casual substance use to SUD. We sought to expand on the current literature by examining the effects of IntA on the ability of cue extinction to reduce cue-induced cocaine seeking, motivation for cocaine on a progressive ratio (PR) schedule, punished cocaine self-administration, and the development of DLS dopamine-dependent cocaine seeking in both males and females. Our results support previous findings that IntA-induced enhanced motivation for cocaine is more prominent in females, provide new evidence that IntA promotes DLS dopamine-dependent, habit-like behavior, and suggest that IntA may be valuable in examining sex differences in the early phases of the progression toward SUDs.

4.2 Methods

4.2.1 Animals

Adult Sprague-Dawley rats (Envigo) age 8-9 weeks upon arrival (n=120; male n=60; female n=60) were used. Rats were given at least 5 days to acclimate to the colony before experimentation. Before surgery, rats were pair-housed in auto-ventilated racks with automated watering in a humidity- and temperature-controlled room with a 12-hour light-dark cycle and had *ad libitum* access to food and water. Rats were individually housed after surgery, and one day before the start of self-administration they were food-restricted to a maintain 90% of their free-feeding body weight. Behavioral experiments were run in the dark cycle under red light and began within 3 hours of the same time of day. Procedures were conducted in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

4.2.2 Drugs

Cocaine hydrochloride (graciously provided by NIDA) was dissolved at 1 mg/ml in 0.9% sterile saline (Thermo Fischer) and filter-sterilized. *Cis*-flupenthixol hydrochloride (Cayman Chemical Company) was dissolved at 20 μg/μl in ddH_2O.

4.2.3 Behavioral apparatus

Experiments were conducted in 24 standard operant conditioning chambers (MedAssociates) using MedPC software (MedAssociates) as previously described (Bender and Torregrossa, 2021). Half of the boxes were equipped with grid floor harnesses that allowed an aversive stimulus generator to pass electrical current through the grid floors which were checked prior to testing with an amp meter (MedAssociates).

4.2.4 Surgery

4.2.4.1 Anesthesia

Rats were fully anesthetized with ketamine (87.5-100 mg/kg, Henry Schein) and xylazine (5 mg/kg, Butler Schein), were administered the analgesic Rimadyl (5 mg/kg, Henry Schein) and 5 ml Lactated Ringer's solution, and surgical sites were prepped as previously described (Bender and Torregrossa, 2021; Rich et al., 2019).

4.2.4.2 Intravenous catheterization

Rats were implanted with a chronic indwelling intravenous catheter (made in-house) into the right jugular vein that was capped to prevent blockages as previously described (Bender and Torregrossa, 2021; Torregrossa and Kalivas, 2008).

4.2.4.3 Intracranial cannulation

Rats used for experiments involving intra-DLS infusions were implanted with bilateral 22-gauge guide cannulae (PlasticsOne) 1mm dorsal to the DLS (in mm from Bregma, anterior and

posterior (AP): +0.8; medial and lateral (ML): +/− 3.0; dorsal and ventral (DV): −4.0)) as previously described (Bender and Torregrossa, 2021) immediately following jugular vein catheterization.

4.2.4.4 Post-operative care

Rats were administered Rimadyl (5 mg/kg) subcutaneously on the two days following surgery, and catheter patency was maintained with a gentamicin (3 mg/ml; Henry Schein) and heparin (30 USP/ml; Henry Schein) mixture in 0.9% sterile saline as previously described (Bender and Torregrossa, 2021).

4.2.5 Behavioral procedures

4.2.5.1 Cocaine self-administration

Rats were trained to self-administer cocaine (0.5 mg/kg/infusion) daily. Each session began with the illumination of the house light, start of the fan, and insertion of the active and inactive lever (counterbalanced between animals). All cocaine infusions were paired with a 10-second audiovisual conditioned stimulus (CS) of a tone and the illumination of a cue light above the active lever and initiated a 10-second time-out period when the house light was extinguished and another infusion could not be obtained. Inactive lever presses were recorded, but had no programmed consequences.

Rats underwent initial acquisition training for 2-10 days, during which cocaine was delivered on a fixed-ratio 1 (FR1) schedule continuously for 125 minutes or until 60 infusions were obtained to reduce overdose risk. Rats met acquisition criteria when they received ≥15

infusions with twice as many active vs. inactive lever presses for two consecutive days, as adapted from previous experiments, adjusting for dose of cocaine (Allain and Samaha, 2019).

After meeting acquisition criteria, rats continued self-administering with either continuous access (ContA) or intermittent access (IntA) to cocaine. IntA sessions consisted of 5 5-minute cocaine-available periods separated by 25 minutes of cocaine-unavailable periods for a total of 125 minutes, a protocol adapted from previous IntA experiments (Allain and Samaha, 2019; Garcia et al., 2020). We used this shortened, 125-minute daily self-administration IntA model because evidence suggests shortened IntA produces similar effects to the original 6-hour IntA paradigm (Allain and Samaha, 2019), and this shortened procedure is more readily applied within labs with limited resources. During cocaine-unavailable periods, the house light was extinguished and levers were retracted, but the fan remained on. The number of total infusions allowed was not restricted, since timeouts and cocaine unavailable periods reduce risk of overdose. In ContA sessions, cocaine was available continuously at the start of the session until a maximum of 30 infusions was reached, after which the house light was extinguished and levers were retracted. We chose to limit the ContA-trained rats to 30 infusions because rats tended to take more cocaine by the end of acquisition (on average, over 40 infusions) than IntA-trained rats. Previous work has shown that the number of cocaine-cue pairings is correlated with the strength of thalamo-amygdala synapses that encode cocaine-cue associations (Rich et al., 2019). Therefore, we aimed to ensure that ContA- and IntA-trained rats received equivalent exposure to cocaine-cue pairings.

4.2.5.2 Pavlovian cue extinction

Rats in experiment 1 (n=72; male n=36; female n=36) underwent Pavlovian cue extinction or a control procedure on the day following the tenth day of ContA or IntA. During cue extinction or a 0-CS control procedure, rats were presented with 0 or 120 audiovisual cues noncontingently

in the context where self-administration previously occurred with levers retracted. Ten-second cues (previously paired with cocaine infusions) were presented every 30 seconds for 1 hour.

4.2.5.3 Cue-induced cocaine-seeking test

Rats in experiment 1 underwent a 1-hour cue-induced cocaine-seeking test on the day following cue extinction. During this test, lever presses resulted in cue presentations and timeouts as previously occurred during self-administration, but no cocaine was delivered.

4.2.5.4 Punished cocaine self-administration

A subset of rats from experiment 1 (n=24; male n=12; female n=12) underwent testing for punished cocaine self-administration after the cue-induced cocaine-seeking test. For 3 days, all rats self-administered cocaine with continuous access in 110-minute sessions. These 3 days allowed IntA-trained rats to learn to revert their cocaine intake back to a load-and-maintain pattern, which prevents binge-like intake from interfering with results of the subsequent punishment testing (Bentzley et al., 2014; James et al., 2019).

The following day, rats underwent punishment testing using a protocol previously described (Bentzley et al., 2014; James et al., 2019). Cocaine self-administration was separated into 10-minute bins. During the first bin, rats reached their desired brain cocaine concentration, and during the second bin they were allowed to maintain this unpunished. For the remaining 9 bins, cocaine infusions (0.5 mg/kg/inf) were paired with a 0.5-second aversive footshock delivered at the time of cocaine infusion, and the intensity of this footshock increased incrementally on a tenth-$\log_{10}$ scale during each bin, starting at 0.13 mA and ending at 0.79 mA. The charge withstood during each bin was calculated by multiplying the infusion number with the duration of the shock (0.5 s) and the shock amplitude as previously described, and maximum shock withstood in any

one bin represents the maximum charge each rat is willing to withstand to maintain their desired brain cocaine concentration, which controls for individual differences in brain cocaine concentration preference (Bentzley et al., 2014).

4.2.5.5 Progressive ratio

A subset of rats from experiment 1 (n=48; male n=24; female n=24) underwent progressive ratio (PR) testing after the cue-induced cocaine-seeking test. Rats were allowed to self-administer cocaine for 1 day on their previous access model, then underwent a PR protocol as previously described (Allain and Samaha, 2019). Rats self-administered cocaine on a PR schedule for 4 days at 4 different doses (0.063, 0.124, 0.25, and 0.75 mg/kg/infusion), with the doses counter-balanced except the largest dose was always on the final day. After each cocaine infusion (paired with the 10-second audiovisual cue and timeout), the number of lever presses required to obtain the next infusion increased on a logarithmic scale. Sessions ended after 5 hours or when 1 hour passed without the next infusion being obtained.

4.2.5.6 Cocaine-seeking tests after intra-DLS drug infusion

Rats in experiment 2 with implanted with DLS guide cannulae (n=48; male n=24; female n=24) and were given a total of 4 cocaine-seeking tests 15 minutes after bilateral infusion of drug (10 μg *cis*-flupenthixol) or vehicle (ddH_2O) through a 28-guage internal cannula extending 1 mm below the guide cannula connected to a syringe pump (Harvard Apparatus) and 10 μl Hamilton syringes. Internal cannulae were left in place for one minute following infusion. Cocaine-seeking tests were identical to self-administration sessions except they were 15 minutes long and cocaine was not delivered upon timeout initiation. Rats immediately entered their daily cocaine self-administration session after each cocaine-seeking test. Tests occurred on slightly different

timelines for two groups (**Figure 18A, 18C**). In both groups (n=24 each), tests 1-2 (after *cis*-flupenthixol or vehicle, counter-balanced) occurred prior to any IntA, and tests 3-4 occurred after IntA, allowing for a within-subjects analysis between drug and vehicle and before and after IntA. The second group was added to ensure 10 days of undisrupted IntA before tests 3-4.

4.2.6 Vaginal cytology

Vaginal cytology was used daily to monitor the estrous cycle of female rats throughout the experiment. Sterile saline (200 μl) was pipetted into the vaginal canal and then placed onto a slide and coverslipped. Males were handled similarly. Slides were visualized at 200× magnification to determine estrous phase (Goldman et al., 2007).

4.2.7 Histology

Rats in experiment 2 were euthanized with CO_2 followed by decapitation, and brains were removed and submerged for at least 24 hours in 10% buffered formalin phosphate (Fischer Chemical). Brains were then cryo-protected with a 30% sucrose solution prior to sectioning and visualization as previously described (Bender and Torregrossa, 2021). Histological misses were characterized by placement more than 2.3 mm anterior to Bregma, less than 0.6 mm anterior to Bregma, dorsal of the striatum, or medial or lateral to the DLS.

4.2.8 Exclusion criteria

Rats were excluded from analysis due to death or illness after surgery (n=4), failure to meet acquisition criteria by 10 days (n=3), loss of catheter patency (determined by a 0.1-0.2 ml intravenous infusion of 10 mg/ml sodium brevital) (n=19), or if they failed to maintain an overall average of 15 daily infusions (n=3). Some rats were included in initial analysis and excluded from later analysis if catheter patency was lost between testing (n=5). For rats with histological misses (n=9), only self-administration data was included. Numbers of subjects reported in the results indicate the number of rats included in analysis after exclusions.

4.2.9 Quantification and statistical analysis

Animals were matched for responding when split into groups, and experimenters were blind to rats' treatment condition when possible. Behavioral data were collected using MedPC Software. All statistical analyses were performed on GraphPadPrism and SPSS Statistics software. The Shapiro-Wilk test was used to determine all data were normally distributed, and the Bartlett's test was used to determine that there were no significant differences between groups in estimated variance. For all statistical analyses, significance was set at $p<0.05$. Infusions and lever presses during the 10 days of ContA or IntA were analyzed using a three-way rmANOVA with time as the repeated measure factor and either access model, sex, or lever as factors where indicated. All other analyses used two- and three-way ANOVAs as necessary, except a mixed-effects model was used to analyze the effect of estrous phase on infusions because not all rats exhibited every phase. When interactions were significant in ANOVA or mixed-effects analysis, Sidak's or Tukey's post-hoc multiple comparisons were used to further analyze differences between groups.

4.3 Results

4.3.1 Continuous vs. intermittent cocaine self-administration produce different patterns of cocaine intake

Self-administration data for all rats used in these studies are reported together (**Figure 16**). Rats were given continuous access to cocaine until acquisition criteria were met (days -10-0), and then were split into ContA and IntA groups for an additional 10 days of self-administration (days 1-10). During acquisition and ContA, rats could continuously self-administer cocaine until the maximum number of infusions were met, but during IntA cocaine availability was restricted to 5 5-minute periods (**Figure 16A**).

Rats (n=91) learned to self-administer cocaine, and during the 10 days after acquisition, there was no main effect of access model ($F_{(1,87)}$=0.04217, p=0.8378) or sex ($F_{(1,87)}$=0.9945, p=0.3214), but there was a main effect of training day ($F_{(9,783)}$=9.860, p<0.0001) and a training day × access model interaction ($F_{(9,783)}$=9.834, p<0.0001) on number of infusions (3-way rmANOVA) (**Figure 16B**). Further post-hoc analyses (Tukey's multiple comparisons) indicated this interaction was driven by fewer infusions in the IntA-trained rats compared to the ContA-trained rats on the first day of IntA or ContA. IntA-trained males took significantly less cocaine than ContA-trained males (p=0.0484, q=5.531) and ContA-trained females (p=0.0374, q=5.635), and IntA-trained females took significantly less cocaine than ContA-trained males (p=0.0407, q=5.601) and ContA-trained females (p=0.0311, q=5.708). ContA-trained males and females (p>0.9999, q=0.3256) and IntA-trained males and females (p>0.9999, q=0.3363) did not differ in cocaine intake on the first day of IntA or ContA (**Figure 16B**). The reduction in infusions on the first day of IntA is likely due to the change in access model, when rats must learn they can only take cocaine in 5-minute

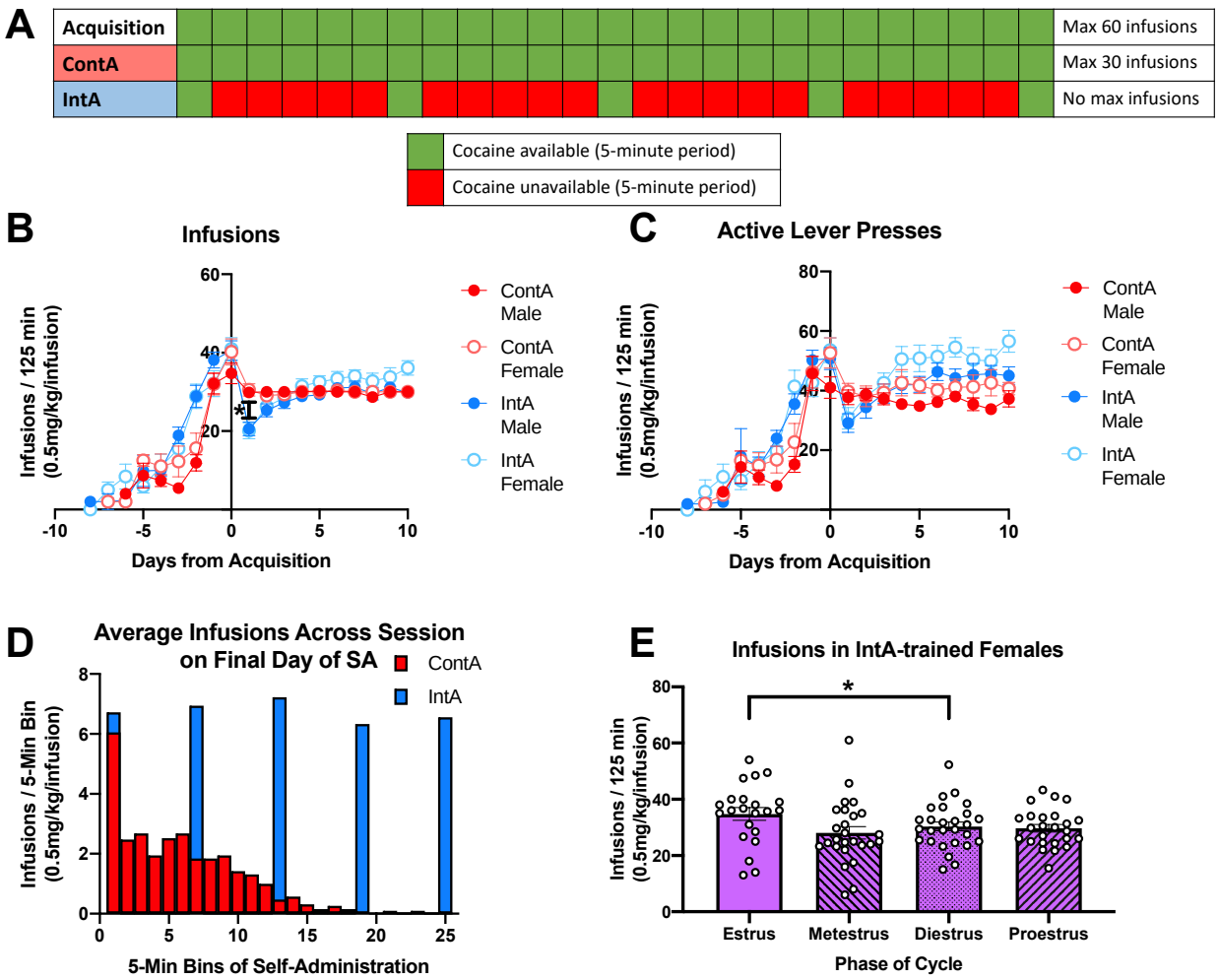

Figure 16: Continuous vs. intermittent cocaine self-administration produce different patterns of cocaine intake

Schematic for acquisition, ContA, and IntA self-administration sessions (**A**). All rats (experiments 1-2) self-administered cocaine for 2-10 days until they met acquisition criteria, then for 10 days with either ContA or IntA to cocaine. During the 10 days of ContA or IntA after acquisition, there was no effect of access model or sex on infusions, but there was a main effect of training day and a training day × access model interaction (**B**). Post-hoc analyses revealed that on the first day of ContA or IntA training, IntA-trained males and females self-administered less cocaine than ContA-trained males and females despite no overall differences in cocaine intake (**B**). During the 10 days of ContA or IntA after acquisition, there was a main effect of training day and access model on active lever presses, and there was a significant training day × access model interaction (**C**). In a subset of rats, the number of infusions during each 5-minute period of the final day of self-administration were plotted in a histogram to show the different patterns of cocaine intake for ContA vs. IntA access models (**D**). The estrous phase in all female rats was monitored via vaginal

cytology throughout self-administration. Although ContA almost always reached the maximum 30 infusions, there was a main effect of estrous phase on the average number of cocaine infusions in IntA-trained rats (**E**). Post-hoc analyses revealed that IntA-trained rats took more cocaine infusions in estrus than in diestrus (**E**). Graphs show group means ± SEM and individual data points. *p<0.05.

periods. Note that we chose to cap ContA-trained rats at a maximum of 30 infusions to ensure that ContA- and IntA-trained rats obtained an equivalent number of cocaine infusions. Therefore, any differences in later tests would be due to the access model and would not be confounded by overall cocaine intake. Most ContA-trained rats reached the maximum within about an hour.

