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Role of the Prefrontal Cortex in Reward Seeking Behaviors

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ROLE OF PREFRONTAL CORTEX IN REWARD SEEKING BEHAVIORS

A Dissertation Presented

by

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DEDICATION

To:

Noemí Feliciano Cintrón, Margarita Cintrón Rodríguez, and my island Puerto Rico.
I owe everything I am to you.

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ABSTRACT

ROLE OF PREFRONTAL CORTEX IN REWARD SEEKING BEHAVIORS

SEPTEMBER 2021

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Disorders associated with compulsive seeking of rewards, like binge-eating, are associated with abnormalities of the prefrontal cortex in humans, which is analogous to the prelimbic (PL) and infralimbic (IL) subregions of the medial prefrontal cortex (mPFC) in rodents. Although studies have examined the role of the mPFC in drug seeking behaviors, studies examining natural reward seeking behaviors (i.e. food and sucrose) are often unclear and contradictory. This dissertation aims to characterize the role of the PL and IL mPFC in operant sucrose seeking behaviors. We used pharmacological and chemogenetic tools to selectively inactivate the PL, IL and PL-nucleus accumbens (NAc) NAc during Fixed Ratio 1 (FR1), extinction, and cue-induced reinstatement. Furthermore, we describe the role of PL projections to the NAc in both highly-motivated rats (food restricted) and low-motivated rats (free fed) in operant sucrose seeking behaviors. Our results demonstrate that the IL subregion of the mPFC plays a role in the execution of reward seeking behaviors during extinction (i.e. well entries) and cue-induced reinstatement (i.e. nose poking). Additionally, our results demonstrate that the PL plays a role in inhibiting reward seeking during FR1 (i.e. nose pokes and rewarded well entries). However, the PL seems to play a role in promoting

reward seeking during extinction (i.e. nose poking and well entries). We also observed that inactivating PL-NAc in food restricted rats during extinction and cue-induced reinstatement suppresses behaviors that do not result in reward delivery (i.e., inactive lever presses). In free fed rats, PL-NAc inhibits reward seeking behaviors (i.e. initiated trials) during cue-induced reinstatement. Our findings support our claim that the mPFC and its projections differentially control reward seeking behaviors depending on the behavioral (e.g., FR1, extinction, or cue-induced reinstatement) and motivational context (e.g., level of satiety) of animals. Understanding the function of the mPFC will give insight to understand and develop specialized therapies to treat and cure disorders like binge-eating, as well as other diseases associated with the mPFC, like substance use disorders.

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CHAPTER 1

GENERAL INTRODUCTION AND BACKGROUND

1.1 Introduction

Our ability to perform complex behaviors is due to the activity of billions of interconnected neurons in the brain. The frontal lobe of the brain is the area that encompasses the highest cognitive capabilities for humans. Specifically, the prefrontal cortex (PFC) is in charge of controlling the appropriate cognitive processes in order to carry out the behavioral response to a stimulus, including attention, working memory, task switching, planning, decision making, and behavioral inhibition (Brown & Bowman, 2002; Dalley et al., 2004; Fuster, 2000; Miller, 2000; Miller & Cohen, 2001; Ongur & Price, 2000; Robbins, 2000). The PFC also plays an important role in mediating goal-directed behaviors. In order to reach a goal, information from the environment needs to be interpreted to implement the appropriate attentional and decision-making processes, and to execute or inhibit the appropriate behavioral response for a given stimulus (de Haan et al., 2018; Miller & Cohen, 2001). Hence, abnormal control of execution and inhibition of behaviors is a major contributor to problems like substance-use disorders, eating disorders, and gambling (Gut-Fayand et al., 2001; Jentsch & Taylor, 1999; Nigg, 2000). There is vast evidence that the rodent PFC, similar to primate PFC, is in charge of cognitive and executive processes regulating execution and inhibition of reward seeking behavior (Brown & Bowman, 2002; Dalley et al., 2004; Kesner & Churchwell, 2011; Sharpe & Killcross, 2018). Therefore, rodent studies are imperative in the advancement

of the field because they allow us to conduct more invasive experiments that can lead to discoveries that are difficult to perform on humans and/or non-human primates.

There is vast evidence demonstrating that there are homologies between the primate and rodent PFC (Seamans et al., 2008; Uylings et al., 2003; Uylings & van Eden, 1991). Additionally, connections between the rodent mPFC and the thalamus have been studied in detail providing further evidence of homologies between the primate and rodent neural circuits (Gabbott et al., 2005; Ko, 2017; Leonard, 1969, 2016; Mailly et al., 2013; Riga et al., 2014; Vertes, 2004). The first paper to confirm afferent projections to the frontal pole from the thalamus and use the terms "prefrontal cortex" and "rat" was published in 1969 (Leonard, 1969). In 1973, a second paper using "prefrontal cortex" and "rat" was published, where stimulation of neurons in the nucleus accumbens led to activation of neurons in the PFC in rats (Rolls & Cooper, 1973). These studies opened the possibility to use rodent models to research the PFC and there are currently over 12,000 papers that use these terms to date. Subdivisions of the medial PFC include a dorsal region composed of a precentral and anterior cingulate cortex (ACC), and a ventral region that includes prelimbic (PL), infralimbic (IL), and medial orbital cortices (Dalley et al., 2004; Groenewegen et al., 1997). A retrograde tracer injection study showed that in rat brain the mediodorsal thalamus (MD) received 9% afferents from PL; 7% afferents from IL; and 8% afferents from ACC (Gabbott et al., 2005). The field has currently shifted the rodent PFC nomenclature to medial prefrontal cortex (mPFC) when referring to studies that include these three subareas: medial agranular or ACC, PL, and IL (Krettek & Price, 1977; Laubach et al., 2018; Perez-Cruz et al., 2007; Vertes, 2004). For

the purpose of this dissertation, we will place special focus on the rodent mPFC and its role in mediating reward seeking behaviors.