For the 10 days after acquisition, there was no main effect of sex ($F_{(1,87)}$=3.159, p=0.0790), but there was a main effect of training day ($F_{(9,783)}$=6.846, p<0.0001) and access model ($F_{(9,87)}$=3.977, p=0.0493), and a training day × access model interaction ($F_{(9,783)}$=6.581, p<0.0001) on active lever presses (3-way rmANOVA) (**Figure 16C**). These data suggest that active lever presses increased as training progressed in IntA-trained rats, again likely due to the change in access model leading to a reduction in lever presses on the first few days of IntA until binge-like cocaine intake was learned. Despite having equivalent cocaine intake to ContA-trained rats, IntA-trained rats made more active lever presses by the end of training. Increased active lever pressing in IntA rats is likely due to increased timeout active lever presses, which occur during the timeout period and thus are counted but do not result in cocaine infusions. Although ContA and IntA-trained rats self-administered equivalent cocaine infusions, their patterns of cocaine intake were unsurprisingly very different. The average number of cocaine infusions during each 5-minute bin on the final day of self-administration was plotted for a subset of rats (n=37) (**Figure 16D**). ContA-trained rats binged in the beginning of the session and then reduced their cocaine intake to maintain their desired brain cocaine concentration until they reached the maximum 30 infusions, whereas IntA-trained rats binged during each 5-minute period of cocaine availability.

Vaginal cytology was used to monitor estrous phase in female rats throughout the study. Because ContA-trained rats almost always reached the maximum 30 infusions, no effect of estrous cycle on infusions could be observed in ContA-trained rats in this study. We examined if there was an effect of estrous cycle on the number of cocaine infusions obtained in IntA-trained females that completed 10 days of IntA (n=28). The average number of daily infusions each rat obtained during each estrous phase was calculated. There was a main effect of estrous phase on average number of cocaine infusions ($F_{(2.070,47.61)}$=3.272, p<0.0450) (mixed-effects analysis) (**Figure 16E**). Further post-hoc analyses (Tukey's multiple comparisons) indicated rats took more infusions during estrus than diestrus (p=0.0439, q=4.047) (**Figure 16E**). There were no other significant differences between phases, but there were nonsignificant trends towards rats taking more infusions in estrus than metestrus (p=0.0633, q=3.793) or proestrus (p=0.0588, q=3.862) (**Figure 16E**). Therefore, although IntA-trained females did not self-administer significantly more cocaine than IntA-trained males, they tended to take more cocaine during estrus than other cycle phases, though this effect was small.

4.3.2 Intermittent cocaine self-administration has sex-specific effects on the efficacy of cue extinction, motivation for cocaine, and punished cocaine self-administration

After 10 days of ContA or IntA, rats in experiment 1 (n=53) underwent behavioral testing for the effects of cue extinction on cue-induced drug seeking (n=53), motivation for cocaine using a PR test (n=30), and punished cocaine self-administration (n=18) (**Figure 17A**). Because we have previously shown that rats trained on second-order schedules of reinforcement are resistant to cue extinction (Bender and Torregrossa, 2021), we first sought to determine if a different pattern of self-administration (such as IntA) could also facilitate this resistance to cue extinction. After the

10th day of ContA or IntA, rats underwent cue extinction, when 120 audiovisual cues (120 CS) were presented non-contingently, or a control 0-CS procedure. The following day, rats underwent a 1-hour cue-induced cocaine-seeking test, and lever presses were recorded and resulted in cues but no cocaine delivery. There was a main effect of cue extinction ($F_{(1,45)}$=8.995, p<0.0044) and access model ($F_{(1,45)}$=5.431, p<0.0243) on active lever presses during the cue-induced cocaine-seeking test, but no main effect of sex ($F_{(1,45)}$=3.680, p=0.0614), cue extinction × access model interaction ($F_{(1,45)}$=0.9398, p=0.3375), cue extinction × sex interaction ($F_{(1,45)}$=2.459, p=0.1239), access model × sex interaction ($F_{(1,45)}$=1.108, p=0.2982), or 3-way interaction ($F_{(1,45)}$=0.6079, p=0.4397) (3-way ANOVA) (**Figure 17B**). These results suggest that overall, cue extinction reduced cue-induced cocaine seeking, and IntA-trained rats showed increased cue-induced cocaine seeking than ContA-trained rats, although it is possible that this could be due to increased baseline lever presses in IntA-trained rats observed during self-administration. Note that this experiment was likely underpowered to detect a three-way interaction between access model, sex, and cue extinction. Additionally, although estrous phases were fairly balanced between groups during cue-induced drug seeking, we collapsed across groups to determine if rats in estrus responded more during this cue-induced drug-seeking test like they did during self-administration. There is was no effect of estrous phase on lever presses ($F_{(3,21)}$=0.8034, p=0.5060) (one-way ANOVA) (data not shown).

A subset of rats from experiment 1 (n=30) underwent PR testing to determine if there was an effect of IntA or sex on motivation for cocaine. For 4 days on 4 different doses of cocaine, the number of lever presses required to obtain the next cocaine infusion increased on a logarithmic scale. The ratio at which rats no longer obtained another infusion, or the breakpoint, is an indicator of motivation (Panlilio and Goldberg, 2007). There was a 3-way dose × access model × sex

interactions ($F_{(3,78)}$=7.952, p=0.0001) for breakpoint (3-way rmANOVA) (**Figure 17C**). Further post-hoc analyses (Sidak's multiple comparisons) indicated that IntA-trained females had a higher breakpoint at the highest dose (0.75 mg/kg/infusion) than ContA-trained males (p<0.0001 t=7.253), ContA-trained females (p<0.0001 t=7.517), or IntA-trained males (p<0.0001 t=7.956). These data indicate that IntA promoted increased motivation for 0.75 mg/kg/infusion cocaine exclusively in females.

Another subset of rats from experiment 1 (n=18) were used to determine if IntA promotes increased punishment-resistant cocaine self-administration. We used a previously-established test where the session is divided into 10-minute bins, and for the first 2 bins rats self-administer unpunished to reach and begin to maintain their desired brain-cocaine concentration (Bentzley et al., 2014). For the remaining 9 bins, the intensity of an aversive footshock paired with cocaine infusions is increased incrementally from 0.13 mA to 0.79 mA (**Figure 17D**).

First, we examined if there were any differences in the average number of unpunished cocaine infusions during the first 2 bins between sexes or access models. There were no main effects of access model ($F_{(1,14)}$=4.448, p=0.0534) or sex ($F_{(1,14)}$=0.1852, p=0.6735) or interaction ($F_{(1,14)}$=0.2369, p=0.6735) for unpunished cocaine infusions (2-way ANOVA) (**Figure 17E**), which suggests that there were no significant differences in cocaine self-administration when infusions were unpunished. Even so, we noted that both IntA-trained males and females did take non-significantly more cocaine unpunished. Therefore, to ensure differences in punished cocaine self-administration were not due to differences in the amount of cocaine self-administered, we used the previously-established method of calculating the maximum charge withstood in each 10-minute bin of punished cocaine self-administration and comparing the maximum charge each rat withstood in any one bin. Similar to a breakpoint, this value represents the amount of punishment

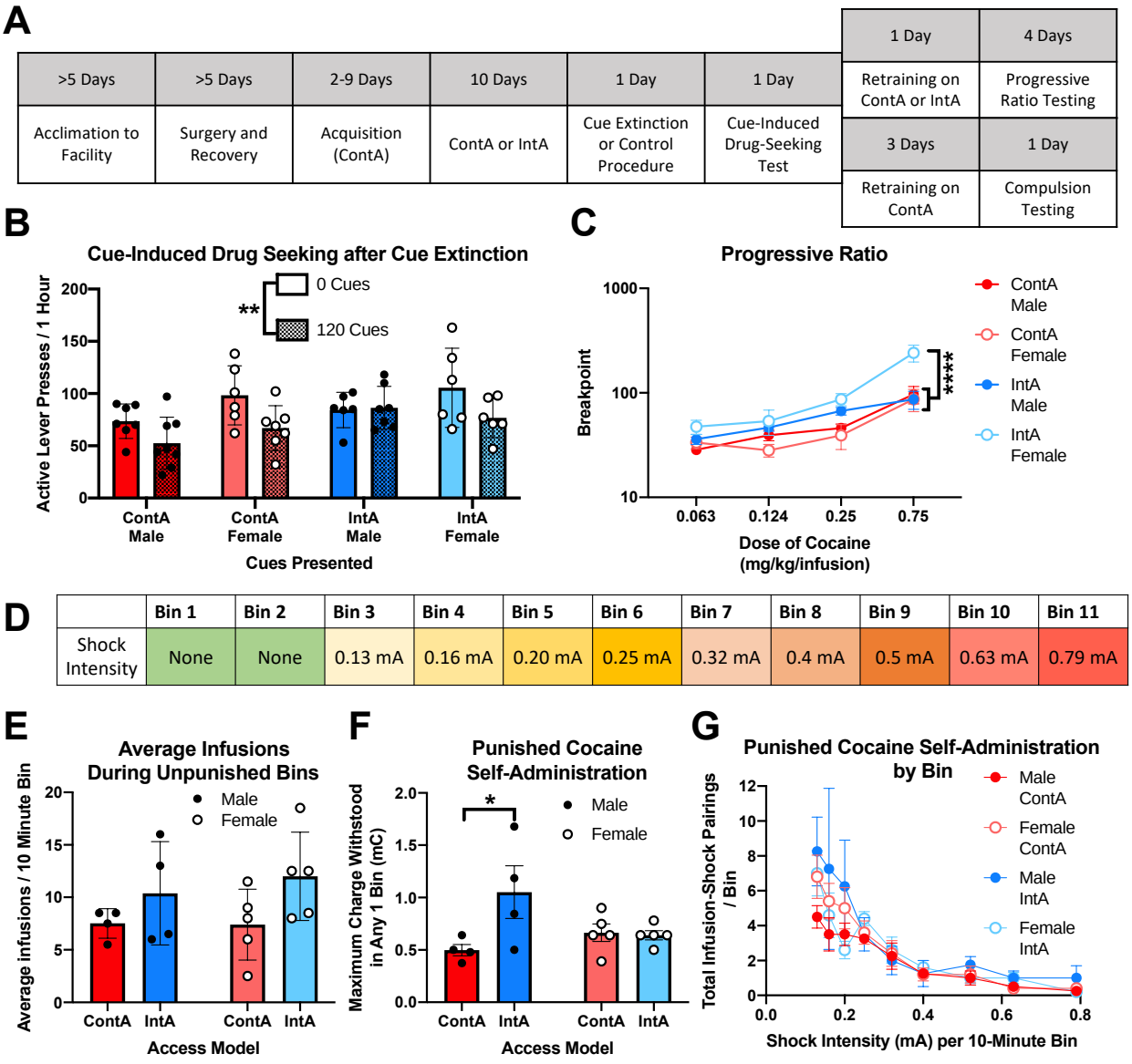

Figure 17: Sex-specific effects of intermittent cocaine self-administration on the efficacy of cue extinction, motivation for cocaine, and punished cocaine self-administration

Experimental timeline for the subset of rats included in experiment 1 (**A**). After 10 days of ContA or IntA, rats underwent cue extinction (120 CS) or a control procedure (0 CS) followed by a 1-hour cue-induced cocaine-seeking test. There was a main effect of cue extinction and access model on active lever presses, and overall rats that underwent 120-CS cue extinction made significantly fewer lever presses than 0 CS control rats (**B**). A subset of rats underwent progressive ratio testing for motivation for cocaine. There was a main effect of dose of cocaine, access model, and sex on the breakpoint in the progressive ratio test, and there were dose × access model, dose × sex, access model × sex,

and dose × access model × sex interactions (**C**). Post-hoc analyses revealed that at the highest dose, IntA-trained females had a higher breakpoint than all other groups (**C**). Another subset of rats underwent a test for punished cocaine self-administration that involves a period of unpunished cocaine self-administration, followed by 10-minute bins where cocaine infusions are paired with a footshock of increasing intensity (**D**). There was no effect of sex or access model on the average number of cocaine infusions taken during unpunished bins (**E**). When the maximum footshock charge withstood in any 1 bin for each rat was calculated, there was an access model × sex interaction (**F**). Post-hoc analyses revealed that IntA-trained males withstood significantly more shock than ContA-trained males, but this was not the case for IntA-trained females vs. ContA-trained females (**F**). There was a main effect of shock intensity on the number of cocaine infusion-shock pairings per bin (**G**). Graphs show group means ± SEM and individual data points. *p<0.05. **p<0.01. ****p<0.0001.

each rat is willing to withstand to maintain their desired brain-cocaine concentration. We found no main effect of access model ($F_{(1,14)}$=4.483, p=0.0526) or sex ($F_{(1,14)}$=0.9766, p=0.3398) on the maximum charge withstood in any 1 bin, but there was an access model × sex interaction ($F_{(1,14)}$=5.332, p=0.0367) (2-way ANOVA) (**Figure 17F**). Further post-hoc analyses (Sidak's multiple comparisons) indicated that IntA-trained males withstood significantly more shock than ContA-trained males (p=0.0202, t=2.969), but IntA-trained females did not withstand more shock than ContA-trained females (p=0.9874, t=0.1439). Additionally, we examined the total cocaine infusion-shock pairings in each bin. There was a main effect of shock intensity ($F_{(2.077,29.08)}$=16.13, p<0.0001) on punished infusions, which indicates rats reduced their infusions as the shock intensity increased (3-way rmANOVA) (**Figure 17G**). There were no main effects of access model ($F_{(1,14)}$=2.742, p=0.1200) or sex ($F_{(1,14)}$=0.01683, p=0.8986), nor any shock intensity × access model ($F_{(8,112)}$=0.3812, p=0.9287), shock intensity × sex ($F_{(8,112)}$=0.2420, p=0.9819), access model × sex ($F_{(1,14)}$=4.339, p=0.0561), or 3-way ($F_{(8,112)}$=1.099, p=0.3691) interactions for punished infusions. Overall, these results suggest that IntA promoted punishment resistant cocaine self-administration exclusively in males. Due to splitting animals into groups and loss of catheter

patency by the end of testing, this experiment is slightly underpowered (n=4-5), so these results should be interpreted cautiously.

4.3.3 Intermittent cocaine self-administration facilitates cocaine seeking dependent on DLS dopamine

Given that we have previously shown that DLS dopamine-dependent, habit-like cocaine seeking is resistant to cue extinction, the observation that IntA-trained males may be resistant to the effects of cue extinction led us to examine if DLS dopamine antagonism impacts cocaine seeking after IntA in experiment 2 (n=29). Rats were infused bilaterally in the DLS with the nonspecific dopamine antagonist *cis*-flupenthixol and vehicle (counterbalanced across 2 days) prior to a 15-minute drug-seeking test at two different timepoints before and after IntA for a total of 4 tests.

Initially, in experiment 2A (n=13), the first set of tests occurred after acquisition and prior to IntA, and the second set occurred after 7-8 days of IntA (**Figure 18A**). All rats received both drug and vehicle and were tested at both timepoints for a within-subjects design. During the cocaine-seeking tests after vehicle or *cis*-flupenthixol infusion, there was no effect of drug ($F_{(1,11)}$=0.7622, p=0.4013), IntA ($F_{(1,11)}$=1.248, p=0.2877), or sex ($F_{(1,11)}$=1.282, p=0.2815) on active lever presses, and there were no interactions between drug × IntA ($F_{(1,11)}$=0.6104, p=0.4498), drug × sex ($F_{(1,11)}$=0.8404, p=0.3789), IntA × sex ($F_{(1,11)}$=0.8404, p=0.3789), or 3-way interaction ($F_{(1,11)}$=1.209, p=0.2951) (3-way ANOVA) (**Figure 18B**). These data suggest that both before and after minimal (7-8) days of IntA, drug seeking is unaffected by DLS dopamine antagonism.

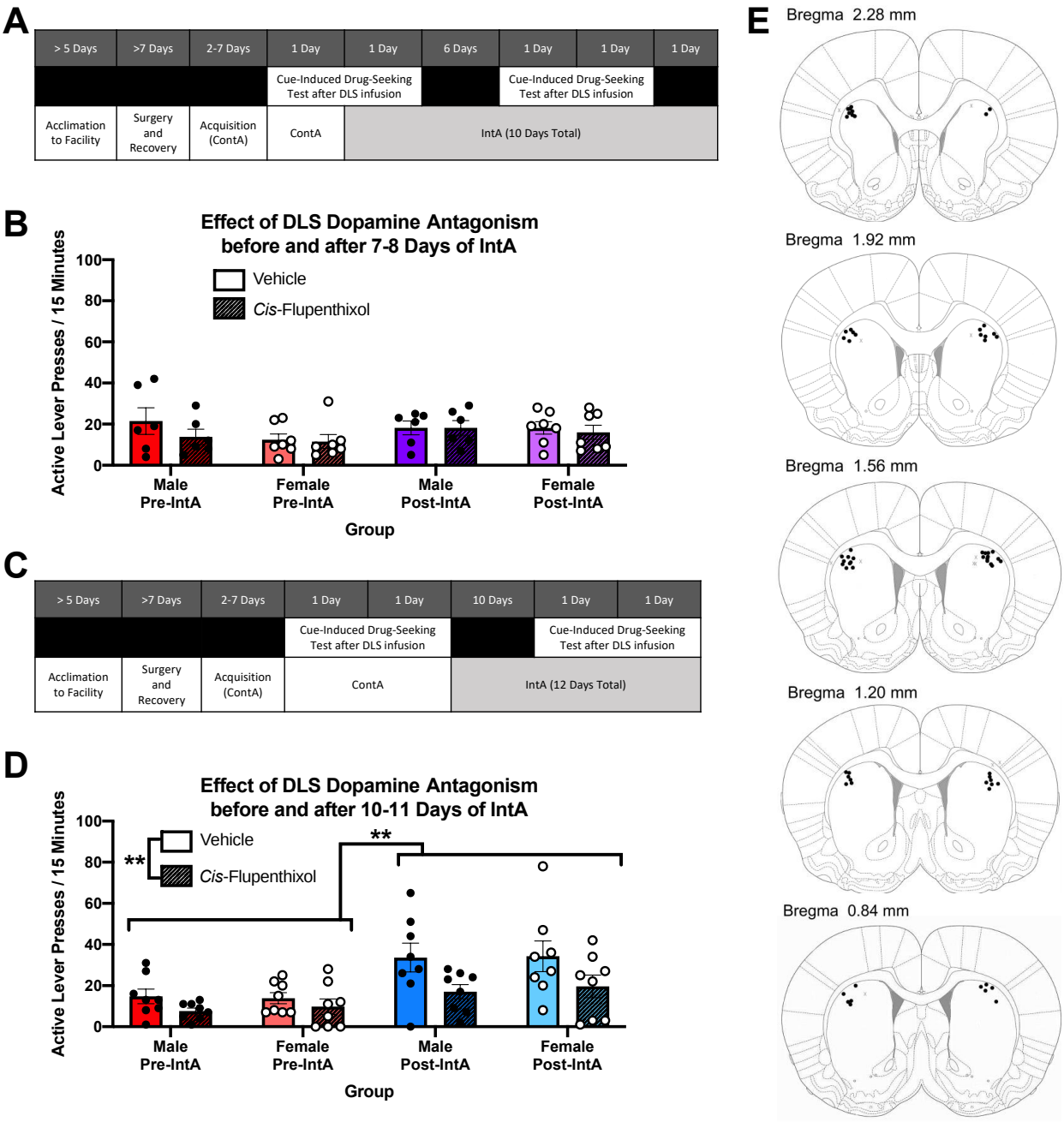

Figure 18: Intermittent cocaine self-administration facilitates cocaine seeking dependent on DLS dopamine

Experimental timeline for the subset of rats in experiment 2A (**A**). After acquisition (before IntA) and after 7-8 days of IntA, rats underwent a 15-minute drug-seeking test after direct DLS infusion of the nonspecific dopamine antagonist *cis*-flupenthixol or vehicle. There was no effect of DLS dopamine antagonism on drug seeking before or after 7-8 days of IntA (**B**). Experimental timeline for the subset of rats in experiment 2B (**C**). There was a main effect of drug and IntA training, as well as a drug × IntA training interaction on active lever presses during the cocaine-seeking tests

before and after 10-11 days of IntA (**D**). Placement of DLS cannulae were histologically evaluated, and each on-target placement is indicated by a black dot, while off-target misses are indicated by gray Xs (**E**). Graphs show group means ± SEM and individual data points. **p<0.01.