1.2 Role of PL, IL and accumbens subregions in natural reward seeking behaviors

The PL and IL mPFC, and PL projections to the nucleus accumbens core (NAcC) have been strongly implicated in the behavioral execution/inhibition balance (Bari et al., 2011; Bari & Robbins, 2013; Hardung et al., 2017; Roitman & Loriaux, 2014; Sharpe & Killcross, 2015). Previous work has shown that the PL promotes the expression of conditioned fear and drug-seeking behavior, i.e., "going," and the IL promotes the inhibition of these behaviors, i.e., "stopping," often demonstrated through extinction learning (Peters et al., 2009). However, a number of studies question this strict PL/IL functional dichotomy and suggest that even though PL and IL may have opposing roles in some behaviors, the contributions of each region are more complex than previously thought (Burgos-Robles et al., 2013; McGlinchey et al., 2016; Meyer & Bucci, 2014; Moorman & Aston-Jones, 2015). In particular, the specific behavioral paradigms and reward employed play a critical role in understanding contributions of each brain region to execution or inhibition. Therefore, if we disregard the numerous variables in play, generalizing the roles of PL and IL in execution and inhibition may be counterproductive.

When assessing reward seeking behaviors, it is important to clearly establish if the behavior was guided by motivation to seek/obtain a reward, or as an automatic response to a cue. A goal directed action is proposed to meet two criteria: 1) a situation/state of being must precede the behavior that leads to the goal/reward, and 2) there is an instrumental contingency between the behavior and the goal/reward

(Dickinson & Balleine, 1993). If a behavior leads to a goal/reward, but does not meet both criteria, the behavior cannot be considered goal directed and can be defined as a response (Dickinson & Balleine, 1993). For example, if an animal learns to receive a food pellet every time they press a lever, it is only considered goal directed if the animal is hungry and if the animal has previously learned that a food pellet will be dispensed when pressing the lever. If the animal is not hungry but presses the lever to receive the food pellet, or if the animal is hungry but presses the lever without previous knowledge that the lever will deliver food, it is not considered a goal directed behavior. Accordingly, a habit is defined as an instrumental behavior performed to reach a goal/reward (action-outcome contingency) which with repeated practice over time becomes a response triggered by the stimuli, independent of the value of the reward (stimulus-response habits) (Barker et al., 2014; Dickinson, 1985).

As mentioned before, the PFC has been demonstrated to play a critical role in cognitive control of reward seeking behaviors and PL/IL have been theorized to play different and opposing roles in terms of "going" and "stopping". However, IL has also been shown to control inflexible reward seeking, also defined as habitual reward seeking (Barker et al., 2014). An IL lesion study using sucrose and food pellet rewards demonstrated that rats with an IL lesion were able to acquire action-outcome contingencies and were also sensitive to outcome devaluation even after extensive training (Killcross & Coutureau, 2003). This same study also found that lesioning the PL blocked the sensitivity to reward devaluation, compared to control rats (Killcross & Coutureau, 2003). In a study using sucrose and chocolate milk as a reward, optogenetically inhibiting the IL blocked expression of habitual reward seeking

behaviors. Moreover, if enough time had passed in order to develop a new habitual behavior, IL inactivation blocked the new habitual behavior and the previously acquired habitual behavior was rescued (Smith et al., 2012). One explanation is that the IL controls reflexive responding and inhibits the goal-directed behaviors mediated by the PL (Killcross & Coutureau, 2003). In a PL inactivation study using Baclofen/Muscimol via cannulae and food pellets as reward, inactivating the PL decreased goal directed reward seeking in minimally trained animals (Shipman et al., 2018). These findings support the theory that PL is important to mediate goal-directed behaviors before they become habitual.

The role the PL and the IL play in regulating acquisition and recall of reward seeking behaviors of food and sucrose is not as clearly established in the field as it is in the drug seeking literature. In a fixed interval food seeking task, inactivation of the PL with $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen increased lever pressing in food restricted rats (Jonkman et al., 2009). Inactivation of ventromedial PFC neuronal ensembles (mostly IL) during a food self-administration task decreased food seeking behaviors (Warren et al., 2016). In a variable interval schedule of reinforcement study (VI-60), neural activity in the PL correlated with delivery of sucrose pellets, but inactivation of these neurons had no effect on behavior (Burgos-Robles et al., 2013). In this same study, neural activity in the IL correlated with collection of the sucrose pellets and inactivation of the IL let to a longer latency to collect the reward (Burgos-Robles et al., 2013). These contradicting results highlight the importance of further elucidating the specific roles PL and IL play under various contexts.