Experiment 2B (n=16) was very similar, but we made some adjustments to the timeline to ensure that no drug-seeking test occurred on the first day of IntA and rats would receive 10 days of undisrupted IntA prior to the second set of drug-seeking tests after DLS dopamine antagonism (**Figure 18C**). There was a main effect of drug ($F_{(1,14)}$=16.75, p=0.0011) and IntA ($F_{(1,14)}$=15.73, p=0.0014) on active lever presses during cocaine-seeking tests, but no effect of sex ($F_{(1,14)}$=0.06260, p=0.8061) (3-way ANOVA) (**Figure 18D**). There was also a drug × IntA interaction ($F_{(1,14)}$=4.667, p=0.0486), but no drug × sex ($F_{(1,14)}$=0.2318, p=0.6376), IntA × sex ($F_{(1,14)}$=0.01838, p=0.8941), or 3-way interactions ($F_{(1,14)}$=0.01167, p=0.9155) (**Figure 18D**). These results suggest that at least 10 days of IntA facilitated DLS dopamine-dependent cocaine seeking. Histological analysis of DLS cannulae placement are shown (**Figure 18E**).

4.4 Discussion

Here, we showed notable sex differences in the effects of IntA on addiction-like behavior. We found that compared to ContA, just 10 days of IntA sessions that were just over 2 hours long were enough to promote several addiction-like behaviors differentially in males and females. Recent studies have utilized these 2-4-hour long IntA sessions (Allain and Samaha, 2019; Cippitelli et al., 2022; Garcia et al., 2020; Kawa et al., 2019), shorter than the traditional 6-8 hours, which are more accessible to labs with limited operant space or time. Interestingly, one study in males suggested that shorter IntA sessions promote more individual differences, where a subset of

rats showed escalated cocaine intake accompanied by increased cue-induced reinstatement and locomotor sensitization to cocaine (Garcia et al., 2020). Despite using a similar protocol, we did not find a subgroup of IntA-trained rats (male or female) expressing escalated cocaine intake, likely because the previous study saw escalation occur mainly on days 10-14, which we did not include (Garcia et al., 2020). Still, we extend these findings by showing short IntA sessions also promote sex differences in the effects of IntA, which indicates that these protocols may be useful in identifying risk factors that contribute to acceleration of SUD development during early stages of drug use.

Although we found no sex differences in cocaine intake, IntA-trained females took more cocaine when in estrus. It is unlikely that this effect is unique to IntA, given that previous findings in a ContA models show a similar effect, but we could not determine if estrous phase had an effect on ContA-trained females in our study because they almost always obtained the maximum number of infusions, and therefore no differences could be observed (M. W. Feltenstein and See, 2007; Roberts et al., 1989). Additionally, our experiments were not powered to determine if estrous phase impacted the effects of IntA on the various aspects of addiction-like behavior we evaluated on test days. Given our findings and previous studies showing that estradiol can influence sensitization to cocaine, which is enhanced in IntA-trained females, future experiments should determine the effect of circulating sex hormones on the ability of IntA to promote addiction-like behaviors in females (Carr et al., 2020; Sell et al., 2002).

We did not find an enhancement of motivation for cocaine in males after IntA compared to ContA, which was surprising given several studies that have shown this enhancement using either PR or behavioral economic testing (Algallal et al., 2020; Calipari et al., 2015; James et al., 2019; Zimmer et al., 2012). Even though evidence suggests that the length of daily IntA does not

impact motivation (Allain and Samaha, 2019), because our IntA training was rather minimal (10 days) compared to these other studies, it is possible that more days of short IntA training are necessary to enhance motivation for cocaine in males. Alternatively, it is possible that motivation for cocaine in males was enhanced but was masked by an over-reliance on the DLS dopamine-dependent, habit-like behavior we identified in IntA-trained males. Both the PR and behavioral economic tests for motivation rely on the assumption that rats are responding in a goal-directed manner and increasing their lever presses when the outcome is of higher value, but if behavior is habit-like and does not rely on the value of the outcome, breakpoint may not be an accurate measure of motivation. Future studies should investigate if restoring goal-directed control could reveal increased motivation in a PR test in IntA-trained males. We did find that IntA-trained females showed increased motivation compared to IntA-trained males, which agrees with previous literature suggesting enhancement of motivation for cocaine after IntA is greater in females than in males (Algallal et al., 2020; Kawa and Robinson, 2019). Additionally, we found that IntA-trained females showed greater motivation for cocaine compared to ContA-trained females, as previously shown (Algallal et al., 2020). Although IntA-trained rats, especially females, showed enhanced lever pressing during self-administration, it is unlikely that this contributed to the increased breakpoint in IntA-trained females because breakpoint was only increased for the highest dose of cocaine. Based on other studies, motivation for cocaine in females may be reduced when females are in proestrus or enhanced during estrus (Kohtz et al., 2022; Roberts et al., 1989), but it is unlikely that circulating hormones in females contributed to this effect in our hands because estrous phases were fairly balanced between ContA and IntA groups during PR testing. Overall, these findings along with the previous literature suggest that IntA promotes increased motivation for cocaine more quickly and more robustly in females, so future studies should examine the role

of sex hormones and biological sex differences on the interaction between sensitization and motivation for cocaine.

Our findings expand on previous research showing that IntA promotes increased punishment-resistant cocaine self-administration in males (James et al., 2019), but we also showed a lack of this effect in females. During punishment-resistant testing, both male and female IntA-trained rats took more unpunished cocaine than ContA-trained rats, but IntA-trained males persisted more when cocaine was punished. These results suggest that IntA promotes increased punishment-resistant cocaine self-administration either more quickly or exclusively in males, and future studies should determine if more days of IntA or longer daily IntA sessions would be sufficient to replicate this effect in females. Interestingly, one previous study showed that there were no sex differences in preference for social reward over cocaine after intermittent cocaine self-administration, which suggests that sex differences may not be present for other aspects of compulsive behavior (Venniro et al., 2021). We also determined that IntA-trained rats reduced active lever presses after DLS dopamine antagonism after a full 10-11 days of IntA, but not before, which suggests the development of habit-like, DLS dopamine-dependent behavior. Although unlikely, it is possible that a floor effect could have masked an effect of DLS dopamine antagonism in the earlier tests conducted. Therefore, this novel finding should be examined further using behavioral analysis of habitual behavior to supplement the pharmacology used here, since no previous studies have examined how IntA may promote the development of habitual behavior. There is evidence that functional disconnection of the OFC and ventrolateral striatum, a circuit shown to be involved in goal-directed behavior, reduces responding on a PR schedule in IntA-trained rats (Gourley et al., 2013; Minogianis et al., 2019), which contradicts our findings by suggesting that self-administration under a PR schedule after IntA remains goal-directed.

However, this experiment did not examine the DLS and only allowed rats to take up to 0.5 mg/kg/infusion during each drug-available period of training, which is well below what most rats would take freely in an IntA binge period (Minogianis et al., 2019). It is possible that a combination of IntA and higher doses of cocaine are required to facilitate DLS dopamine-dependent behavior, and this should be further investigated.

We also examined the ability of Pavlovian cue extinction to reduce drug seeking after ContA or IntA. We did show overall increased lever presses in IntA-trained compared to ContA-trained rats, in accordance with previous studies showing increased cue-induced drug seeking in IntA-trained rats (Aragona et al., 2009; James et al., 2019; Kawa et al., 2019). Additionally, we found a main effect of cue extinction to reduce lever pressing in a cue-induced drug-seeking test, providing new evidence that despite enhanced cue-mediated responding, drug seeking in IntA-trained rats is still susceptible to extinction of the drug-cue association. Our previous findings indicated that DLS dopamine-dependent behavior is resistant to cue extinction after training on a second-order schedule, which suggests that different reinforcement schedules and patterns of drug exposure may have different effects (Bender and Torregrossa, 2021). However, it should be noted that the cue extinction effect in IntA-trained rats was driven by the females and was not visually apparent in males, which suggests the study was underpowered to detect a 3-way interaction. There is conflicting evidence that IntA may or may not promote persistence of lever pressing during operant extinction, when cues are not presented, so this persistence of drug-seeking behavior and the role of the drug-associated cue should be examined further (Garcia et al., 2020; James et al., 2019).

Interestingly, because we found increased PR breakpoint exclusively in IntA-trained females, but increased punishment resistance exclusively in IntA-trained males, our results suggest

that punishment-resistant cocaine self-administration and motivation for cocaine are promoted independently. Additionally, because the IntA-trained males showed the most robust DLS dopamine-dependent behavior, it may be that the development of habit-like behavior coincides with the degree of punishment-resistant cocaine seeking, and this should be examined further. Moreover, that IntA may promote habit-like behavior, increased motivation for cocaine, and punishment-resistance in a sex-specific manner lends credence to its usefulness in modeling addiction-like behavior in rodents and examining the different contributions of these drug effects on behavior. Importantly, the sex differences we identified in these experiments occurred after rather limited IntA. We may be capturing a timepoint at which these addiction-like behaviors may be developing in some rats at different rates based on sex and other individual differences, a possibility that is further supported by previous work suggesting that genetically male mice are more prone to form ethanol-seeking habits than females (Barker et al., 2010), though the opposite is true for the seeking of a food reward (Quinn et al., 2007). It is possible that additional short IntA training, such as 10-20 additional days, would promote enhanced motivation, punishment resistant cocaine self-administration, resistance to cue extinction, and reliance on DLS dopamine across sexes, and future experiments should examine this. Overall, we expanded on the current literature by providing new insights into how IntA may differentially promote addiction-like behaviors in males and females. Our findings suggest that IntA may be uniquely suited to identify sex differences in how cocaine impacts dopamine modulation, motivation, and punishment resistance, especially during the early stages of drug use when sensitization occurs.

5.0 General Discussion

The studies presented in this dissertation investigated the role of Pavlovian cocaine-cue associations in cocaine-seeking behavior that is goal-directed, habitual, or resistant to punishment. We used FR and SO schedules of reinforcement to facilitate goal-directed and habitual cocaine-seeking, respectively. We examined how these training schedules influenced the dorsal striatum, a key brain region for the learning and execution of goal-directed and habitual behavior, and evaluated how extinction of the Pavlovian drug-cue associations impacted behavior and the dorsal striatum (**Chapters 2 & 3**). Additionally, we employed IntA to facilitate punishment-resistant cocaine self-administration and determined how IntA affected habitual behavior reliant on DLS dopamine as well as the efficacy of cue extinction (**Chapter 4**).

Through these experiments, we identify distinct patterns of dorsal striatal protein expression, as well as calcium and dopamine responses to behavior that delivered drug-associated cues, in FR- and SO-trained rats, findings that support previous literature describing distinct roles of the DMS and DLS in goal-directed and habitual behavior. We show that both SO-schedule training and IntA promote drug-seeking behavior that is dependent on DLS dopamine. Our findings also provide evidence that the facilitation of cocaine-seeking habits involves disengagement of the DMS. Additionally, we show that extinction of Pavlovian drug-cue associations affects DMS calcium and dopamine activity, but does not impact the DLS and does not affect cocaine seeking in SO-trained rats unless goal-directed control is restored. Finally, our results suggest that IntA enhances punishment-resistant cocaine self-administration exclusively in males, and males also appear to be unaffected by cue extinction. Overall, these findings support the hypothesis that punishment-resistant and habitual cocaine-seeking behaviors and their

underlying neural circuitry are unaffected by Pavlovian cue extinction. In the following discussion, we propose additional questions to be addressed, note some limitations of these findings, and discuss the implications of these results for SUD treatment.

5.1 Distinct roles of dopamine in striatal sub-regions for reward-related learning and memory

Although the striatum's importance for reward-related learning and the execution of motivated behavior is clear, the specific contributions of striatal sub-regions have been debated (Liljeholm and O'Doherty, 2012). The role of dopamine in the NAc in Pavlovian associative learning has been thoroughly described; as a stimulus becomes associated with a reward, the dopamine release in the NAc that initially follows reward delivery shifts to occur upon stimulus onset (Day et al., 2007; Flagel et al., 2011; Shnitko and Robinson, 2015). Furthermore, how this dopamine encodes reward prediction error (RPE), particularly in the NAc, has been extensively described (Lerner et al., 2021). However, dopamine's role appears to extend beyond RPE, especially with respect to the dorsal striatum (Barter et al., 2015; Lerner et al., 2021). Indeed, we show that, in SO-trained rats behaving habitually, DLS dopamine increases when the performance of a behavior is followed by cue presentation, and this response is greater than responses to lever presses or cue presentations alone (**Chapter 3**). Although we were not the first to show that DLS dopamine increases during cue-induced drug-seeking behavior in SO-trained rats, our results expanded on a previous study by determining that DLS dopamine release is specifically time-locked to cue-reinforced lever presses (Ito et al., 2002). Additionally, another study that used variable interval schedules of alcohol and sucrose self-administration, which also promote habitual

behavior, identified similar dopamine responses to cue-reinforced lever presses that were specific to the DLS (Shnitko and Robinson, 2015). Therefore, our findings, along with the literature, suggest that DLS dopamine represents the occurrence of stimulus-response pairing, which supports the theory that DLS dopamine release is important for stimulus-response learning.

It remains to be determined neurobiologically why SO schedules and other schedules of reinforcement, such as variable interval schedules, facilitate the DLS dopamine release that seemingly promotes rapid stimulus-response control of behavior. SO-trained rats receive many stimulus-response pairings, and these are also often not accompanied by cocaine reward delivery. In contrast, FR-trained rats only receive one stimulus-response pairing per cocaine infusion, and each stimulus is always accompanied by cocaine. Interestingly, we found that SO training facilitates a DLS dopamine response to cue-reinforced lever presses relatively quickly; this response occurred during the first five days of SO-schedule training and did not further increase with an additional 5 days of SO-schedule training (**Chapter 3**). The rapidity of this transition is further supported by our finding that DLS dopamine antagonism reduced drug-seeking after just two days of SO training (**Chapter 2**). We also determined that IntA, which involves bursts of responding over short periods of time, promotes DLS dopamine-dependent behavior (**Chapter 4**). Therefore, future experiments could examine whether it is the number of stimulus-response pairings, a rapid rate of these pairings, a lack of certainty that reward will accompany these pairings, or a combination of these factors that promote an enhanced DLS dopamine response.

We were initially surprised that, in FR-trained rats for which behavior is presumably dependent on DMS dopamine (Murray et al., 2012), there was not an enhanced DMS dopamine response to cue-reinforced lever presses compared to lever presses that had no consequence (**Chapter 3**). We did not record DMS dopamine responses to lever presses that resulted in reward

delivery due to technical limitations as well as complications involved in cocaine's effects on dopamine, but one previous study did show that DMS calcium activity in dopamine neuron terminals accompanies a sucrose-reinforced lever press in a task where no discrete cue accompanied reward delivery (Seiler et al., 2022). In contrast, another study did not show enhanced DMS dopamine release in response to lever presses that resulted in a cue that signaled reward delivery (Shnitko and Robinson, 2015). Therefore, it remains to be determined if DMS dopamine release would be enhanced for lever presses that result in cocaine infusions, and it would be interesting to determine if this release occurs in both the presence and absence of additional cue reinforcement.

Another outstanding question is how striatal dopamine release contributes differentially to learning and behavioral output, and how this contribution changes as learning progresses. Generally, inhibition of dopamine in striatal sub-regions inhibits the execution of behaviors promoted by associations encoded in each region, which indicates a clear role of dopamine in movement and behavioral execution (Blaiss and Janak, 2009; Murray et al., 2012; Palmiter, 2008; Parkinson et al., 1999). Interestingly, there is evidence that associative learning can still occur in the absence of dopamine in dopamine-depleted mice (Palmiter, 2008). However, even if striatal dopamine may not necessarily be essential for associative learning, there is ample evidence that it facilitates learning (Liljeholm and O'Doherty, 2012). Additionally, we also show small dopamine responses to novel stimuli in both the DMS and DLS (**Appendix**), which suggests that dorsal striatal dopamine is not only involved in behavior, and may also play a more complex role than simply encoding stimulus-response or response-outcome associations. Because dopamine release from SN neurons, which project to the dorsal striatum, in response to novel stimuli has been implicated in Pavlovian learning and conditioned responding (Morrens et al., 2020), future

experiments could manipulate dopaminergic projections to distinct striatal regions during learning and behavior to answer some of these questions.

Indeed, studies using optogenetic techniques to stimulate phasic dopamine release from VTA neurons have shown that this stimulation, in the place of a reward, is sufficient to promote conditioned responding (Tsai et al., 2009; Witten et al., 2011). Therefore, a careful examination of how dopamine release in the DMS and DLS impacts learning would be interesting. However, designing and interpreting the results of these experiments can be difficult, because dopamine depletion can inhibit general locomotor activity, optogenetic activation of midbrain dopamine neurons can impact the release of other neuromodulators, and dopaminergic neurons in the midbrain have extensive collateralizations (Dagher et al., 2022; Palmiter, 2008; Yu et al., 2022). Overall, our findings and those of related literature support a role of dorsal striatal dopamine in learning in addition to execution of behavior, as has been previously suggested (Atallah et al., 2007).

Dopamine's neuromodulatory effects that can impact synaptic plasticity are likely responsible for its enhancement of learning (Lovinger, 2010; Nicola et al., 2000). We showed that SO-trained rats had increased membrane insertion of the GluA1 AMPA receptor subunit in the DLS compared to FR-trained rats (**Chapter 2**). Other studies have shown that D1 dopamine receptor activation increases localization of GluA1-containing AMPA receptors to the post-synaptic membrane of striatal neurons, and subsequent activation of calcium-permeable AMPA receptors results in insertion into the post-synaptic membrane through a mechanism dependent on protein kinase C (PKC) (Sun et al., 2005; Tukey and Ziff, 2013). Therefore, it would be interesting to determine if PKC inhibition in the DLS would prevent the increase in GluA1 membrane insertion in the DLS in SO-trained rats. Additionally, given that IntA also promotes behavior that

is sensitive to DLS dopamine antagonism (**Chapter 4**) and IntA promotes sensitization of the dopamine transporter (Calipari et al., 2015, 2013), it would also be interesting to examine if sensitization in the NAc or DLS causes changes in receptor trafficking or is required for the ability of IntA to promote DLS dopamine-dependent drug seeking.

5.2 Divergent circuits for goal-directed and habitual cue-induced drug seeking

In addition to differences in dorsal striatal dopamine, goal-directed and habitual behaviors are characterized by differential excitatory and inhibitory input to the DMS and DLS (Gourley and Taylor, 2016; Shiflett and Balleine, 2011). The dorsal striatum receives inputs from the thalamus, amygdala, and several cortical regions that mediate the initiation of behavior (Alloway et al., 2017; Smith and Laiks, 2017). However, the specific inputs to the DLS that undergo plasticity during habit learning have not been thoroughly characterized (O'Hare et al., 2018). Experimenters have examined neural activity in the dorsal striatum and its inputs during various types of habit learning, including motor skill learning on a rotarod, T-maze reward-seeking tasks, and operant behavioral tasks. Our examination of dorsal striatal calcium activity during goal-directed or habitual cocaine seeking (**Chapter 3**), as well as the changes in the expression of plasticity-related proteins induced in the dorsal striatum by these behaviors (**Chapter 2**), expand upon these previous findings about habit learning and the divergent circuits involved in goal-directed and habitual behavior. Additionally, by examining Pavlovian cue extinction, our results provide new insights into the influence of cocaine-cue associations on these circuits.

In addition to its role in stimulus-response learning, the DLS has been implicated in general motor output and learning of sequential behavior (Graybiel and Grafton, 2015; O'Hare et al.,

2018). Motor-skill learning can be measured in rodents as they learn to balance on an accelerating rotating rod (rotarod), and sustained training on this task is generally believed to promote learning of motor habits (Yin et al., 2009). Early on in rotarod training, more DMS neurons increase their firing rate when the rodent is running (Yin et al., 2009). Conversely, after extensive rotarod training, more DLS neurons increase their firing rate during running, which suggests these regions are differentially engaged during early learning and when the task is well-learned (Yin et al., 2009). Additionally, a T-maze task, where rats learn to turn left or right at a pathway junction to receive a reward, has also been used to examine DMS and DLS activity during habit learning (Yin and Knowlton, 2004). Differential engagement of the DMS and DLS occurs throughout different stages of the maze task, and activity changes as the task is learned (Thorn et al., 2010).

It is possible that different kinds of behavior, such as rotarod and maze running compared to drug-seeking lever presses, involve different types of sensory processing of stimuli associated with a behavioral response, so habit learning in operant reward-seeking tasks may be most applicable to drug-seeking behavior. General investigations of DMS and DLS neural activity during operant habit learning indicate distinct patterns of activity in these regions that change as learning progresses, but these patterns often differ depending on the specific parameters of the operant task (Fanelli et al., 2013; Kimchi et al., 2009; Vandaele et al., 2019; Vandaele and Janak, 2022). We used fiber photometry to record calcium activity simultaneously in the DMS and DLS during goal-directed or habitual cocaine seeking (**Chapter 3**). Given recent evidence that dorsal striatal calcium signals collected via fiber photometry primarily reflect dendritic calcium (Legaria et al., 2022), changes in the calcium signals we recorded in the dorsal striatum likely reflect changes in the balance of excitatory and inhibitory input into the dorsal striatum, and could also reflect changes in neuronal excitability or the membrane insertion of calcium-permeable receptors

in dorsal striatal neurons (Legaria et al., 2022). We showed that whereas both groups showed enhanced calcium responses to cue-reinforced lever presses in both the DMS and DLS during FR training, continued FR training maintained these responses, but SO training resulted in a lack of DMS calcium response (**Chapter 3**). A lack of DMS calcium response in SO-trained rats suggests a disengagement of the DMS when cocaine seeking becomes habitual. The mechanism by which the DMS becomes disengaged during habitual cocaine self-administration has yet to be determined, but past literature investigating DMS disengagement during habit formation provides some possible mechanisms.

5.2.1 DMS disengagement during habitual cocaine seeking

Reduced excitability of DMS neurons or reduced synaptic strength of DMS inputs after habitual behavior develops could be mechanisms by which the DMS is disengaged during habitual cocaine seeking. Engagement of the DMS and DLS in early and late rotarod training, respectively, is accompanied by plasticity at glutamatergic synapses in these regions (Yin et al., 2009). Recent evidence also suggests that this plasticity occurs differentially in subpopulations of dorsal striatal medium spiny neurons (MSNs) with unique outputs (Perez et al., 2022). Mice trained on a habit-promoting random-interval schedule of reinforcement in a food self-administration task showed enhanced synaptic plasticity in a subpopulation of MSNs in the DLS, which suggests these findings may be applicable to both motor-skill learning and operant habitual behavior (Shan et al., 2015).

Therefore, it has been demonstrated that habit learning is accompanied by changes in dorsal striatal synaptic plasticity, and our findings agree with these previous studies. We found that SO training resulted in altered expression of plasticity-related glutamate receptor subunits compared to FR training, which indicates altered dorsal striatal synaptic plasticity after habitual cocaine

seeking (**Chapter 2**). SO-trained rats showed increased membrane insertion of the calcium-permeable GluA1 AMPA receptor subunit in the DLS, which compliments previous findings that habit learning induces plasticity in the DLS. Additionally, we showed reduced membrane insertion of the calcium-impermeable GluA2/3 in the DMS of SO-trained rats, which suggests reduced DMS plasticity after habit formation, in accordance with previous studies. Although we were initially surprised by this increased GluA2/3 insertion in the DMS in SO-trained rats, an increased reliance on calcium-impermeable AMPA receptors may partially contribute to the reduced DMS calcium response to cue-reinforced lever presses we showed in SO-trained rats, and the relationship between these findings would be interesting to explore in future experiments. Moreover, one study showed that habitual cocaine self-administration led to an upregulation and downregulation in the DLS and DMS, respectively, of L-type calcium channels (Shen et al., 2018), a category of voltage-gated dendritic calcium channels regulated by dopamine receptors (Surmeier et al., 2007), so it would be interesting to determine if altered expression of these receptors contributes to reduced calcium activity in the DMS of rats habitually self-administering cocaine.

Another potential mechanism for DMS disengagement is through reduced excitatory and/or increased inhibitory input from regions that project to the DMS. PL projections to the DMS are only engaged during early rotarod training and become disengaged after extensive training (Kupferschmidt et al., 2017). Direct OFC-DMS projections are important for goal-directed operant responding, and inhibition of these projections inhibits goal-directed memory learning and retrieval (Gremel et al., 2016; Li et al., 2022). The DMS also receives sparse excitatory input from the BLA, but it is unclear if the BLA-DMS connectivity important for goal-directed behavior is direct or indirect, potentially via the OFC or PL (Corbit et al., 2013; Fisher et al., 2020; Zimmermann et al., 2017). Finally, projections from the parafascicular nucleus of the thalamus to

the DMS are also important for goal-directed behavior (Brown et al., 2010; Stayte et al., 2021). Therefore, reduced excitatory input from the PL, OFC, BLA, or thalamus could contribute to reduced DMS engagement in rats habitually seeking cocaine. Although the IL also sends direct projections to the DMS, the IL actually becomes more engaged during T-maze habit learning (Smith and Graybiel, 2013). Moreover, optogenetic inhibition of the IL inhibits habitual responding in a T-maze task, and IL lesions or inactivation prevent habitual learning in an operant task (Coutureau and Killcross, 2003; Killcross and Coutureau, 2003; Smith et al., 2012). Therefore, because activity of excitatory IL-DMS projections that promote habitual behavior would presumably increase DMS calcium activity, it seems unlikely that these projections would be directly involved in reduced DMS calcium activity after SO training, though it is possible the IL is involved through indirect projections, such as those to the nucleus accumbens or amygdala (Barker et al., 2013). Increased inhibitory input to the striatum, potentially from the cortex, could contribute to DMS disengagement (Melzer et al., 2017; Rock et al., 2016). Given that the CeA is important for the expression of habitual cocaine seeking, CeA projections could also directly or indirectly influence DMS activity (Murray et al., 2015; Smith and Laiks, 2017). Examining the activity of these many projections throughout training would be an interesting route for future investigation.

5.2.2 Early engagement of the DLS during goal-directed cocaine seeking

Another interesting finding from our studies was early DLS engagement when behavior was still goal-directed. Cue-reinforced lever presses increased DLS calcium activity after just 7 days of FR training, and could have occurred in the first week of training before we began photometry recordings (**Chapter 3**). Although behaviors are initially goal-directed and promoted

by response-outcome associations, it is still somewhat unclear when stimulus-response associations are learned (Smith and Laiks, 2017). DMS lesions before operant training do not prevent learning but do result in behavior insensitive to contingency degradation, which suggests stimulus-response learning occurs early and can guide behavior in the absence of goal-directed learning (Yin et al., 2005b). During an operant food-reward task that facilitates habitual behavior, DLS activity occurs during early training, and this activity is reduced but sustained through extended training (Vandaele et al., 2019). Similarly, during motor skill learning, primary motor cortex (M1)-DLS projections are engaged during early learning and this engagement decreases, but is sustained, after extensive training (Kupferschmidt et al., 2017). However, for an operant task, DMS activity was not disengaged after extended training, in contrast to our findings (Vandaele et al., 2019). Therefore, it remains to be determined if DMS disengagement is required for the transition to habitual cocaine self-administration, or if it is a byproduct of certain training parameters.

The presence of a calcium response to cue-reinforced lever presses in the DLS early in training, before behavior is habitual, also raises questions about what causes the transition to DLS control. Given that DLS dopamine is required for the transition toward habitual control to occur (Faure et al., 2005), it is possible that the DLS calcium signal represents initial stimulus-response learning, but enhanced DLS dopamine release that occurs under certain conditions (such as SO training, as shown in **Chapter 3**) facilitates strengthening of this learning, through the induction of synaptic plasticity, by signaling motivation for or salience of the stimulus-response associations. DLS synaptic plasticity, which would presumably be caused by temporally linked dopaminergic and glutamatergic input to these neurons, potentially along with DMS suppression through other mechanisms, could be responsible for the transition to habitual behavior, but this remains to be

thoroughly examined. Optogenetic suppression of DLS dopamine terminals during the one second after cue-reinforced lever presses, the timepoint during which DLS dopamine release occurs under SO-trained cocaine self-administration, would be one way to test this hypothesis.

5.2.3 Impact of extinction of cocaine-cue associations

In addition to examining differences between goal-directed and habitual cocaine seeking, we were also interested in the effects of Pavlovian cue extinction on these different behaviors. Our results provide novel evidence that habitual cocaine seeking is unaffected by cue extinction, whereas cue extinction prevents an increase in cue-induced cocaine seeking that occurs in goal-directed control rats (**Chapter 2**). A lack of effect of cue extinction on habitual cocaine seeking does not appear to be due to impaired Pavlovian extinction learning, because restoration of goal-directed control reveals an effect of cue extinction, and cue extinction also extinguishes the conditioned-reinforcing effects of the audiovisual cue in a new, presumably goal-directed task (**Chapter 2**). Furthermore, IntA facilitates both DLS dopamine-dependent behavior and punishment-resistant cocaine self-administration in male rats, which also appear to be resistant to cue extinction (**Chapter 4**). Finally, we show that cue extinction impacts both dopamine and calcium activity in the DMS during subsequent drug seeking, but has no effect on the DLS (**Chapter 3**).

The mechanisms by which cue extinction reduces the DMS calcium response to cue-reinforced lever presses in rats exhibiting goal-directed behavior could be similar to the mechanisms by which the DMS is suppressed or disengaged after SO-schedule training, discussed above. However, the reduced DMS calcium response after cue extinction in FR-trained rats is subtler than the outright disengagement that occurs after SO training. Given that cue extinction's

reduction of drug seeking relies on depotentiation of thalamic projections to the BLA, it is likely that the BLA influences DMS calcium activity through either direct BLA-DMS projections or indirect projections via other regions such as the PL or OFC (Alloway et al., 2017; Corbit et al., 2013; Fisher et al., 2020; Kita and Kitai, 1990; Rich et al., 2019; Zimmermann et al., 2017). The depotentiation of thalamo-lateral amygdala synapses could also influence DMS dopamine release through the BLA's projections to the NAc that have been shown to influence dorsal striatal dopamine release (Murray et al., 2015). Both of these hypotheses remain to be tested, and a direct connection could be drawn by examining the effects of optogenetic depotentiation of thalamo-lateral amygdala synapses, which mimics the behavioral and lateral amygdala synaptic effects of cue extinction (Rich et al., 2019), on DMS dopamine and calcium.

5.2.4 Extinction of stimulus-response associations

One remaining question is why repeated noncontingent cue presentations, in the absence of both the behavioral response and outcome of cocaine, appear to extinguish cocaine-cue, but not stimulus-response associations. A potential explanation for this phenomenon is that the light and tone cue that accompanies cocaine delivery is only a small component of the set of stimuli that become associated with cocaine-seeking behavior. Indeed, we showed that removal of the audiovisual cue was not sufficient to reduce cocaine self-administration after SO training (**Chapter 2**), which suggests that the well-established cocaine-seeking habit may not rely on the audiovisual cue alone, but it is also possible that cocaine seeking became goal-directed in the absence of the audiovisual cue. Of course, it is also true that the audiovisual cue is not the only stimulus that forms Pavlovian associations with cocaine. Pavlovian associations also form between cocaine and the context, or the operant chamber in this case (Bouton, 2002). Indeed, when

Pavlovian cue extinction occurs outside of the context of self-administration, drug-seeking in the original context is not reduced (Bechard and Knackstedt, 2019; Torregrossa et al., 2010). However, administration of D-cycloserine (DCS) in the NAc immediately after cue extinction in a new context or direct optogenetic depotentiation of thalamo-amygdala synapses outside of the self-administration context both resulted in reduced cue-induced drug-seeking behavior in the original context (Rich et al., 2019; Torregrossa et al., 2010). It is somewhat unclear if the context dependency of cue extinction occurs because returning to the context of self-administration renews the cocaine-cue memory, if the extinction learning is specific to the context it occurs in, or if the cocaine-cue association fails to be extinguished in a different context without additional intervention.

Therefore, it is possible that stimulus-response extinction learning would require the presentation of additional stimuli not presented during our cue extinction protocol. Importantly, our cue extinction procedure occurred with levers retracted, but levers were extended during self-administration. Because lever insertion signaled the end of the timeout-period and availability to perform the drug-seeking behavior, it would be interesting to determine if adding lever insertion to cue extinction would result in reduced habitual drug seeking. Indeed, lever insertion may be a particularly relevant stimulus for rats that undergo IntA, where levers are retracted during periods of cocaine unavailability (**Chapter 4**). Interestingly, SO-trained rats show reduced DLS dopamine responses to cue-reinforced lever presses that do not result in lever retraction (**Appendix**), which further supports the theory that lever retraction/insertion may be a component of the stimulus-response association. Unfortunately, testing this hypothesis would be technically challenging, as the rats would need to be physically separated from the levers during cue extinction to prevent additional instrumental extinction from complicating interpretation of results.

Overall, our results suggest that although Pavlovian cue extinction, in its current design, affects the DMS and goal-directed cue-induced drug seeking, it does not affect the DLS or habitual cue-induced drug seeking. Therefore, it appears that the circuit mechanisms by which stimuli promote habitual, stimulus-response behavior are unique from the circuit mechanisms by which drug-associated stimuli promote goal-directed drug seeking. Future experiments could examine what inputs to the DLS provide sensory information about stimuli that become associated with drug-seeking behavior, and evidence suggests these inputs could arise from the thalamus, primary and secondary somatosensory cortices, or anterior insula (Alloway et al., 2017; Haggerty et al., 2022; Smith and Laiks, 2017).

5.3 Habitual behavior, DLS dependence, and punishment resistance

In addition to evaluating the effects of cue extinction on goal-directed and habitual behavior, we were interested in its effects in rats expressing punishment-resistant cocaine self-administration, an important component of compulsive behavior, because the role of Pavlovian drug-cue associations in punishment-resistant drug seeking is unclear. We used IntA, an access model of cocaine self-administration that facilitates binge-like patterns of self-administration, because it has been shown to enhance motivation for cocaine and punishment-resistant cocaine self-administration (James et al., 2019; Kawa et al., 2016). We showed that IntA enhanced compulsive cocaine self-administration exclusively in males, and, although the study was underpowered to detect a 3-way interaction between access model, sex, and cue extinction, IntA males also appeared to be resistant to cue extinction, which suggests a reduced role of drug-cue associations in behavior that is resistant to punishment (**Chapter 4**).

Evaluating how Pavlovian drug-cue associations are involved in punished behavior is complicated by the experimental design of many preclinical studies. Often, the reward-seeking action, or lever press, that results in cue and reward delivery, is punished via delivery of a footshock, such as in our experimental design in **Chapter 4**. In this case, the punishment likely becomes associated with the behavior, cue, and outcome. One way to isolate punishment of drug-seeking behavior is to use a seeking-taking chain, where rats learn to press a seeking lever to gain access to a separate taking lever, and to press the taking lever for cue and reward delivery. Punishment of the seeking, but not taking, lever ensures that punishment does not become directly associated with the cue or reinforcer. Interestingly, BLA lesions (but not lesions of other regions including the anterior cingulate cortex, anterior insular cortex, OFC, IL, or PL) enhance persistence of footshock-punished cocaine seeking in a seeking-taking chain, which suggests that the BLA may suppress punished cocaine seeking, even when punishment is not directly associated with the drug-associated cue (Pelloux et al., 2013). However, BLA lesions generally inhibit associative learning, which complicates the interpretation of these results (Pelloux et al., 2013). Future experiments could examine how BLA inhibition, specifically during punished drug seeking, impacts drug-seeking in the presence or absence of drug-associated cues, which would determine if behavior guided by drug-associated cues is more sensitive to punishment.

Additionally, because there is disagreement in the field about the relationship between habitual behavior, punishment resistance, and motivation, we also determined if IntA promoted DLS dopamine-dependent behavior or affected motivation for cocaine. Somewhat conflicting theories propose that compulsive drug-seeking arises from increased reliance on habitual behaviors that are insensitive to changes in outcome value, impaired cognitive control of decision-making, or enhanced motivation that drives goal-directed drug choice over other, competing options

(Everitt, 2014; Hogarth, 2020; Verdejo-García et al., 2020; Wood and Neal, 2007). The latter theory, proposing enhanced goal-directed control or motivation in compulsive drug seeking, seems at odds with the idea that an over-reliance on habits is involved. Below we discuss some evidence for each of these theories and examine how our findings provide some insights and raise additional questions.

A recent study examining the role of dopaminergic projections to the DMS and DLS in punishment-resistant sucrose self-administration, using a random interval schedule that typically promotes habitual behavior, provides some evidence for the latter theory that compulsive behavior arises from enhanced goal-directed motivation (Seiler et al., 2022). Enhanced punishment resistance was correlated with greater activity at dopamine terminals in the DMS, but not the DLS, in response to rewarded nosepokes (Seiler et al., 2022). Additionally, optogenetic excitation or inhibition of DMS dopamine terminals augmented or inhibited punishment resistance, respectively, while optogenetic excitation of DLS dopamine terminals had no effect (Seiler et al., 2022). Overall, this study provides evidence that DMS dopamine is involved in punishment resistance, at least for this sucrose self-administration task, and suggests that punishment resistance involves goal-directed circuitry. However, their experimental design did not include a discrete Pavlovian reward-paired audiovisual cue, so it would be interesting to determine if different results would be obtained in a task involving a salient reward-paired stimulus.

In contrast, there is converging evidence that impaired cognitive control of action selection, or a reliance on habits and an inability to use updated outcome values to change behavior, is a major component of compulsive reward seeking (Smith and Laiks, 2017). In mice trained to habitually self-administer food, IL infusion of a dopamine D1 receptor antagonist or D2 receptor agonist restored goal-directed control, measured by sensitivity to contingency degradation, and

also reduced compulsive reward seeking by increasing the time it took for mice to enter the reward-paired chamber where they had recently received footshocks (Barker et al., 2013). Therefore, this study suggests overlapping roles of the IL in both habitual and punishment-resistant reward seeking and supports the theory that impaired cognitive control may be involved.