Another important area that has been demonstrated to play an important role in instrumental conditioning is the NAc, which is thought to play an important role in relating reward to action-outcome association (Corbit et al., 2001; Dickinson & Balleine, 1994). The NAc receives glutamatergic inputs from limbic structures, including the PFC (Powell & Leman, 1976). It is also a major component of the mesolimbic dopamine system, which plays an important role in regulating reward and desire of food (Aitken et al., 2016; Biesdorf et al., 2015; Powell & Leman, 1976). Further characterization of PL to NAc circuitry is needed to understand the link between PL and NAc in mediating reward seeking behaviors. It has been shown that NAc neurons fire during both acquisition and maintenance of goal-directed sucrose seeking behaviors in food restricted rats (Gillis & Morrison, 2019). In an instrumental conditioning task using sucrose and food pellets as reward, inactivating PL projections to NAc did not have any effect on goal-directed learning (Hart et al., 2018). However, a study using a Fixed-Ratio-1 discriminative stimulus task found that NAc Core neurons fired when the reward cue was presented (Ishikawa et al., 2008a). By the time of the recordings, these food restricted rats had already learned to associate cue (lever and tone) with reward delivery (10% sucrose) (Ishikawa et al., 2008a). Additionally, inactivating dorsomedial PFC in those same animals decreased NAc Core firing and also decreased lever pressing (Ishikawa et al., 2008a). An important detail to note is that for both of the previously mentioned studies, the rats were food restricted. This detail is important because it allows for a better comparison of palatable food reward seeking behaviors, especially because it has been shown that neurons in the NAc are associated with palatable food or sucrose cues when rats are hungry (Ahn & Phillips, 1999; Aitken et al., 2016).

1.3 PL/IL/NAc and extinction

Extinction is defined as a new memory, where a previously learned action-outcome association is inhibited in response to the newly formed memory (Barker et al., 2014). In terms of instrumental conditioning, when a response-contingent reward or reinforcer is omitted, expression of operant behavior decreases (Skinner, 1938). The IL has been shown to mediate extinction learning, specifically for inhibition of cocaine seeking behaviors and inhibition of conditioned fear expression (Augur et al., 2016; Barker et al., 2014; Peters, LaLumiere, et al., 2008; Peters et al., 2009; Sierra-Mercado et al., 2011). In terms of cocaine, the NAc, as well as IL projections to the NAc shell are implicated in extinction and withdrawal (Millan et al., 2011; Warren et al., 2019). A microdialysis study found that levels of DA in the NAc shell decrease during extinction, when food-contingent cue was not followed by a food reward (Biesdorf et al., 2015). However, the role the mPFC plays in extinction of natural rewards is unclear. The IL has been shown to play an important role in Pavlovian conditioning, but not in instrumental conditioning (Mendoza et al., 2015). Inactivation of NMDA receptors in the IL using a sucrose self-administration task where rats were fed *ad libitum* disrupted extinction consolidation, but not performance during extinction training (Peters & De Vries, 2013).

1.4 PL/IL/NAc and reinstatement

Campbell and Jaynes were the first to introduce the term reinstatement into the scientific literature, and defined it as partial practice or repetition of an experience that maintains its effects over time (Campbell & Jaynes, 1966). They specifically described context induced reinstatement using footshock, introducing rats into a double chamber

where one side was paired with footshock and the other side was "safe" (Campbell & Jaynes, 1966). Rats that had previously experienced footshock in the footshock context spent more time in the "safe" side when re-introduced to the environment (Campbell & Jaynes, 1966). However, rats that had not been previously trained to associate the "non-safe" side with footshock, did not spend as much time in the "safe" side (Campbell & Jaynes, 1966). We can describe these findings as a strengthening between a conditioned stimulus and a conditioned response in previously exposed rats. The stimulus remains neutral for rats that have not learned to associate a context to a conditioned response. Stimuli can be context (an environment paired with a footshock) or cue (tone, lever, etc.) which, depending on the conditioned stimulus, can trigger freezing behavior as a fear response or reward seeking behavior in response to foodor drug cues.

Several brain regions have been associated with reinstatement of drug-seeking behavior, including the PL, the NAc core, the ventral tegmental area (VTA), and the ventral pallidum (VP) (Fuchs et al., 2004; McFarland & Kalivas, 2001). In a baclofen/muscimol inactivation study, it was shown that VTA-PL-NAc core- VP are important for reinstatement of cocaine seeking behaviors, however, inactivation of PL-NAc core did not have any effect on food reinstatement using non-contingent food delivery (McFarland & Kalivas, 2001). In this study, rats were food restricted to maintain 90% free feeding weight, which is typically considered mild food restriction and not enough to induce hunger (D'Cunha et al., 2013). It has also been shown that contralateral projections of PL to NAc core neurons play a role in cocaine cue-induced reinstatement, but not sucrose or food (McFarland et al., 2003). However, PL neurons showed an increase in Fos for both sucrose and cocaine reinstatement in free fed rats (James et al.,

2018). Another study that looked at Fos levels in PL to NAc core during cocaine and sucrose cue-induced reinstatement found that there was an increase in Fos for cocaine, but not sucrose cues (McGlinchey et al., 2016). This study also maintained rats in *ad libitum* feeding schedule, which raises the question as to how qualitatively comparable cocaine and sucrose reward are in terms of motivation when using sucrose or food as a reward in satiated rats.

1.5 Summary

It is clear that PL/IL have a "going" vs "stopping" role in terms of fear and cocaine seeking behaviors (Alvarez-Jaimes et al., 2008; Bossert et al., 2012; Carelli & West, 2014; Jaramillo et al., 2018; Moorman & Aston-Jones, 2015; Peters et al., 2009; Sierra-Mercado et al., 2011; Stefanik et al., 2016; Warren et al., 2019). However, in terms of food and sucrose reward, results are varied and often reveal contradicting evidence (Chen et al., 2013; Eddy et al., 2015; Gutman et al., 2017; Ishikawa et al., 2008a, 2008b; McFarland & Kalivas, 2001; McGlinchey et al., 2016; Moorman & Aston-Jones, 2015; Rhodes & Killcross, 2007; Rhodes & Killcross, 2004; Sangha et al., 2014; Trask et al., 2017). When comparing sucrose and cocaine rewards, it has been shown that free fed rats, housed in groups of two or three, prefer sugar over cocaine (Lenoir et al., 2007). This data suggests that sucrose should be more motivating than cocaine. But, in the previously mentioned study, sucrose was more pleasurable than cocaine in animals that were pair housed and free fed; this is not the case in animals that are single housed (Nicolas et al., 2016). These findings highlight the complexity that encompasses the role of the mPFC in controlling reward seeking behaviors. It also highlights the importance to

define and assess the hedonic value of the reward that is being used and to specify emotional/satiation state of the animal when taking into consideration the results of the findings.