Furthermore, studies implicating the DLS in punishment-resistant reward seeking also support the theory that compulsive behavior arises from reliance on habitual behavior. In alcohol-preferring rats (P rats), DLS dopamine antagonism reduced drug seeking in all rats trained on a seeking-taking chain to self-administer alcohol, but only significantly reduced alcohol seeking that was unpredictably punished with a footshock exclusively in rats with increased resistance to punishment (Giuliano et al., 2019). Additionally, P rats with increased punishment resistance were less sensitive to outcome devaluation (Giuliano et al., 2021). Together, these results suggest both engagement of the DLS and habitual behavior, along with a failure to re-engage the DMS, play a role in compulsive alcohol seeking. In a similar paradigm, punishment-resistant rats also had increased motivation for alcohol on a PR schedule, but it is unclear if this increased motivation contributed to punishment resistance (Giuliano et al., 2018).

Interestingly, in all three of these experiments, rats underwent an intermittent 2-bottle choice procedure between alcohol and sucrose solutions, so it would be interesting to determine how this intermittent alcohol access may contribute to increased motivation or punishment resistance. Binge-like alcohol drinking also induced plasticity at anterior insula-DLS synapses exclusively in male mice (Haggerty et al., 2022). Even though alcohol and cocaine have very different neurobiological effects, we also showed that IntA enhanced punishment-resistant cocaine seeking exclusively in males (**Chapter 4**), so it would be interesting to examine the impact of IntA cocaine self-administration on the plasticity of DLS projections from the anterior insula.

Punishment-resistant cocaine self-administration has also been examined. In rats trained to self-administer cocaine on a seeking-taking chain, inactivation of the DLS, but not a more ventral region (termed the midlateral striatum), with a combination of baclofen and muscimol reduced unpredictably punished cocaine seeking, which further implicates the DLS in punishment-resistant cocaine self-administration (Jonkman et al., 2012). However, male rats given IntA to cocaine via a puzzle-solving procedure that promotes sustained reliance on goal-directed behavior and a lack of reliance on DLS dopamine also showed enhanced resistance to punishment, which suggests that habitual behavior and reliance on DLS dopamine is not required for enhanced punishment resistance (Singer et al., 2018). In other studies using only male rats, IntA to cocaine both enhanced punishment-resistant cocaine self-administration and motivation for cocaine, but it is possible that these effects occurred independently of one another (James et al., 2019; Kawa et al., 2016).

Using a shorter IntA protocol, we observed sex differences in the effects of IntA that coincidentally have implications on these conflicting theories of compulsive cocaine-seeking behavior. First, we determined that IntA enhanced punishment-resistant cocaine self-administration exclusively in males, whereas IntA enhanced motivation for cocaine exclusively in females (**Chapter 4**). The dissociation between punishment resistance and increased motivation for cocaine between sexes does not support theories that compulsive drug-seeking behavior arises from enhanced motivation for cocaine. Interestingly, after 10-11 days of IntA, DLS dopamine antagonism reduced drug seeking in both males and females, which suggests reliance on habitual behavior in both sexes. Therefore, although habitual behavior coincided with punishment-resistant behavior in males, females also showed reliance on DLS dopamine in the absence of enhanced punishment resistance or resistance to cue extinction, which complicates interpretations of how these systems interact. Given that dependence on DLS dopamine appears to arise early in habit

learning (**Chapter 2**), it is possible that DLS dopamine impacts behavior in females even though habitual and compulsive behavior is not yet well-established, but more analysis is required to clarify these complex findings.

5.4 Considerations and limitations

One major consideration for the interpretation of these results is that we chose to identify habitual cocaine seeking through its sensitivity to DLS dopamine antagonism (**Chapters 2 & 4**), which has been previously established and relies on the assumption that a behavior inhibited by DLS dopamine antagonism is under habitual control (Murray et al., 2015, 2012). However, habits are typically defined behaviorally through their insensitivity to changes in outcome value or contingency (Ostlund and Balleine, 2008; Smith and Laiks, 2017; Vandaele and Ahmed, 2020). Habitual responding is unchanged when the reinforcer is devalued through specific satiety or pairing with an aversive outcome or when the subject learns that reward is delivered regardless of behavior (Ostlund and Balleine, 2008; Smith and Laiks, 2017; Vandaele and Ahmed, 2020). Still, it has been widely demonstrated that lesions of the DLS or dopaminergic projections to the DLS, as well as DLS infusion of the inhibitory baclofen/muscimol cocktail, dopamine receptor antagonists, or the AMPA inhibitor NBQX, inhibit behaviorally defined habitual responding for rewards that are delivered orally, such as food pellets, sucrose, and alcohol (Corbit et al., 2014b, 2012; Faure et al., 2005; Yin et al., 2006, 2004). Therefore, it is clear that the DLS plays an essential role in behaviorally defined habits, but our findings do not necessarily identify habitual cocaine seeking in its traditional behavioral definition.

However, these behavioral protocols also have limitations, in that their lack of effect could indicate impaired goal-directed learning and their application to cocaine is complicated by the intravenous route of self-administration (Everitt and Robbins, 2013; Houwer, 2019; Ostlund and Balleine, 2008; Smith and Laiks, 2017). For example, behavioral insensitivity to response-outcome contingency degradation could reflect either reliance on habitual behavior or an inability to learn the new lack of contingency (Watson and de Wit, 2018). Additionally, because cocaine is delivered intravenously and its effects are likely perceived gradually and sustained, it may be difficult for rats to learn that the response-outcome contingency is degraded (Everitt and Robbins, 2013; Ostlund and Balleine, 2008; Smith and Laiks, 2017). Still, one study did attempt to apply contingency degradation to intravenously self-administered cocaine and found that while rats did reduce cocaine-seeking after contingency degradation after 2 days of self-administration on an FR schedule, some rats no longer changed their behavior after contingency degradation after extended self-administration (7+ days) on a habit-promoting fixed-interval schedule (Shen et al., 2018). However, it is possible that training on a fixed-interval schedule, which results in a much higher rate of responding, could make learning the degradation of the response-outcome contingency more difficult, so it is unclear if reduced sensitivity was due to reliance on habitual behavior or impaired learning. Using rats trained to self-administer cocaine on a seeking-taking chain, some studies have devalued the taking lever through instrumental extinction, which also relies on the assumption that habitual behavior is insensitive to changes in the response-outcome contingency (Olmstead et al., 2001; Zapata et al., 2010). Only after extended training, devaluation of the taking link of the seeking-taking chain did not reduce cocaine seeking, unless the DLS was inhibited with lidocaine, which provides further evidence that DLS antagonism inhibits habitual cocaine seeking (Zapata et al., 2010).

Another method of devaluation involves pairing the reward with a negative outcome, such as gastric malaise induced by LiCl injection. A few studies have applied this method to intravenous cocaine self-administration by using LiCl to induce gastric malaise after noncontingent cocaine infusions (Leong et al., 2016; Root et al., 2009). In one such study, cocaine seeking in the self-administration context was insensitive to devaluation, unless a discriminative stimulus that signaled cocaine availability was present, which suggests that responding was habitual until the discriminative stimulus indicated cocaine availability, at which point goal-directed control was restored (Root et al., 2009). In a similar study, rats were given intraperitoneal injections of LiCl after noncontingent cocaine infusions, and results indicated that LgA, but not ShA, produced cocaine self-administration that was insensitive to LiCl devaluation (Leong et al., 2016). Importantly, both of these studies devalued cocaine out of the self-administration context, which is important for preventing associations between gastric malaise and the cues and contexts from being learned (Leong et al., 2016; Root et al., 2009). However, it is unclear if changes in motivation for cocaine, which are induced by long access, can also reduce the extent to which cocaine can be devalued by gastric malaise. Together, these studies suggest that LiCl devaluation may be an inconsistent method of devaluation, especially if there could be changes in motivation for cocaine (such as those induced by IntA) or differences in the presence of cocaine-paired cues (such as during SO training).

Experimental designs utilizing specific satiety, or the pre-consumption of the reward prior to testing, are complicated by cocaine's stimulant effects that promote responding (Everitt, 2014; Smith and Laiks, 2017). However, rats typically maintain a specific level of brain-cocaine concentration throughout self-administration sessions, and experimenter administration above this concentration prevents cocaine self-administration (Tsibulsky and Norman, 1999). A recent study

employed these properties and showed that training on habit-promoting interval schedules, but not ratio schedules, of reinforcement rendered cocaine-seeking insensitive to outcome devaluation via experimenter administration of a presumably satiety-inducing dose of cocaine prior to testing (Jones et al., 2022). Moreover, DLS lesions prevented insensitivity, and DMS lesions induced insensitivity to specific satiety (Jones et al., 2022). Therefore, the application of this specific satiety protocol to SO-trained rats or rats that undergo IntA could improve the behavioral validity of the experiments outlined in this dissertation.

Another limitation of these findings is that we did not use any cell-type-specific manipulations. In our fiber photometry experiment, we expressed the calcium indicator RCaMP under the synapsin promotor, which targets neurons, to gain an understanding of general dorsal striatal activity. However, there are various types of neurons throughout the dorsal striatum that serve different functions. In rodents, about 95% of neurons are inhibitory projection neurons called medium spiny neurons (MSNs), named for their extensive spiny dendrites (Kreitzer, 2009). Given their extensive dendritic arbors and the high proportion of MSNs in the rodent dorsal striatum, our photometry recordings presumably reflect mostly MSN calcium activity (**Chapter 3**). Even so, there are two major categories of MSNs: striatonigral or direct-pathway MSNs (dMSNs) that project to the substantia nigra *pars reticulata* (SNr) and striatopallidal indirect-pathway MSNs (iMSNs) that project to the globus pallidus (Kreitzer, 2009). Working together as the primary output of the dorsal striatum, MSNs are important for the execution of coordinated movement and behavior (Cui et al., 2013). In addition to projecting to different targets, direct- and indirect-pathway MSNs have differential expression of dopamine receptor types, play different roles in synaptic plasticity, and have different functions in the regulation of goal-directed and habitual behavior (Kreitzer, 2009; O'Hare et al., 2016; Surmeier et al., 2007). The dorsal striatum also

contains various types of interneurons, including fast-spiking interneurons, which are important for habitual behavior, as well as cholinergic interneurons, which regulate dopamine release and synaptic plasticity (Cover et al., 2019; Muñoz-Manchado et al., 2018; O'Hare et al., 2017). In non-human primates, additional sub-types of MSNs have been identified, and non-human primates also have a greater proportion of interneurons compared to rodents (Graveland and Difiglia, 1985). Therefore, these MSN subtypes and local inhibitory networks may be more important for action selection in humans, and how they regulate habit learning and expression remains to be fully characterized.

Additionally, because we examined drug seeking one day after cue extinction, our results do not necessarily represent relapse-like behavior after extended abstinence. One study showed that abstinence after the establishment of habitual cocaine self-administration paradoxically promotes subsequent relapse-like behavior mediated by enhanced DMS activity (Fouyssac et al., 2022). Experimenters concluded that abstinence enhanced "negative urgency" to perform the drug-seeking behavior in a goal-directed manner (Fouyssac et al., 2022), which provides some evidence that additional days of abstinence may alter the role of the dorsal striatum in drug-seeking behavior. It is also important to consider that in our cocaine self-administration model, rodents only have access to cocaine, and no other reinforcers are present in the operant chamber. Depending on the response requirements, rats often choose other reinforcers, such as social interaction or sucrose, over cocaine, and alternative choices can be under habitual control (Vandaele et al., 2022; Venniro et al., 2021). Therefore, in some cases, habit formation could be protective against the development of SUDs. The availability of an alternative choice, such as sucrose, also reduces punishment-resistant cocaine self-administration (Pelloux et al., 2015). Therefore, it would be interesting to examine how abstinence, either forced or by voluntary methods such as alternative choice or

avoidance of an electric barrier, would impact the balance between goal-directed and habitual behavior (Fredriksson et al., 2021).

Finally, the translational validity of IntA as a model has been questioned because IntA promotes sensitization to cocaine, whereas individuals with SUDs or extensive histories of drug use usually show marked tolerance to cocaine (Calipari et al., 2013; Carr et al., 2020; Mendelson et al., 1998; Wang et al., 1999). Although this is an important consideration, rodent self-administration studies are not necessarily equipped to examine the long-term effects of SUDs and repeated drug use. Our results suggest that different patterns of cocaine exposure during early drug use, presumably before the transition to SUD, may affect learning and addiction-like behaviors that increase the risk of the eventual development of SUD (**Chapter 4**). Moreover, we also identified sex differences in the effects of IntA (**Chapter 4**). Therefore, IntA may be uniquely useful in examining how patterns of drug exposure can influence learning during early drug use and facilitate the progression to uncontrolled and compulsive drug use.

5.5 Implications for SUD treatment

The results of the experiments outlined in this dissertation have several implications for future studies evaluating the treatment of SUDs. Somewhat recently, researchers have begun to characterize the many sex differences in factors that contribute to SUDs in humans, including differences in drug metabolism, effects of circulating hormones, and neurobiological structure and function (McHugh et al., 2018). Females and males exhibit differences in peak plasma levels of cocaine and brain regions activated by cocaine-associated cues (McHugh et al., 2018). Illicit drug use and SUD diagnoses are more prominent among men, but these gaps have been closing in recent

years (McHugh et al., 2018). Interestingly, although we did not necessarily uncover any sex differences in habit formation, we showed that IntA has sex-specific effects on addiction-like behaviors, especially punishment resistance and motivation for cocaine (**Chapter 4**). Whereas both SO training and IntA promoted habitual DLS dopamine-dependent behavior in both sexes, IntA led to increased punishment-resistant cocaine self-administration in males, but increased motivation for cocaine in females, in agreement with previous preclinical studies indicating that increased motivation for cocaine induced by IntA is enhanced in females (Algallal et al., 2020; Kawa and Robinson, 2019). These results suggest that there are sex differences in how early drug use affects learning and motivation, which could have large-scale implications for how genetic, physical, and environmental risk factors for the transition from casual drug use to SUDs could affect males and females differently.

We also show that cue extinction may not affect habitual cocaine seeking, which has major implications for the application of cue exposure therapy to the treatment of SUDs. Although CET has been shown to reduce subjective reports of drug craving, it has generally been unsuccessful in improving treatment outcomes (Carter and Tiffany, 1999; Conklin and Tiffany, 2002; Mellentin et al., 2017; O'Brien et al., 1990). It is generally assumed that cues promote relapse through their association with the drug, which increased drug craving. However, cue exposure also activates the putamen, the human brain structure that most closely relates to the rodent DLS, and this activation is correlated with subjective reports of cocaine craving (Prisciandaro et al., 2013a; Wong et al., 2006). Therefore, it is possible that stimuli exposure also promotes drug craving or the urge to perform drug-seeking behavior through habitual stimulus-response associations, and targeting stimulus-response associations alongside cocaine-cue associations may lead to better treatment outcomes. Our results suggest that information about stimuli associated with drug-seeking

behavior impacts DLS activity early in learning, and conditions that facilitate a DLS dopamine response to cues, such as SO training, lead to a rapid transition to habitual behavior (**Chapters 2 & 3**). Although inhibition of DLS dopamine may prevent habit formation, manipulation of dopamine during early drug use in humans to prevent the formation of drug-seeking habits is unrealistic for several reasons. Depletion of dorsal striatal dopamine would not only impair movement but would also generally inhibit the formation of adaptive habits (Faure et al., 2005; Palmiter, 2008; Yu et al., 2022). Additionally, once individuals are diagnosed with a SUD, they have presumably already undergone extensive learning, so treatment would need to involve the manipulation of existing memories. Therefore, manipulation of the projections carrying cue information to the dorsal striatum may be a better target.

Strategies that have been proposed to weaken Pavlovian drug-cue associative memories could potentially be applied to stimulus-response memories. When memories are learned, they are stabilized through the process of consolidation (Milton, 2013; Milton and Everitt, 2012; Rich and Torregrossa, 2018; Torregrossa and Taylor, 2013). Exposure to a conditioned stimulus (CS) associated with cocaine can reactivate the cocaine-cue memory, and it enters a labile phase before undergoing reconsolidation (Torregrossa and Taylor, 2013). Reconsolidation requires several specific cellular functions and proteins, including protein synthesis, protein kinases, transcription factors, and neurotransmitter receptors, and interfering with the function or expression of these proteins can impair reconsolidation and weaken the drug-cue memory (Alberini, 2005; Brown et al., 2007; Dunbar and Taylor, 2016; Lee et al., 2006; Monsey et al., 2017; Rich and Torregrossa, 2018). Reactivation of stimulus-response memories and the subsequent interference with reconsolidation may be an interesting route for future research.

Importantly, we show that restoration of goal-directed control increases drug-seeking in the absence of additional cue extinction (**Chapter 2**), which indicates that weakening or extinguishing drug-seeking habits alone may have negative consequences. Additionally, in rats behaving in a goal-directed manner, cue extinction reduces cue-induced drug seeking as well as DMS dopamine and the DMS calcium response to cues (**Chapters 2 & 3**), and future experiments could examine if this reflects a reduced goal-directed drive to seek cocaine because the cue no longer signals cocaine availability. Therefore, combined restoration of goal-directed control and a reduction in cue-induced behavior may be the most successful strategy for reducing drug-seeking behavior.

The simultaneous enhancement of extinction and interference with reactivation of cocaine-cue associations has been proposed to further augment the effects of memory manipulation on behavior, and one recent study identified a single molecular target for strengthening both of these processes (Merlo et al., 2014; Rich et al., 2020; Rich and Torregrossa, 2018). Additionally, because drug-cue and stimulus-response associations may consist of similar stimuli, it is possible that reactivation and extinction of both of these types of memories could occur by presenting stimuli in the absence of both the drug and the behavior. However, the reactivation of stimulus-response memories may be technically challenging; our data suggest that in rodents, the stimulus that promotes drug-seeking may be a set of contextual and discrete stimuli and not just the audiovisual cue, so these stimuli may need to be presented together. Interestingly, the presentation of multiple cocaine-associated stimuli may enhance the extinction of cocaine-cue associations as well (Janak et al., 2012), which suggests that the presentation of multiple contextual and discrete stimuli together could lead to extinction of stimulus-response associations and enhance extinction of drug-cue associations. However, the presentation of complex environmental and discrete stimuli, which

can include objects, locations, and even people, presents a major challenge because treatment for SUDs often occurs in clinical settings. Recent advances in virtual reality technology could overcome these challenges. Indeed, the use of virtual reality has been shown to enhance cue exposure therapy for the treatment of anxiety-related disorders, and the application of these methods to SUD treatment has yet to be fully evaluated (Powers and Emmelkamp, 2008; Tsamitros et al., 2021).

Overall, our findings suggest that cue extinction involving individual cocaine-paired cues may reduce goal-directed behavior, but have no impact on habitual behavior due to differences in how cocaine-cue associations impact the circuits encoding the associative memories that guide goal-directed and habitual behavior. Results of studies examining various ways to enhance cue exposure therapy should therefore be interpreted in the light of these findings. Additionally, future studies may examine therapeutic methods that target stimulus-response and CS-cocaine associations to affect the ability of cues to promote relapse.