Therefore, the purpose of this dissertation is to characterize the role of the PL, IL, and PL projections to NAc in controlling execution and inhibition of sucrose reward seeking behaviors. **I hypothesize that the role that PL, IL, and PL-NAc play in controlling sucrose seeking behaviors is dependent on the hedonic value of the reward and cue.** In order to test my hypothesis, I developed two projects: 1) Examine the role of PL and IL in sucrose seeking FR1, extinction, cue-induced reinstatement, and progressive ratio using pharmacological inactivation, and 2) Examine whether PL-NAc plays a different role in sucrose FR1, extinction, cue-induced reinstatement, and Fixed-Ratio-5 (FR5) depending on levels of satiation using chemogenetic inactivation. We use 15% and 12% sucrose, respectively, as a reward because of its highly palatable properties to rats (Nissenbaum & Sclafani, 1987). Our findings support our claim that the mPFC and its projections differentially control reward seeking behaviors depending on the task and motivational value of the reward. This dissertation bridges the gap in the reward seeking literature by providing evidence that the complexity of the mPFC needs to be taken into consideration when creating targeted therapies towards substance abuse disorders and binge eating disorders.

CHAPTER 2

DIFFERENTIAL EFFECTS OF DORSAL AND VENTRAL MEDIAL PREFRONTAL CORTEX INACTIVATION DURING NATURAL REWARD SEEKING, EXTINCTION, AND CUE-INDUCED REINSTATEMENT

Caballero, J. P., Scarpa, G. B., Remage-Healey, L., & Moorman, D. E. (2019). Differential Effects of Dorsal and Ventral Medial Prefrontal Cortex Inactivation during Natural Reward Seeking, Extinction, and Cue-Induced Reinstatement. *Eneuro*, *6*(5), ENEURO.0296-19.2019. https://doi.org/10.1523/ENEURO.0296-19.2019

2.1 Abstract

Rodent dorsal medial prefrontal cortex (mPFC), typically prelimbic cortex, is often described as promoting actions such as reward seeking, whereas ventral mPFC, typically infralimbic cortex, is thought to promote response inhibition. However, both dorsal and ventral mPFC are necessary for both expression and suppression of different behaviors, and each region may contribute to different functions depending on the specifics of the behavior tested. To better understand the roles of dorsal and ventral mPFC in motivated behavior we pharmacologically inactivated each area during operant fixed ratio 1 (FR1) seeking for a natural reward (sucrose), extinction, cue-induced reinstatement, and progressive ratio sucrose seeking in male Long-Evans rats. Bilateral inactivation of dorsal mPFC, but not ventral mPFC increased reward seeking during FR1. Inactivation of both dorsal and ventral mPFC decreased seeking during extinction. Bilateral inactivation of ventral mPFC, but not dorsal mPFC decreased reward seeking during cue-induced reinstatement. No effect of inactivation was found during progressive

ratio. Our data contrast sharply with observations seen during drug seeking and fear conditioning, indicating that previously established roles of dorsal mPFC = going vs. ventral mPFC = stopping are not applicable to all motivated behaviors and/or outcomes. Our results indicate that dichotomous functions of dorsal vs. ventral mPFC, if they exist, may align better with other models, or may require the development of a new framework in which these multifaceted brain areas play different roles in action control depending on the behavioral context in which they are engaged.

2.2 Introduction

The rodent medial prefrontal cortex (mPFC) plays a key role in numerous behaviors and cognitive functions, including action control, emotional regulation, attention, memory, and decision-making, among others (Barker et al., 2014; Cassaday et al., 2014; Dalley et al., 2004; Eichenbaum, 2017; Euston et al., 2012; Ko, 2017; Moorman & Aston-Jones, 2015; Vertes, 2006). Multiple studies have demonstrated that dorsal mPFC (typically prelimbic cortex) and ventral mPFC (typically infralimbic cortex) have opposing roles that facilitate the execution and inhibition, respectively, of behaviors (Gass & Chandler, 2013; Gourley & Taylor, 2016; Peters et al., 2009). These differences have been observed during drug seeking, fear-associated behaviors, and certain studies of natural reward seeking. For example, dorsal mPFC inactivation reduces reinstatement of drugs of abuse such as cocaine or heroin (Fuchs et al., 2005; LaLumiere & Kalivas, 2008; McFarland & Kalivas, 2001; McLaughlin & See, 2003). In contrast, ventral mPFC inactivation increases cocaine seeking during extinction, and activation of ventral mPFC decreases reinstatement of cocaine and other drugs of abuse (LaLumiere & Kalivas,

2008; Muller Ewald & LaLumiere, 2018; Peters, Vallone, et al., 2008). In studies of auditory fear conditioning and extinction, dorsal mPFC inactivation decreases fear expression and ventral mPFC inactivation impairs extinction learning and recall (Maren & Quirk, 2004; Peters et al., 2009; Sierra-Mercado et al., 2011). Dorsal and ventral mPFC may also have opposing roles with respect to natural reward seeking: inactivation of dorsal and ventral mPFC decreases and increases in reward seeking, respectively, in certain behavioral paradigms (Eddy et al., 2015; Ishikawa et al., 2008a, 2008b; Rhodes & Killcross, 2007, 2004; Sangha et al., 2014; Trask et al., 2017). However, these dorsal vs. ventral dichotomies are not always observed, and in some cases opposing functions have been described (Moorman et al., 2015). For example, inhibition of dorsal mPFC in models of cocaine dependence result in increased punishment-resistant drug seeking (Chen et al., 2013). Some studies have found an effect of mPFC manipulation on cocaine, but not natural reward seeking (Gutman, Nett, et al., 2017; McFarland & Kalivas, 2001; McGlinchey et al., 2016). In a discriminative stimulus-driven reward seeking task, both dorsal and ventral mPFC neurons fired during reward seeking and extinction, and inactivation of dorsal or ventral mPFC did not result in specific deficits in execution and extinction of reward seeking (Moorman & Aston-Jones, 2015). In a variable interval sucrose seeking task, dorsal mPFC neurons fired during reward delivery and inactivating this region did not alter reward seeking, whereas ventral mPFC neurons fired during reward collection and inactivating the ventral mPFC delayed the collection of reward (Burgos-Robles et al., 2013). Dorsal mPFC has also been associated with goal directed behaviors, attention, or spatial location representation, and ventral mPFC has been associated with habitual behaviors and emotional regulation, among multiple other

functions (Cassaday et al., 2014; Dalley et al., 2004; Euston et al., 2012; Gourley & Taylor, 2016; Killcross & Coutureau, 2003; K. S. Smith et al., 2012; K. S. Smith & Graybiel, 2013). This diversity of results indicates not only that these areas play complex roles in shaping behavior, but also that there may be differences depending on the tasks used to probe mPFC function. Surprisingly, there has been limited characterization of dorsal vs. ventral mPFC contributions to self-initiated instrumental reward seeking and, analogous to described models of drug seeking, extinction and reinstatement. Here we used pharmacological inactivation to characterize the roles of mPFC subregions during these tasks and during a progressive ratio task to assess motivation. We also performed a preliminary assessment of whether or not individual mPFC hemispheres differentially regulate reward seeking, as seen in other behaviors (Sullivan & Gratton, 2002a, 2002b) and we performed physiological and behavioral controls to verify the effects of our pharmacological manipulations. Despite observing differential effects of dorsal vs. ventral mPFC inactivation on reward seeking, our findings do not align with previous observations of go/stop dichotomies. Instead they indicate that these brain areas likely perform multiple functions, befitting their complex integrative nature, and that behavioral context, such as the task employed, dictates the contributions of these regions to the behaviors studied.

2.3 Materials and Methods

2.3.1 Animals

Male Long-Evans rats (~9 weeks old and 275-300g upon arrival; Charles River; N = 80) were used in behavioral studies (sucrose self-administration N = 40; extinction N = 16; cue-induced reinstatement progressive ratio N = 16; spontaneous locomotion, N = 8). Two additional male Long Evans rats were used for in vitro electrophysiology studies (see below for details). All rats were single-housed on a reversed light cycle (7:00am on and 7:00pm off) and allowed free access to food and water. Experiments were conducted during active cycle (lights off). All animal procedures were performed in accordance with the University of Massachusetts Amherst animal care committee's regulations.

2.3.2 Surgery

Rats were anesthetized with isoflurane in a closed container (5%) and transferred to a stereotaxic frame where they received isoflurane through a nosecone (1.5%-2%). Rats were given systemic antibiotic (0.1 mL cefazolin) and analgesic (1mg/kg meloxicam), and incisions were treated with local anesthetic (0.3mL, 2% lidocane). Bilateral craniotomies were made above the mPFC, and double guide cannulae (26 gauge, Plastics One, Roanoke, VA) were implanted in either dorsal mPFC (+3.0 mm AP; +/- 0.6 mm ML; -2.5 mm DV) or ventral mPFC (+3.0 mm AP; +/-0.6 mm ML; -4.0 mm DV). Three screws were implanted to secure cannulae with dental cement. Rats were allowed 1 week to recover following surgery. Rats tested in the spontaneous locomotor assay (see below) received comparable surgeries, but bilateral guide cannulae were

implanted in the shell/core border of the nucleus accumbens (NAc; +1.5 mm AP; +/-1.2 mm ML; -5.4 mm DV).

2.3.3 Baclofen/Muscimol Infusions

Rats were bilaterally injected with 0.3 μL of either artificial cerebrospinal fluid (aCSF) or a 1.0 nmol/0.1 nmol mixture of the GABA-A and -B receptor agonists baclofen and muscimol (BM; Tocris Bioscience, Avonmouth, Bristol, UK) dissolved in aCSF. Injection cannulae (33 gauge, Plastics One) were inserted bilaterally and protruded 1mm below the guide cannulae. Solutions were delivered over the course of 1 minute using a microinfusion pump (UMP3/Micro 4, World Precision Instruments, Sarasota, FL), and the injection cannulae were maintained in place for an extra minute to allow diffusion of the fluid. For the NAc locomotion task, injection cannulae extended 2mm beyond guide cannulae. Rats were tested at least 5 minutes after the injection cannulae were removed.