5.6 Summary and conclusions

In summary, this dissertation provides new insights into the divergent circuits underlying cue-induced drug seeking that is goal-directed compared to when it is habitual and resistant to punishment. We expand upon previous literature that has identified a major role of the DMS in goal-directed behavior and of the DLS in habitual behavior. When behavior is goal-directed, we show that cue-reinforced cocaine seeking results in increased calcium activity in the DMS and DLS, presumably due to changes in synaptic plasticity and because of an increase in the ratio of excitatory to inhibitory input to both of these regions (**Figure 19A**). When behavior becomes

habitual, this increased calcium activity only occurs in the DLS and is accompanied by increased dopamine release in the DLS, and the DMS calcium response is no longer present, which suggests either reduced excitatory input, increased inhibitory input, or a combination of these mechanisms (**Figure 19B**). Extinction of the Pavlovian drug-cue association in rats behaving in a goal-directed manner reduces the DMS calcium response to cue-reinforced behavior, reduces DMS dopamine, and results in reduced responding compared to controls (**Figure 19C**). Cue extinction in rats behaving habitually has no effect on the DLS calcium or dopamine response to cue reinforced lever presses, and although it does reduce DMS dopamine, it has no effect on subsequent behavior (**Figure 19D**). We also provide evidence for a role of habitual behavior in persistence of drug self-administration in the face of punishment. Overall, these findings suggest that although extinction of Pavlovian drug-cue associations may reduce goal-directed drug seeking induced by cues, cues and other related stimuli can promote habitual and compulsive, punishment-resistant behavior via distinct neural circuitry independent of the association between these stimuli and cocaine. Therefore, future therapeutics that simultaneously target these circuits will likely be the most successful in preventing cue-induced relapse in individuals with SUDs.

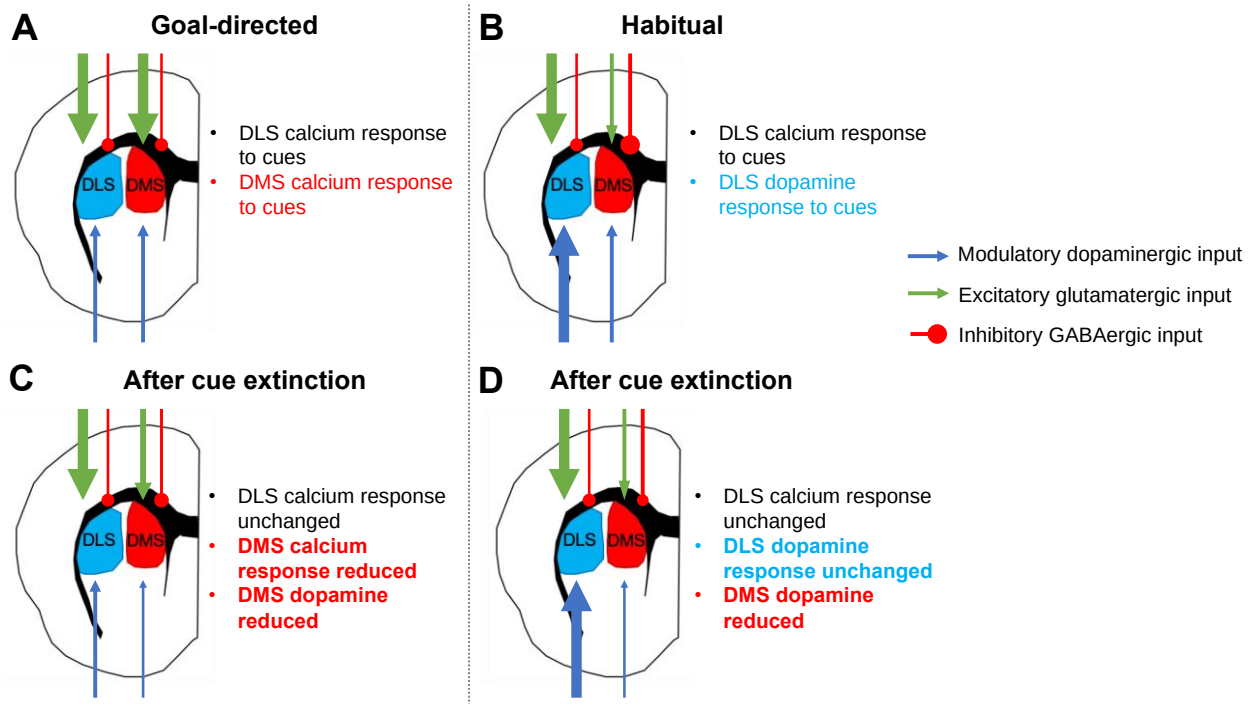

Figure 19: Summary of major conclusions

Whereas the DMS (red) is important for the initiation of goal-directed behavior, the DLS (blue) is important for habitual behavior. These regions receive dopaminergic input from the midbrain (blue arrows) as well as excitatory (green arrows) and inhibitory (red arrows) input from several regions, including the thalamus, amygdala, and cortical regions such as the prefrontal cortex, orbitofrontal cortex, insula, somatosensory cortex, premotor cortex, and other regions. In rats trained to self-administer cocaine in a goal-directed manner, cue-reinforced cocaine seeking results in increased calcium activity in the DMS and DLS (**A**). In rats trained to self-administer cocaine habitually, this increased calcium activity only occurs in the DLS and is accompanied by increased dopamine release in the DLS, and the DMS calcium response is no longer present, which suggests either reduced excitatory input, increased inhibitory input, or a combination of these mechanisms (**B**). After cue extinction, when Pavlovian drug-cue associations are extinguished in rats behaving in a goal-directed manner, the DMS calcium response to cue-reinforced behavior and DMS dopamine are reduced, which results in reduced drug-seeking behavior compared to controls (**C**). In rats behaving habitually, cue extinction has no effect on the DLS calcium or dopamine response to cue reinforced lever presses, and although DMS dopamine is reduced, there is no effect on subsequent behavior (**D**).

6.0 Appendix: Supplementary results and figures

6.1 No effect of sex or estrous phase on dorsal striatal calcium or dopamine responses to lever presses

Because we used both male and female rats in these experiments, we wanted to determine if there were any effects of sex or estrous phase on dorsal striatal activity during drug seeking. During the early phase of training, before rats were split into FR- and SO-trained groups, we compared activity between males and females using 2-way ANOVAs. For DLS calcium peak amplitude, there was a main effect of cue reinforcement ($F_{(1,10)}$=10.96, p=0.0079), but no effect of sex ($F_{(1,10)}$=3.416, p=0.0943) or cue reinforcement × sex interaction ($F_{(1,10)}$=2.403, p=0.1521) (**Figure 20A**). Similarly, for DMS calcium peak amplitude, there was a main effect of cue reinforcement ($F_{(1,11)}$=12.42, p=0.0048), but no effect of sex ($F_{(1,11)}$=3.703, p=0.0806) or cue reinforcement × sex interaction ($F_{(1,11)}$=0.7044, p=0.4192) (**Figure 20B**). There was no main effect of cue reinforcement ($F_{(1,11)}$=0.003257, p=0.9555; $F_{(1,11)}$=0.0007764, p=0.9103) or sex ($F_{(1,11)}$=0.4337, p=0.5237; $F_{(1,11)}$=0.02260, p=0.6880) or cue reinforcement × sex interaction ($F_{(1,11)}$=0.5442, p=0.4761; $F_{(1,11)}$=0.1285, p=0.7268) for DLS (**Figure 20C**) or DMS (**Figure 20D**) dopamine peak amplitude. There was a main effect of cue reinforcement ($F_{(1,10)}$=36.38, p=0.0001; $F_{(1,11)}$=23.37, p=0.0005), but no effect of sex ($F_{(1,10)}$=0.9574, p=0.3509; $F_{(1,11)}$=1.860, p=0.1998) or cue reinforcement × sex interaction ($F_{(1,10)}$=0.3662, p=0.5585; $F_{(1,11)}$=1.865, p=0.1993) for DLS (**Figure 20E**) and DMS (**Figure 20F**) calcium AUC. There was no main effect of cue reinforcement ($F_{(1,11)}$=4.356, p=0.0609; $F_{(1,11)}$=2.843, p=0.1199) or sex ($F_{(1,11)}$=0.3945, p=0.5428;

$F_{(1,11)}$=0.02097, p=0.8875) or cue reinforcement × sex interaction ($F_{(1,11)}$=0.3112, p=0.1277; $F_{(1,11)}$=0.3112, p=0.5881) for DLS (**Figure 20G**) or DMS (**Figure 20H**) dopamine AUC.

Most females (n=6 of 7) were in estrus during one day of the 3 days of the early training phase, so we also examined if calcium or dopamine responses to lever presses differed when females were in estrus or another phase of the estrous cycle (non-estrus). For these analyses, mixed effects analyses were used to accommodate one rat not being in estrus and another not making a cue-reinforced lever press while in estrus. For DLS (**Figure 20I**) and DMS (**Figure 20J**) calcium peak amplitude, there was a main effect of cue reinforcement ($F_{(1,5)}$=9.646, p=0.0267; $F_{(1,6)}$=8.109, p=0.0293), but no effect of estrous phase ($F_{(1,5)}$=0.01962, p=0.8941; $F_{(1,6)}$=0.5886, p=0.4721) or cue reinforcement × estrous phase interaction ($F_{(1,2)}$=2.386, p=0.2624; $F_{(1,3)}$=2.399, p=0.2192). For DLS (**Figure 20K**) and DMS (**Figure 20L**) dopamine peak amplitude, there was no main effect of cue reinforcement ($F_{(1,5)}$=0.5567, p=0.4892; $F_{(1,6)}$=0.0004565, p=0.9836) or estrous phase ($F_{(1,5)}$=0.1196, p=0.7436; $F_{(1,6)}$=2.614, p=0.1570) or cue reinforcement × estrous phase interaction ($F_{(1,2)}$=11.41, p=0.0776; $F_{(1,3)}$=0.3294, p=0.6062). For DLS (**Figure 20M**) and DMS (**Figure 20N**) calcium AUC, there was a main effect of cue reinforcement ($F_{(1,5)}$=26.75, p=0.0035; $F_{(1,6)}$=20.64, p=0.0039), but no effect of estrous phase ($F_{(1,5)}$=0.1281, p=0.7351; $F_{(1,6)}$=0.4512, p=0.5268) or cue reinforcement × estrous phase interaction ($F_{(1,2)}$=1.252, p=0.3795; $F_{(1,3)}$=1.870, p=0.2649). There was no main effect of cue reinforcement ($F_{(1,5)}$=0.3965, p=0.5565; $F_{(1,21)}$=3.932, p=0.0606) or estrous phase ($F_{(1,5)}$=0.3025, p=0.6060; $F_{(1,21)}$=0.05453, p=0.8176) or cue reinforcement × estrous phase interaction ($F_{(1,2)}$=3.826, p=0.1896; $F_{(1,21)}$=0.2593, p=0.6159) for DLS (**Figure 20O**) and DMS (**Figure 20P**) dopamine AUC. Overall, these data indicate that there was no effect of sex or estrous phase on dorsal striatal calcium or dopamine responses to lever presses, and we therefore collapsed results across sex throughout analyses in the main text.

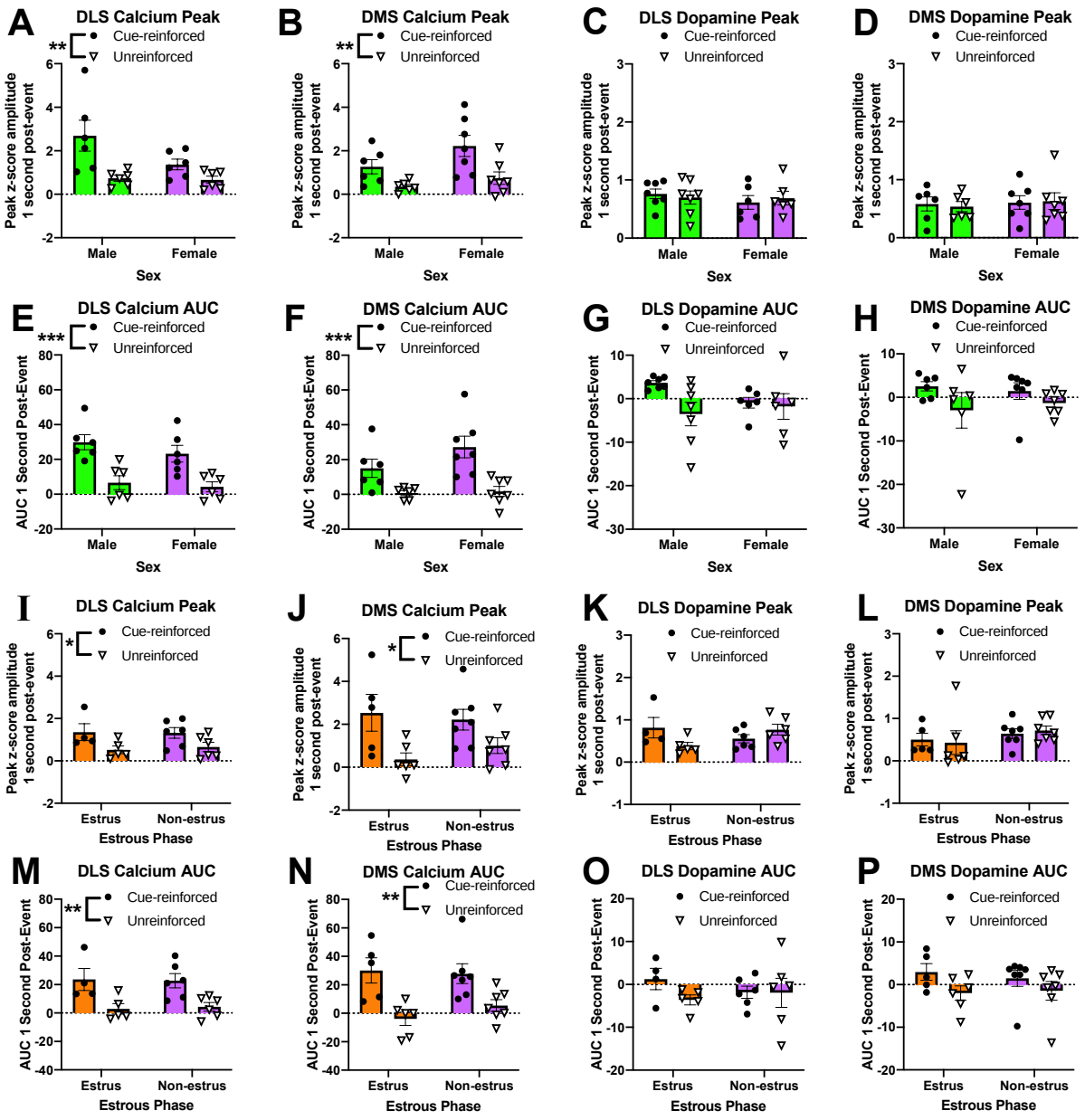

Figure 20: No effect of sex or estrous phase on dorsal striatal calcium or dopamine responses to lever presses

Because male and female rats were used for these experiments, we evaluated if sex (**A-H**) or estrous phase in females (**I-P**) had an effect on dorsal striatal calcium and dopamine responses to lever presses during the early phase of training, because we could collapse across future training schedule. For DLS calcium peak (**A**) and AUC (**E**) and DMS calcium peak (**B**) and AUC (**F**), there was a main effect of cue reinforcement but no effect of sex or cue reinforcement × sex interaction. For DLS dopamine peak (**C**) and AUC (**G**) and DMS dopamine peak (**D**) and AUC (**H**), there was no effect of cue reinforcement, sex, or interaction. For DLS calcium peak (**I**) and AUC (**M**) and DMS calcium peak (**J**)

and AUC (**N**), there was a main effect of cue reinforcement but no effect of estrous phase or cue reinforcement × estrous phase interaction. For DLS dopamine peak (**K**) and AUC (**O**) and DMS dopamine peak (**L**) and AUC (**P**), there was no effect of cue reinforcement, estrous phase, or interaction. Graphs show group means ± SEM and individual data points. *p<0.05; **p<0.01; ***p<0.001.

6.2 During early training, cue-reinforced lever presses result in increased AUC for DLS calcium and dopamine and DMS calcium

During early training, prior to being split into FR- and SO-trained groups, we found that calcium peak amplitude in both the DLS and DMS was greater for cue-reinforced lever presses, but this was not the case for dopamine peak amplitude (**Figure 12**). Additionally, there were no differences in peak amplitude between rats that would later be split into FR- and SO-trained groups (**Figure 12**). In addition to peak amplitude data, we also calculated the AUC in the 1 second after lever press. Results were overall similar, with a few differences. There was a main effect of cue reinforcement ($F_{(1,8)}$=35.23, p=0.0003) as well as future training schedule ($F_{(1,8)}$=7.727, p=0.0239) for DLS calcium AUC, but no interaction ($F_{(1,8)}$=0.3938, p=0.5478) (2-way ANOVA) (**Figure 21A**). These data suggest that rats later placed in the SO-trained group may have by chance had greater calcium responses to lever presses in the DLS during early training (rats were split into groups randomly and photometry analysis occurred at the end of the experiment). Importantly, this difference was not present for peak analysis (**Figure 12A**) and did not persist throughout late training, and this difference does not greatly impact our overall interpretation of results. For DMS calcium AUC, there was a main effect of cue reinforcement ($F_{(1,9)}$=16.81, p=0.0027), but no effect of future training schedule ($F_{(1,9)}$=0.05188, p=0.8249) or interaction ($F_{(1,9)}$=0.2703, p=0.6157) (**Figure 21B**). For DLS dopamine AUC, there was a main effect of cue reinforcement ($F_{(1,9)}$=7.931,

p=0.0202), but no effect of future training schedule ($F_{(1,9)}$=1.017, p=0.3397) or interaction ($F_{(1,9)}$=2.256, p=0.1673) (**Figure 21C**). The effect of cue was not present for DLS dopamine peak amplitude during early training (**Figure 12C**), likely because peak analysis isn't sensitive to the mainly negative AUC in response to unreinforced lever presses shown here. There was no main effect of cue ($F_{(1,9)}$=2.516, p=0.1513) or future training schedule ($F_{(1,9)}$=1.282, p=0.2903) or interaction ($F_{(1,9)}$=4.229, p=0.0738) for DMS dopamine AUC (**Figure 21D**). Though only statistically significant for the DLS, these results suggest that in this early training phase there appears to be a trend toward a reduction in dorsal striatal dopamine in response to unreinforced lever presses. Interestingly, these recordings took place during the first few days of FR3 training, when animals were for the first time making active lever presses that did not result in cue reinforcement, which previously occurred on an FR1 schedule. Therefore, these results may reflect an aspect of reward prediction error, where a lever press that didn't result in the expected reinforcing outcome resulted in a reduction in dorsal striatal dopamine.

6.3 Average daily lever presses during the early phase of training are not correlated with calcium or dopamine responses to cue-reinforced lever presses

Because rats were later trained on schedules of reinforcement that required increasing numbers of lever presses, we wanted to determine if there was a correlation between number of lever presses and dorsal striatal calcium and dopamine activity. We found no correlation between average daily active lever presses during early training and DLS calcium peak amplitude (r=-0.1212, p=0.7213) (**Figure 21E**), DMS calcium peak amplitude (r=0.07310, p=0.8309) (**Figure 21F**), DLS dopamine peak amplitude (r=0.2325, p=0.4914) (**Figure 21G**), DMS dopamine peak

amplitude (r=-0.5745, p=0.0645) (**Figure 21H**), DLS calcium AUC (r=-0.1935, p=0.5686) (**Figure 21I**), DMS calcium AUC (r=0.08215, p=0.8102) (**Figure 21J**), DLS dopamine AUC (r=0.3760, p=0.2544) (**Figure 21K**), or DMS dopamine AUC (r=-0.2970, p=0.3751) (**Figure 21L**) (Pearson's correlation).

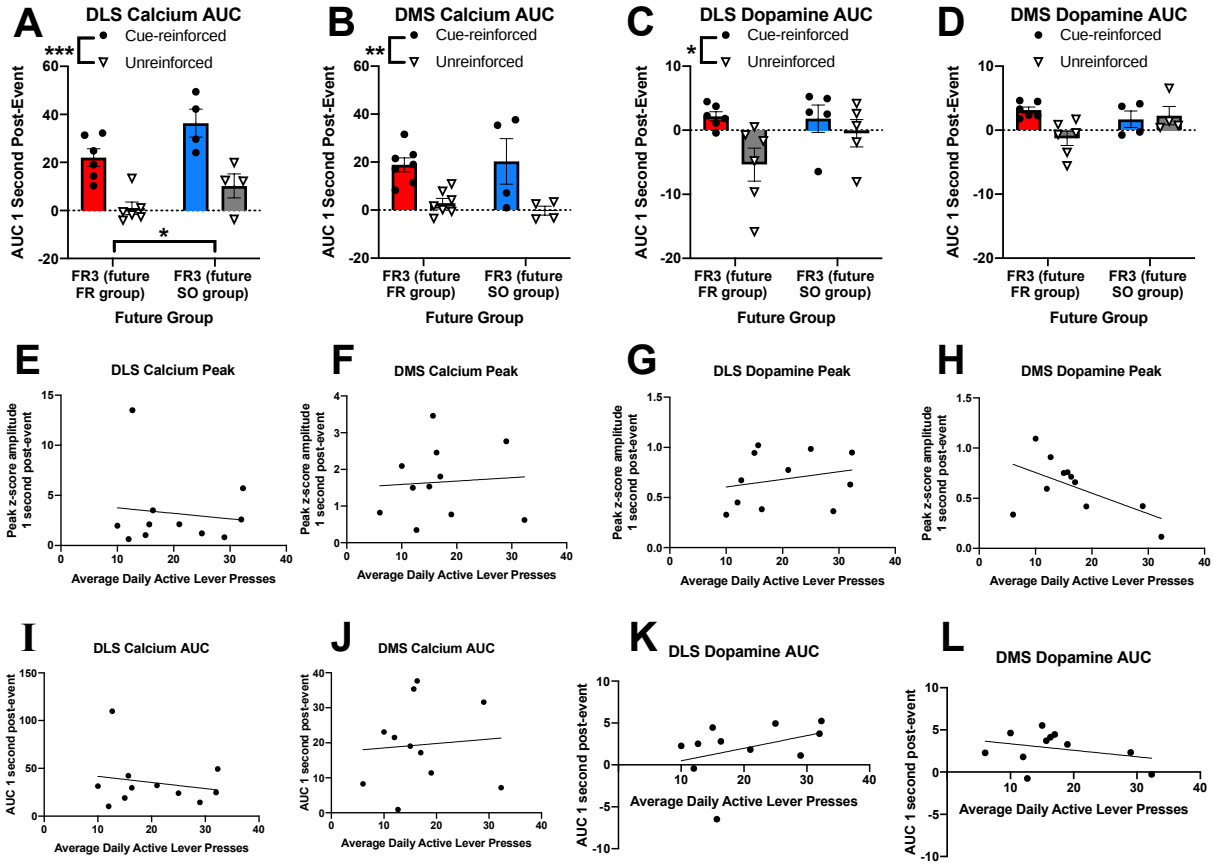

Figure 21: During early training, cue-reinforced lever presses resulted in greater AUC for DLS calcium and dopamine and DMS calcium, and there were no correlations between average daily active lever presses and calcium or dopamine responses to cue-reinforced lever presses

Initial fiber photometry recordings took place during the early phase of training, when all rats were on an FR3 schedule and had not yet been split into FR- and SO-trained groups. During this early phase of training, there was a main effect of cue reinforcement and future training schedule on DLS calcium AUC in the 1 second after lever press, but no cue reinforcement × future training schedule interaction (**A**). For DMS calcium AUC, there was a main effect of cue reinforcement, but no effect of future training schedule or interaction (**B**). There was a main effect of cue reinforcement

on DLS dopamine AUC, but no main effect of future training schedule or interaction (**C**). There was no main effect of cue reinforcement or future training schedule or interaction for DMS dopamine AUC (**D**). There was no significant correlation between average daily active lever presses during early training and DLS calcium (**E**), DMS calcium (**F**), DLS dopamine (**G**), or DMS dopamine (**H**) peak amplitudes or with DLS calcium (**I**), DMS calcium (**J**), DLS dopamine (**K**), or DMS dopamine (**L**) AUC. Graphs show group means ± SEM and individual data points. *p<0.05; **p<0.01; ***p<0.001.

6.4 Dorsal striatal calcium and dopamine AUC after cue-reinforced lever presses differ between FR-trained and SO-trained rats

During middle and late training, AUC in the 1 second after lever presses for calcium and dopamine in the DLS and DMS were compared between groups across training phases using 3-way ANOVAs. For DLS calcium AUC, there was a main effect of cue reinforcement ($F_{(1,9)}$=20.34, p=0.0015) and training schedule ($F_{(1,9)}$=6.695, p=0.0293) as well as a training schedule × phase of training interaction ($F_{(1,9)}$=6.972, p=0.0269), but there was no main effect of phase of training ($F_{(1,9)}$=3.892, p=0.0821) or cue reinforcement × training schedule ($F_{(1,9)}$=2.381, p=0.1572), cue reinforcement × phase of training ($F_{(1,9)}$=0.9052, p=0.3662) or 3-way interaction ($F_{(1,9)}$=2.882, p=0.1238) (**Figure 22A**). These results slightly differ from those for DLS calcium peak amplitude, where there was only a main effect of cue reinforcement (**Figure 13A**), and suggest that SO-trained rats had a greater DLS calcium AUC for cue-reinforced and unreinforced lever presses than FR-trained rats during the middle phase of training. However, this difference cannot necessarily be attributed to SO training, because these rats also showed greater calcium AUC responses to lever presses during early training, before training schedules differed between groups. For DMS calcium AUC, there was a main effect of cue reinforcement ($F_{(1,9)}$=9.318, p=0.0137) as well as a training

schedule × phase of training interaction ($F_{(1,9)}$=20.77, p=0.0014), but no main effects of training schedule ($F_{(1,9)}$=0.01153, p=0.9168), phase of training ($F_{(1,9)}$=2.825, p=0.1271) or cue reinforcement × training schedule ($F_{(1,9)}$=4.775, p=0.0567), cue reinforcement × phase of training ($F_{(1,9)}$=0.03256, p=0.8608) or 3-way interaction ($F_{(1,9)}$=0.05292, p=0.8076) (**Figure 22B**). These results suggest that although there was an overall effect of cue reinforcement on DMS calcium AUC for both groups, DMS calcium AUC after any lever press was reduced in SO-trained rats in the late phase of training. Although these results slightly differ than those for DMS calcium peak amplitude (**Figure 13B**), the overall interpretation that DMS calcium activity was reduced after extended SO training is supported.

For DLS dopamine AUC, there was a main effect of cue reinforcement ($F_{(1,9)}$=13.67, p=0.0049) as well as a cue reinforcement × phase of training ($F_{(1,9)}$=8.994, p=0.0150) and training schedule × phase of training interaction ($F_{(1,9)}$=6.873, p=0.0277), but no main effect of training schedule ($F_{(1,9)}$=2.696, p=0.1350) or phase of training ($F_{(1,9)}$=0.2766, p=0.6117), and there were no cue reinforcement × training schedule ($F_{(1,9)}$=1.648, p=0.2313) or 3-way interactions ($F_{(1,9)}$=0.2454, p=0.6322) (**Figure 22C**). These data suggest that while both groups showed enhanced DLS dopamine AUC to cue-reinforced lever presses versus unreinforced lever presses during the middle phase of training, overall DLS dopamine AUC was greater for SO-trained rats in the middle phase of training. DLS dopamine AUC results differed from those for DLS dopamine peak (**Figure 13C**), which suggest that DLS dopamine peak amplitude may be more related to SO-training, whereas DLS dopamine AUC may be more related to phase of training as well as training schedule. For DMS dopamine AUC, there was no main effect of cue reinforcement ($F_{(1,9)}$=3.556, p=0.0920), training schedule ($F_{(1,9)}$=0.1157, p=0.7416), or phase of training ($F_{(1,9)}$=0.06244, p=0.8083), and there were no cue reinforcement × training schedule ($F_{(1,9)}$=0.02924, p=0.8680),

cue reinforcement × phase of training ($F_{(1,9)}$=0.1923, p=0.6713), training schedule × phase of training ($F_{(1,9)}$=0.1933, p=0.6705), or 3-way interactions ($F_{(1,9)}$=0.2752, p=0.6125) (**Figure 22D**).

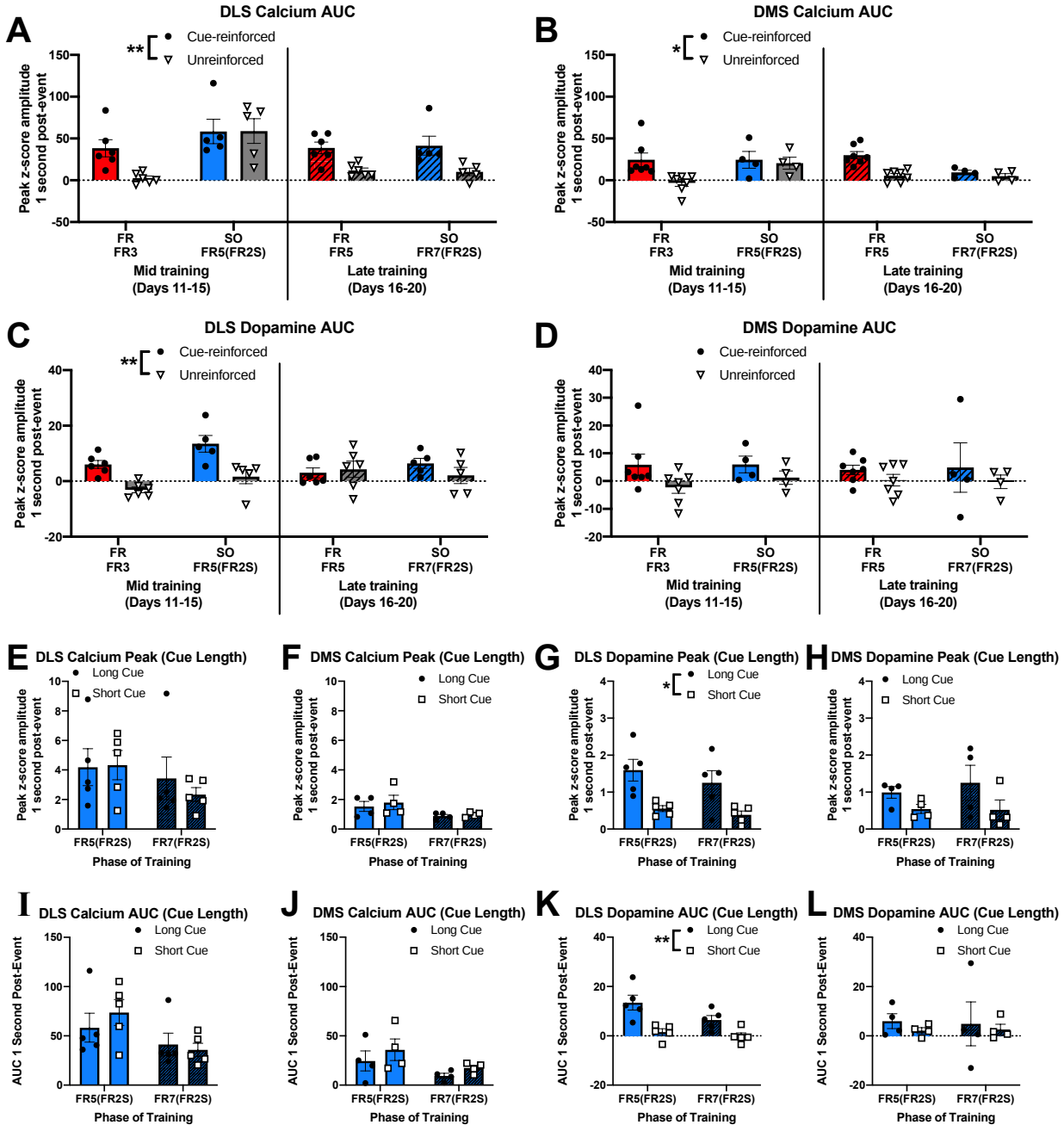

Figure 22: Dorsal striatal calcium and dopamine AUC after cue-reinforced lever presses differ between FR-trained and SO-trained rats, and SO-trained rats have different DLS dopamine responses to short cues vs long cues

During the middle and late phases of training, AUC in the 1 second after cue-reinforced and unreinforced lever presses were compared between groups. There was a main effect of cue reinforcement and training schedule on DLS calcium AUC as well as a training schedule × phase of training interaction, but there was no main effect of phase of training or other interactions (**A**). There was a main effect of cue reinforcement on DMS calcium AUC as well as a training schedule × phase of training interaction, but there was no main effect of training schedule or phase of training or other significant interactions (**B**). For DLS dopamine AUC, there was a main effect of cue reinforcement as well as cue reinforcement × phase of training and training schedule × phase of training interactions, but there were no other main effects or interactions (**C**). For DMS dopamine AUC, there were no main effects of cue reinforcement, training schedule, or phase of training, nor were there any interactions (**D**). During SO training, rats received both short, 1-second cues upon completion of the first-order schedule and long, 20-second cues accompanied by timeout upon completion of the second-order schedule. Therefore, we compared dorsal striatal calcium and dopamine responses to lever presses that resulted in short or long cue presentation in SO-trained rats. There was no main effect of phase of training, cue length, or phase of training × cue length interaction for DLS (**E**) or DMS (**F**) calcium peak amplitude. There was a main effect of cue length on DLS dopamine peak amplitude (**G**), but no effect of phase of training or interaction. There were no main effects of phase of training, cue length, or phase of training × cue length interaction for DMS dopamine peak amplitude (**H**), DLS calcium AUC (**I**), or DMS calcium AUC (**J**). There was a main effect of cue length, but no effect of phase of training or interaction, for DLS dopamine AUC (**K**), but there were no main effects or interactions for DMS dopamine AUC (**L**). Graphs show group means ± SEM and individual data points. *$p<0.05$; **$p<0.01$.

6.5 SO-trained rats have different DLS dopamine responses to short cues (first-order schedule completion) compared to long cues (second-order schedule completion)

During drug-seeking tests, SO-trained rats received both short, 1-second cues upon completion of the first-order schedule and long, 20-second cues accompanied by timeout upon completion of the second-order schedule. These cues did differ slightly: Although 1-second cues

were accompanied by extinguishing of the houselight, levers were not retracted as they were during the 20-second timeout. Therefore, we thought it possible that SO-trained rats could distinguish between these short and long cues even during the 1 second after cue initiation, so we compared the dorsal striatal calcium and dopamine responses to short and long cues in SO-trained rats using 2-way ANOVAs. For both DLS (**Figure 22E**) and DMS (**Figure 22F**) calcium peak amplitude in the 1 second after lever press, there was no effect of phase of training ($F_{(1,4)}$=1.517, p=0.2855; $F_{(1,3)}$=2.931, p=0.1854) or cue length ($F_{(1,4)}$=0.9993, p=0.3714; $F_{(1,3)}$=0.8924, p=0.4145), and there was no phase of training × cue length interaction ($F_{(1,4)}$=0.3243, p=0.5995; $F_{(1,3)}$=0.3635, p=0.5891). There was a main effect of cue length on DLS dopamine peak amplitude ($F_{(1,4)}$=15.97, p=0.0162), but no main effect of phase of training ($F_{(1,4)}$=1.748, p=0.2567) or phase of training × cue length interaction ($F_{(1,4)}$=0.1882, p=0.6868) (**Figure 22G**). For DMS dopamine peak, there were no main effects of phase of training ($F_{(1,3)}$=0.1510, p=0.7235) or cue length ($F_{(1,3)}$=8.058, p=0.0657), and there was no phase of training × cue length interaction ($F_{(1,3)}$=1.271, p=0.3415) (**Figure 22H**). For both DLS (**Figure 22I**) and DMS (**Figure 22J**) calcium AUC, there was no effect of phase of training ($F_{(1,4)}$=3.948, p=0.1178; $F_{(1,3)}$=3.370, p=0.1637) or cue length ($F_{(1,4)}$=0.2685, p=0.6317; $F_{(1,3)}$=2.432, p=0.2168), and there was no phase of training × cue length interaction ($F_{(1,4)}$=0.9497, p=0.3850; $F_{(1,3)}$=0.1045, p=0.7677). There was a main effect of cue length on DLS dopamine AUC ($F_{(1,4)}$=37.49, p=0.0036), but no main effect of phase of training ($F_{(1,4)}$=3.394, p=0.1392) or phase of training × cue length interaction ($F_{(1,4)}$=3.401, p=0.1389) (**Figure 22K**). For DMS dopamine AUC, there were no main effects of phase of training ($F_{(1,3)}$=0.006646, p=0.9402) or cue length ($F_{(1,3)}$=0.5815, p=0.5011), and there was no phase of training × cue length interaction ($F_{(1,3)}$=0.04359, p=0.8480) (**Figure 22L**). These results suggest that SO-trained rats had different DLS dopamine responses to short cues compared to long cues.

Therefore, because SO-trained rats could distinguish between long and short cues by at least one measure, throughout analyses we only compared long cue responses to those of FR-trained rats to ensure group differences were based on training schedule and not different cue responses.

6.6 Dorsal striatal calcium AUC responses to noncontingent cues decrease throughout cue extinction

During cue extinction, we showed that the calcium peak response to noncontingent cues reduced significantly throughout the session in the DMS (**Figure 14**). When we evaluated calcium AUC in response to noncontingent cues during cue extinction, there was no main effect of bin on DLS calcium AUC ($F_{(1.801,18.01)}$=3.362, p=0.0617) (one-way rmANOVA) (**Figure 23A**). For DMS calcium AUC, there was a main effect of bin on AUC ($F_{(2.013,20.13)}$=, p=0.0012), and post-hoc analyses indicated that DMS calcium AUC for cues 1-30 was significantly greater than for cues 61-90 (p=0.0059, q=6.255) and for cues 91-120 (p=0.0136, q=5.492) (Tukey's multiple comparisons) (**Figure 23B**). These results, along with very similar calcium peak amplitude data during cue extinction, indicate a reduction of cue-induced calcium activity in the dorsal striatum throughout cue extinction.