2.3.4 Apparatus

All operant testing was conducted in Med Associates chambers housed in sound attenuation cubicles (Med Associates, Fairfax, VT). Nose pokes were located on the left and right walls of the operant chambers. Beneath the right nose poke was a well where reward (0.1 ml of 15% sucrose solution) was dispensed. Each chamber was illuminated by a house light, and a fan provided approximately 60 dBA background noise. The same boxes were used for extinction, cue-induced reinstatement, and PR experiments, although the inactive nose poke was inaccessible during extinction sessions. For the NAc

locomotion experiments, rats were placed in a plexiglass chamber (39.4x 39.4 x 52.1 cm) with black colored walls and a stainless-steel grid floor. A digital camcorder (Canon VIXIA HF R52) was mounted above the box to record locomotor activity.

2.3.5 Behavioral test groups

Three operant test groups were used in these studies. The first group received inactivation during FR1 sucrose seeking. The second group received inactivation during early and late extinction. The third group received inactivation during cue-induced reinstatement and progressive ratio sessions. The FR1 group received bilateral and unilateral inactivation. Because no major effects were found with unilateral inactivation, the extinction and cue-induced reinstatement/progressive ratio groups received only bilateral inactivation. The FR1 group also received inactivation during extinction, cue-induced reinstatement, and progressive ratio. In this group, we observed no significant effects of manipulation in any of these tests, leading us to consider the possibility that multiple infusions during self-administration resulted in long-lasting damage occluding any potential effects of regional inactivation. Thus, separate groups were run for extinction and cue-induced reinstatement/progressive ratio sessions. Details on testing are below.

2.3.6 Sucrose self-administration

Before surgery, rats were trained to self-administer sucrose on a fixed-ratio 1 (FR1) schedule. A 10-15 sec house light illumination signaled the time-out, during which nose poking in the left (inactive) and right (active) nose pokes were recorded but did not

elicit any consequences. Upon house light offset, nose poking in the right nose poke elicited a tone (15 kHz, 74 dBA, 1 sec) and delivery of 0.1 ml 15% sucrose in the well beneath the nose poke. The first active poke after the time-out was counted as a "trial initiation" to distinguish these pokes from other (e.g., time-out) active nose pokes. Trials in which the rat exited the nose poke and entered the well in less than 1 sec after sucrose was dispensed were counted as "rewarded well-entries". Surgeries were performed after rats reached at least 100 rewarded well-entries and met criteria of 80% rewards collected within 1 sec of delivery. After recovery, rats were retrained for 3 to 10 days (Figure 2.1C). After re-training, rats received a sham infusion in which the injector cannula was inserted and left in place for one minute, but nothing was infused. Testing started the following day. Rats were tested on an FR1 schedule for eight days in total after sham infusion test day. Sessions lasted one hour or until the rat performed 160 trials. During testing, each rat received four separate infusions in counterbalanced order across days: 1) bilateral BM, 2) bilateral aCSF, 3) BM in left hemisphere and 4) aCSF in the right hemisphere, and aCSF in the right hemisphere and BM in the left hemisphere (Figure 2.1C). In between infusion days, rats were run on FR1 with no infusion in order to avoid potential rebound effects and to maintain task performance.

2.3.7 Extinction

A second cohort of rats was trained to reliably respond for sucrose under the FR1 schedule described above (Figure 2.2A). After stable FR1 performance (100 rewarded well-entries and 80% rewards collected within 1 sec), rats received inactivation tests during early and late extinction sessions (Figure 2.2B). Rats received one of two

conditions on the first day of early extinction BM or aCSF). They were then retrained on FR1 for two days, and received a second day of early extinction during infusion with the opposite drug or vehicle combination. We included two days of FR1 retraining in between each early extinction day in order to allow paired analysis of early extinction within rats. Rats were then extinguished until they responded with fewer than 20 nose pokes per session for two continuous sessions. On the last two days of extinction (late extinction) rats received counterbalanced BM/aCSF treatments as in early extinction.

2.3.8 Cue-Induced Reinstatement

A third cohort of rats was trained to reliably respond for sucrose under the FR1 schedule described above and then extinguished to the point of responding with fewer than 20 nose pokes per session for two continuous sessions (Figure 2.3B). Rats were then tested in cue-induced reinstatement sessions. During reinstatement, nose pokes on an FR1 schedule elicited a tone but no sucrose delivery. Rats were bilaterally infused with either BM or aCSF on two separate reinstatement days in a counterbalanced fashion. Reinstatement tests were separated by extinction sessions until rats reached criteria of two sessions with fewer than 20 nose pokes.

2.3.9 Progressive ratio

After cue-induced reinstatement, the same rats that were tested on reinstatement were tested on a progressive ratio (PR) sucrose seeking task. The PR test environment was the same as for FR1, but the number of nose pokes required to receive reward increased on each trial based on the equation: Response ratio (rounded to the nearest

integer) = [5e $^{(\text{injection number x } 0.2)}$] – 5 (Richardson & Roberts, 1996). The highest reward number acquired was considered the breakpoint and was analyzed, along with nose pokes and well entries, as a measure of motivation. Rats were bilaterally infused with BM and aCSF prior to testing on separate PR testing days. PR testing lasted either six hours or until 60 minutes of no nose pokes occurred. PR test days were separated by two consecutive days of FR1 training.

2.3.10 Spontaneous Locomotion

In order to verify the behavioral effects of BM, we tested the effect of NAc inactivation during a spontaneous locomotor assay. Methods were based on those described previously (Fuchs et al., 2004). A new cohort of rats was infused with either BM or aCSF in NAc and placed into a novel box 10 minutes after the infusion (Figure 2.6). Behavior was video recorded for one hour and later analyzed using ANY-maze software (ANY-maze, Wood Dale, IL), in which we divided the chamber in 8 zones and counted numbers of line crosses into each zone.