6.7 Dorsal striatal dopamine responses to noncontingent cues are unaffected by cue extinction

During cue extinction, there was no main effect of bin on DLS dopamine peak amplitude ($F_{(2.299,22.99)}$=1.564, p=0.2295) (**Figure 23C**), DLS dopamine AUC ($F_{(2.154,21.54)}$=2.960, p=0.0698) (**Figure 23D**), DMS dopamine peak amplitude ($F_{(1.936,19.36)}$=1.581, p=0.2313) (**Figure 23F**), or DMS dopamine AUC ($F_{(1.642,16.42)}$=1.245, p=0.3060) (**Figure 23G**) (one-way rmANOVA). Overall, these data suggest that noncontingent cue presentations had little effect on dorsal striatal dopamine, and this did not change throughout the cue extinction session.

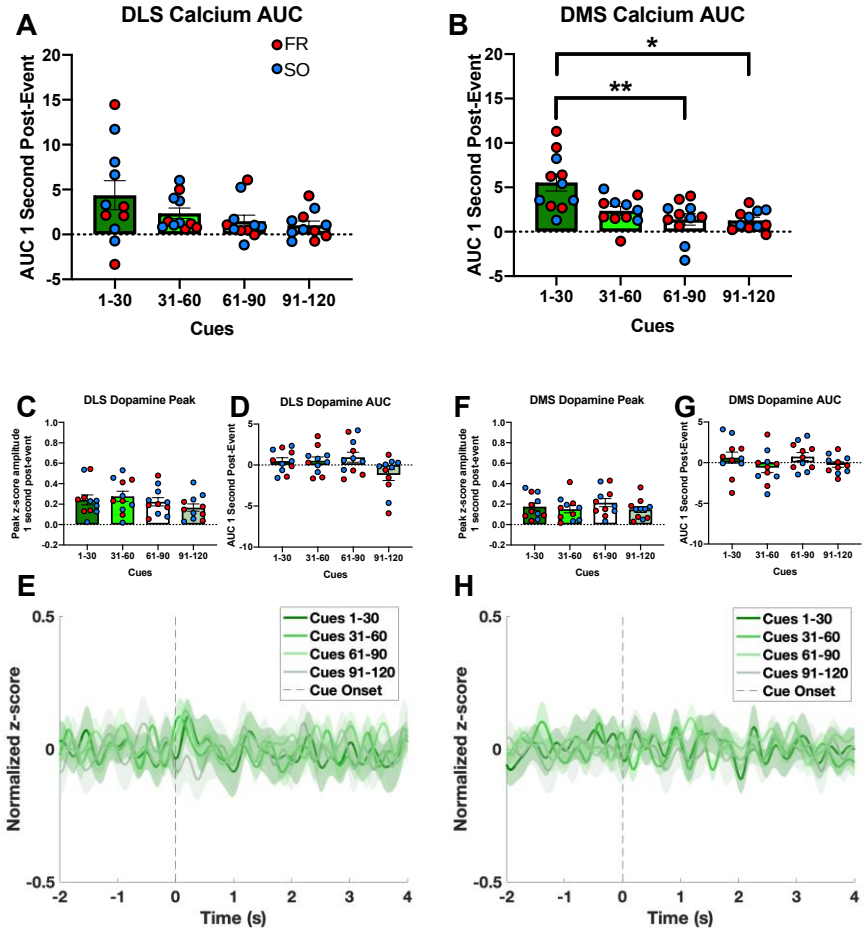

Figure 23: Dorsal striatal calcium AUC responses to noncontingent cues decrease throughout cue extinction, whereas dorsal striatal dopamine responses are unaffected

During cue extinction, 120 noncontingent cue presentations were separated into 30-cue bins. There was no effect of bin on DLS calcium AUC (**A**). For DMS calcium AUC, there was a main effect of bin, and post-hoc analyses indicated that DMS calcium AUC for cues 1-30 was significantly greater than for cues 61-90 and for cues 91-120 (**B**). There was no effect of bin on DLS dopamine peak amplitude or AUC (**C-E**) or on DMS dopamine peak amplitude or AUC (**F-H**). Graphs show group means ± SEM and individual data points with dashed vertical lines indicating cue onset. Traces show overall average trace for each event for each group aligned to behavioral events with SEM shown with shading and dashed vertical lines indicating cue onset. *p<0.05; **p<0.01.

6.8 Cue extinction does not impact dorsal striatal calcium or dopamine AUC responses to lever presses

Dorsal striatal calcium and dopamine AUC in response to cue-reinforced and unreinforced active lever presses were compared during drug-seeking tests during the late phase of training (pre-ext) and after cue extinction (post-ext) using 3-way ANOVAs. For DLS calcium AUC, there was a main effect of cue reinforcement ($F_{(1,7)}$=14.26, p=0.0069) and training schedule ($F_{(1,7)}$=11.47, p=0.0117), but there was no main effect of cue extinction ($F_{(1,7)}$=0.8767, p=0.3803) or cue reinforcement × training schedule ($F_{(1,7)}$=0.2842, p=0.6105), cue reinforcement × cue extinction ($F_{(1,7)}$=0.03958, p=0.8480), training schedule × cue extinction ($F_{(1,7)}$=3.445, p=0.1058), or 3-way interaction ($F_{(1,7)}$=0.2140, p=0.6577) (**Figure 24A**). For DMS calcium AUC, there was a main effect of cue reinforcement ($F_{(1,7)}$=42.11, p=0.0003) and there was also a cue reinforcement × training schedule interaction ($F_{(1,7)}$=24.83, p=0.0016), but there were no main effects of training schedule ($F_{(1,7)}$=1.783, p=0.2235) or cue extinction ($F_{(1,7)}$=1.545, p=0.2535) or cue reinforcement × cue extinction ($F_{(1,7)}$=0.1873, p=0.6782), training schedule × cue extinction ($F_{(1,7)}$=4.800, p=0.0646), or 3-way interactions ($F_{(1,7)}$=0.001162, p=0.9738) (**Figure 24B**). For both DLS (**Figure**

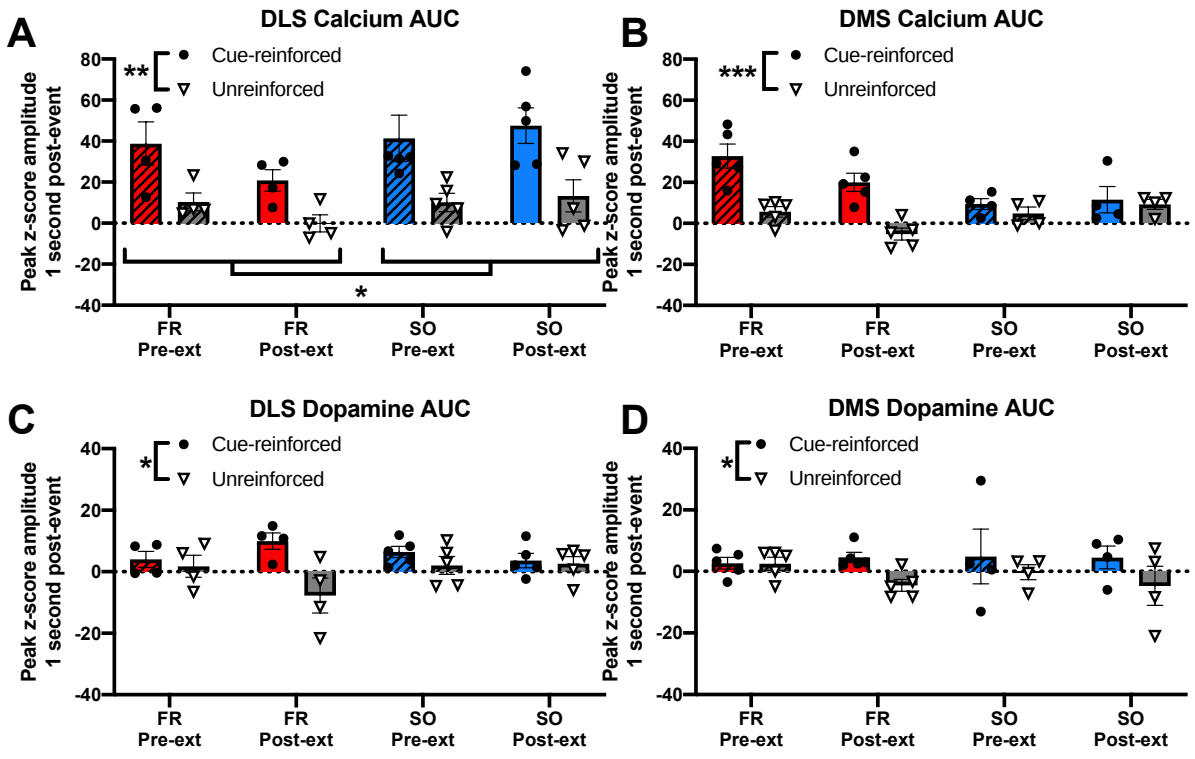

Figure 24: Cue extinction does not impact dorsal striatal calcium or dopamine AUC responses to lever presses

Dorsal striatal calcium and dopamine AUC after lever presses were compared between drug-seeking tests during the late phase of training (pre-ext) and after cue extinction (post-ext). There was a main effect of cue reinforcement and training schedule on DLS calcium AUC, but there was no main effect of cue extinction or cue reinforcement × training schedule, cue reinforcement × cue extinction, training schedule × cue extinction, or 3-way interaction (**A**). There was a main effect of cue reinforcement and a cue reinforcement × training schedule interaction for DMS calcium AUC, but no other main effects or interactions (**B**). There was a main effect of cue reinforcement on DLS dopamine AUC (**C**) and DMS dopamine AUC (**D**), but no other main effects or interactions. Graphs show group means ± SEM and individual data points. *p<0.05; **p<0.01; ***p<0.001.

24C) and DMS (**Figure 24D**) dopamine AUC, there was a main effect of cue reinforcement ($F_{(1,7)}$=9.386, p=0.0182; $F_{(1,7)}$=10.31, p=0.0148), but no main effects of training schedule ($F_{(1,7)}$=0.3980, p=0.5481; $F_{(1,7)}$=0.002485, p=0.9616), cue extinction ($F_{(1,7)}$=0.6542, p=0.4452; $F_{(1,7)}$=1.620, p=0.2437), and there were no cue reinforcement × training schedule ($F_{(1,7)}$=3.054, p=0.1241; $F_{(1,7)}$=0.4782, p=0.5115), cue reinforcement × cue extinction ($F_{(1,7)}$=2.205, p=0.1812;

$F_{(1,7)}$=1.304, p=0.2910), training schedule × cue extinction ($F_{(1,7)}$=0.03134, p=0.8645; $F_{(1,7)}$=0.001651, p=0.9687), or 3-way interactions ($F_{(1,7)}$=5.232, p=0.560; $F_{(1,7)}$=0.1821, p=0.6824). Together, there was no effect of cue extinction or interaction between cue extinction and another factor on calcium or dopamine AUC in the DLS or DMS, which indicates that cue extinction may not impact AUC in the DMS despite its effects on DMS calcium and dopamine peak amplitudes (**Figure 15**).

6.9 Prior to operant behavioral training, novel stimulus presentation induces increases in dorsal striatal calcium and dopamine activity

In a subset of rats (n=5), we evaluated if novel stimuli presented noncontingently prior to operant behavioral training impacted dorsal striatal calcium or dopamine activity. Rats underwent 2 days of 15-minute testing sessions in which novel stimuli were presented for 10 seconds (houselight, cue light, audio tone, levers inserted, or simultaneous cue light and audio tone) 4 times each. The z-score for each trace for each event was averaged for each rat, and the peak amplitude and average AUC per second were compared between the 3 seconds before stimulus onset (baseline) and 10 seconds during stimulus presentation using 2-way ANOVAs. For DLS calcium peak amplitude, there was a main effect of stimulus presentation ($F_{(1,4)}$=439.3, p<0.0001), but no main effect of stimulus type ($F_{(2.311,9.245)}$=3.013, p=0.0937) or stimulus presentation × stimulus type interaction ($F_{(1.723,6.891)}$=1.454, p=0.2923) (**Figure 25A**). There was a significant stimulus presentation × stimulus type interaction ($F_{(1.647,6.588)}$=9.868, p=0.0121) for DLS calcium AUC, and post-hoc analyses revealed a significant effect of simultaneous cue light and tone presentation (p=0.0014, t=12.06) (Sidak's multiple comparisons) (**Figure 25B**). For DMS calcium peak

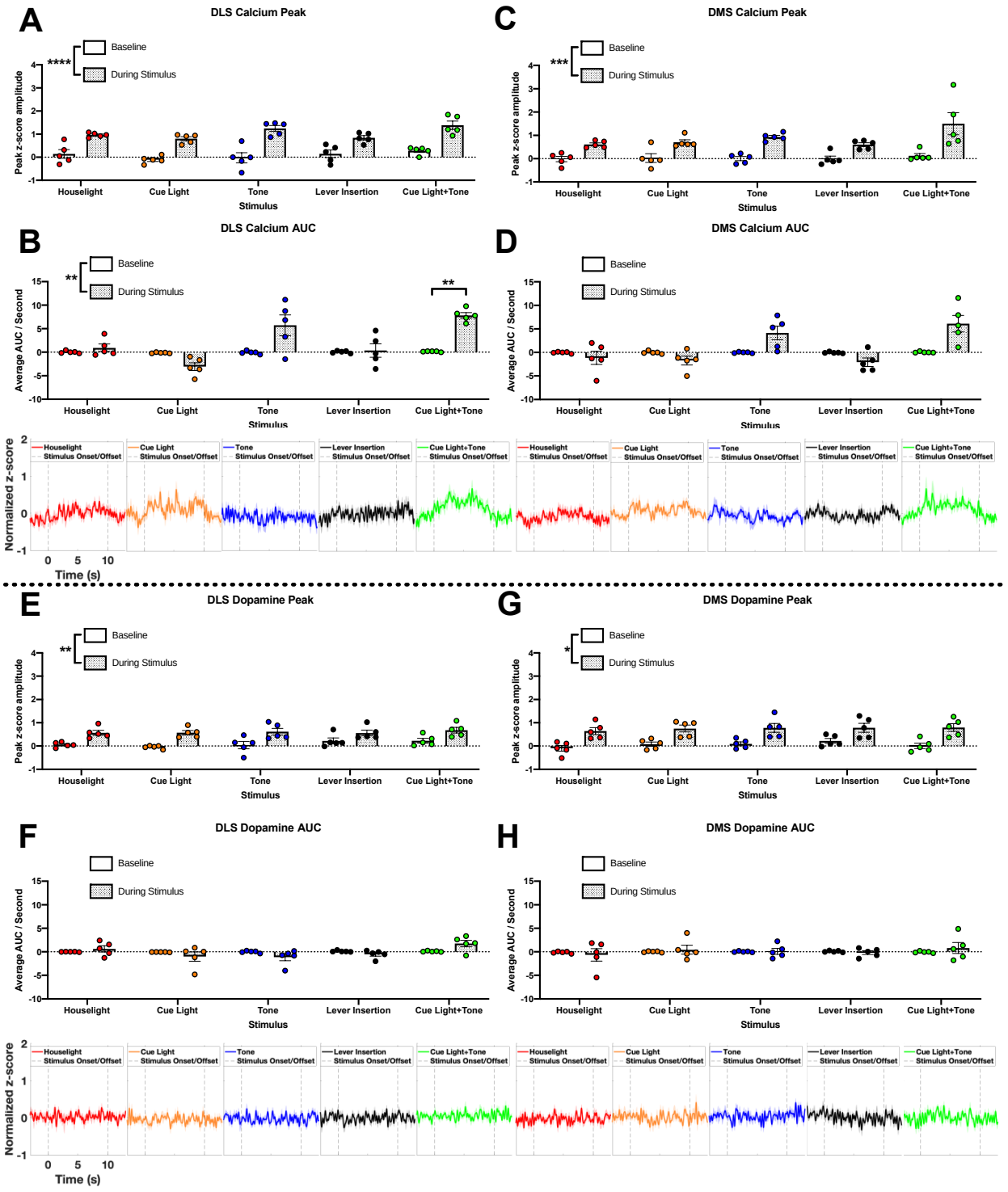

Figure 25: Prior to operant behavioral training, novel stimulus presentation induces increases in dorsal striatal calcium and dopamine activity

A subset of rats (n=5) were exposed to novel stimuli (houselight, cue light, audio tone, levers inserted, or simultaneous cue light and audio tone) prior to operant behavioral training. The effect of these stimuli on dorsal striatal peak amplitude and average AUC per second were compared between the 3 seconds before stimulus onset and 10 seconds during stimulus onset. For DLS calcium peak amplitude, there was a main effect of stimulus presentation, but no main effect of stimulus type or stimulus presentation × stimulus type interaction (**A**). For DLS calcium AUC, there was a significant stimulus presentation × stimulus type interaction, and post-hoc analyses revealed a significant effect of simultaneous cue light and tone presentation on DLS calcium AUC (**B**). There was a main effect of stimulus presentation on DMS calcium peak amplitude, but no main effect of stimulus type or stimulus presentation × stimulus type interaction (**C**). For DMS calcium AUC, there was a significant stimulus presentation × stimulus type interaction, but post-hoc analyses did not reveal any additional effects (**D**). There was a main effect of stimulus presentation, but no main effect of stimulus type or interaction, on DLS dopamine peak amplitude (**E**), but there were no main effects or interactions for DLS dopamine AUC (**F**). Similarly, there was a main effect of stimulus presentation, but no main effect of stimulus type or interaction, on DMS dopamine peak amplitude (**G**), but there were no main effects or interactions for DMS dopamine AUC (**H**). Graphs show group means ± SEM and individual data points. Traces show overall average trace for each event aligned to behavioral events with SEM shown with shading and dashed vertical lines indicating stimulus onset and offset. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

amplitude, there was a main effect of stimulus presentation ($F_{(1,4)}$=78.51, p=0.0009), but no main effect of stimulus type ($F_{(1.382,5.311)}$=2.636, p=0.1626) or stimulus presentation × stimulus type interaction ($F_{(1.291,5.163)}$=2.515, p=0.1735) (**Figure 25C**). For DMS calcium AUC, there was a significant stimulus presentation × stimulus type interaction ($F_{(2.383,9.527)}$=7.638, p=0.0087), but post-hoc analyses did not reveal any additional effects (Sidak's multiple comparisons) (**Figure 25D**). For both DLS (**Figure 25E**) and DMS (**Figure 25G**) dopamine peak amplitude, there was a main effect of stimulus presentation ($F_{(1,4)}$=51.27, p=0.0020; $F_{(1,4)}$=19.08, p=0.0120), but no main effect of stimulus type ($F_{(2.156,8.623)}$=3.002, p=0.1003; $F_{(1.950,7.798)}$=1.990, p=0.2005) or stimulus presentation × stimulus type interaction ($F_{(1.369,5.477)}$=1.137, p=0.3577; $F_{(2.271,9.084)}$=0.4416, p=0.6796). For both DLS (**Figure 25F**) and DMS (**Figure 25H**) dopamine AUC, there were no

main effects of stimulus presentation ($F_{(1,4)}$=0.4533, p=0.5377; $F_{(1,4)}$=0.4211, p=0.5517) or stimulus type ($F_{(2.425,9.698)}$=2.449, p=0.1323; $F_{(1.311,5.234)}$=0.2945, p=0.6694), nor and there was no stimulus presentation × stimulus type interaction ($F_{(2.277,9.109)}$=2.236, p=0.1594; $F_{(1.302,5.208)}$=0.2718, p=0.6828). Interestingly, these data indicate that novel stimulus presentation may induce some dorsal striatal calcium and dopamine activity.

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