2.3.11 Whole-Cell Patch-Clamp

To verify the physiological effects of BM, we recorded the activity of mPFC neurons in vitro during bath application of BM. Seven neurons from two male Long-Evans rats, approximately 25 days old, were included in this analysis. Rats were deeply anesthetized with isoflurane and sacrificed using rapid decapitation, and brains were removed and immersed in ice-cold cutting solution (in mM: 250 Glycerol, 26 NaHCO3, 2.5 KCl, 1.2 NaH2PO4, 11 Glucose, 2.4 CaCl2, and 1.2 MgCl2; 310 mOsms; pH = 7.4

when saturated with 95% O2/5% CO2). 300 μm coronal sections were obtained using a vibrating blade microtome (VT1000S, Leica Biosystems Inc., Buffalo Grove, IL), and were immediately transferred to artificial cerebral spinal fluid (aCSF; 37°C; in mM: 250 Glycerol, 26 NaHCO3, 2.5 KCl, 1.2 NaH2PO4, 11 Glucose, 2.4 CaCl2, and 1.2 MgCl2; 310 mOsms; pH = 7.4 when saturated with 95% O2/5% CO2). After 30 minutes under these conditions, slices were kept in bubbled aCSF at room temperature for the remainder of the experiment. Glass pipettes were pulled from borosilicate glass tubes (1B150F-4, World Precision Instruments, Sarasota, FL) using a two-stage, vertical puller (PC-10, Narishige International USA, East Meadow, NY), and were backfilled with internal solution (in mM: 110 K-Gluconate, 8 NaCl, 30 KCl, 1 MgCl2, 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 0.5 GTP; 298 mOsms; pH = 7.4). When filled, pipettes had a tip resistance of 5-8 MΩ. Once whole-cell configuration was achieved, cells were allowed to stabilize for at least 5 minutes before recordings proceeded. Spontaneous post-synaptic currents (sPSCs) were recorded in voltage clamp mode from pyramidal neurons held at -70 mV in the medial wall of the prefrontal cortex. Recordings were taken before (range: 3-11 min), during (range: 3-13 min), and after (range: 4-30 min) application of BM. Series resistance was monitored throughout the recordings. Recordings were concatenated offline in Igor Pro (Wavemetrics, Lake Oswego, OR) to create one contiguous file, which was then exported to Spike2 (Cambridge Electronic Design Limited, Science Park, Cambridge, UK) where it was low-pass filtered above 100 Hz. Timestamps were obtained in Spike2 through waveform-based template matching. For both the pre-treatment and treatment segments, the length of each recording was standardized to that of the shortest recording

by exclusively including the last 3 minutes, and PSC frequency was tabulated for three minute periods before, during, and after BM treatment.

2.3.12 Histology

After final test sessions, rats were deeply anesthetized with Ketamine/Xylazine (80 mg/kg: 10 mg/kg i.p.), and brains were extracted, stored in 4% paraformaldehyde overnight, and transferred to 20% (wt/vol) solution of sucrose/0.1% sodium azide in phosphate buffer at 4 °C. Coronal sections 40 μm thick were cut using a cryostat, mounted on slides, stained with neutral red and cover slipped. Cannula placements were verified by comparing cannula damage to a rat brain atlas (Paxinos & Watson, 2007). Two ventral mPFC rats in the FR1 group, one ventral mPFC rat in the extinction group, and one dorsal and one ventral mPFC rat in the reinstatement group were excluded from analysis due to blocked cannulae or excessive tissue damage. Two rats were excluded from the locomotion task because of cannula misplacements. Cannula placements are shown in Figures 1-3 for rats in operant testing groups and in Figure 2.6 for rats in spontaneous locomotor tests.

2.3.13 Analysis

Data were analyzed using Prism (GraphPad Software, La Jolla, CA). Total numbers and rates (total number divided by the time taken to complete the task) of active and inactive nose pokes and well entries for the FR1 task were calculated and differences were assessed using one-way repeated measure (RM) ANOVA followed by planned Dunnett's test for multiple comparisons to compare each treatment to bilateral aCSF. In

addition to number of responses, we also measured response rate during FR1 as some rats finished the task before the one hour of duration of the task. Total numbers of nose pokes and well entries for extinction, cue-induced reinstatement, and progressive ratio data were analyzed using one-way ANOVA and paired t-tests. Number of nose pokes during FR1, early extinction, late extinction, and cue-induced reinstatement were divided into quartiles and data were analyzed using paired two-way ANOVA (treatment x quartile). Locomotion was analyzed using a two-way ANOVA comparing an interaction between 10-minute bins of time and infusion condition. Simple effects for locomotion data, as well as patch clamp data were analyzed using a one-way RM ANOVA. Means and standard error of the mean were presented as (mean ± SEM).

2.4 Results

2.4.1 Dorsal, but not ventral mPFC inactivation increased reward seeking during FR1 sucrose self-administration

All rats were highly motivated to perform the FR1 sucrose seeking task (Figure 2. 1). Bilateral inactivation of dorsal mPFC significantly increased nose poking and well entry activity (Figure 2.1D, E). RM ANOVA did not reveal significant differences among groups for number of nose pokes ($F(3,19) = 2.37$, $p=0.08$). However, planned Dunnet's tests revealed an increase in total number of nose pokes when dorsal mPFC was bilaterally inactivated (Figure 2A; $p<0.05$, Dunnett's). Bilateral inactivation also increased overall rate of nose pokes ($F(3,19=2.76$, $p= 0.050$, RM ANOVA across all manipulations; $p<0.05$, Dunnett's for bilateral BM vs bilateral aCSF), and in rate of time

out nose pokes ($F(3,19)=2.31$, $p=0.086$, RM ANOVA; $p<0.05$ Dunnett's). Bilateral dorsal mPFC inactivation increased number of rewarded well entries, defined as entering the well in less than 1 second after sucrose was dispensed, compared to aCSF (Figure 2.1E; $F(3,19)=2.40$, $p=0.077$, RM ANOVA; $p<0.05$ Dunnett's). We also observed a significant increase in the number of initiated trials ($F(3,19=3.13$, $p=0.033$), but Dunnett's tests did not reveal any significant differences compared to bilateral aCSF ($p>0.05$). Unilateral inactivation of dorsal mPFC had no significant effect on numbers or rate of nose pokes or well entries (all $p>0.05$, Dunnett's). Ventral mPFC inactivation, bilateral or unilateral, had no significant effects on number or rate of nose pokes or well entries (Figure 2.1F, G; all $p>0.05$, RM ANOVA and Dunnett's). There were also no effects of inactivation on latency to initiate trials or collect reward after dorsal or ventral mPFC inactivation (all $p>0.05$, RM ANOVA and Dunnett's). Inactive nose poke responses were low and there were no effects of manipulation on inactive responses (range means 1.6 to 5.3, all $p>0.05$, RM ANOVA and Dunnett's)

2.4.2 Dorsal and ventral mPFC inactivation decreased reward seeking during extinction

Fifteen rats received bilateral inactivation of dorsal (n = 8) or ventral (n = 7) mPFC during early (days 1 and 2) and late (2 days of <20 nose pokes) extinction sessions (Figure 2.2). There were no effects of inactivation of dorsal or ventral mPFC during early extinction. However, inactivation of dorsal mPFC significantly reduced both nose pokes ($t(7) = 4.00$, $p=0.0052$) and well entries ($t(7) = 2.38$, $p=0.049$) (Figure 2.2E, F). Inactivation of ventral mPFC significantly decreased well entries ($t(6) = 2.86$, $p=0.029$)

(Figure 2.2J) and, although it appeared that nose pokes were reduced (Figure 2.2I), this effect was not significant ($t(6) = 1.01$, $p=0.35$).

2.4.3 Ventral, but not dorsal mPFC inactivation decreased reward seeking during cue-induced reinstatement

After aCSF treatment on cue-induced reinstatement tests, rats exhibited a significantly increased number of nose pokes compared to the last day of extinction (dorsal mPFC: Figure 2.3D; $t(6)=3.44$, $p=0.014$; ventral mPFC: Figure 2.3I; $t(6)=3.88$, $p=0.008$, paired t-test). Bilateral inactivation of ventral mPFC significantly decreased total number of reinstatement nose pokes (Figure 2.3I; $t(6)=3.05$, $p=0.023$, paired t-test) relative to aCSF treatment. There was also a decrease in number of time-out nose pokes (Figure 2.3J; $t(6)=2.57$, $p=0.042$; paired t-test) and number of initiated trials (Figure 2.3K; $t(6)=3.71$, $p=0.010$). There were no significant effects of bilateral inactivation of dorsal mPFC on nose pokes or well entries (Figure 2.3D-G; all $p>0.05$, paired t-test). There were also no significant effects of either dorsal or ventral mPFC inactivation on inactive nose pokes (all $p>0.05$, paired t-test). Of note the effects on ventral mPFC inactivation observed here were directionally consistent with those observed during reinstatement in our first test group (see Methods). Although the effects in that group were milder and not significant (likely due to 8 prior cannula infusions), the directional consistency across study groups combined with the significant effects observed here strongly supports the reliability of these findings.

2.4.4 Neither dorsal or ventral mPFC inactivation affected reward seeking during progressive ratio sucrose self-administration

Rats demonstrated reliably high levels of sucrose seeking during progressive ratio as measured by nose pokes, well entries, and breakpoints (Figure 2.4). There was no effect of either dorsal or ventral mPFC inactivation on numbers of total active nose pokes, initiated trials, time-out nose pokes, well entries, breakpoint values, or inactive nose pokes (all $p>0.05$, paired t-tests).

2.4.5 Within-session analysis of inactivation effects

One possible outcome of inactivation may have been a transient effect during part of the session that was not overall apparent by comparing total numbers of nose pokes (e.g., effects only early or late during a session). To address this, we divided sessions into four quartiles and compared nose poking during BM vs. aCSF sessions using a repeated measures two-factor ANOVA (treatment x quartile). The results of these analyses are shown in Figure 2.5 for FR1, early and late extinction, and cue-induced reinstatement. Analyses were performed for progressive ratio as well, but there were no significant effects either overall or within sessions. As expected there were overall significant main effects of treatment for dorsal mPFC inactivation during FR1 ($F(1, 76)=7.71$, $p=0.007$) and late extinction ($F(1, 28)=9.27$, $p=0.005$). Post-hoc multiple comparisons (Sidak's MCT) revealed significant differences during the second quartile during FR1 ($t=3.11$, $p=0.011$) and during the first quartile during late extinction ($t=2.97$, $p=0.024$). Despite a significant main effect of treatment after ventral mPFC inactivation during cue-induced reinstatement ($F(1, 24)=5.22$, $p=0.03$), there were no significant

treatment effects in any quartile, indicating consistent small reductions throughout the entire session. There were no effects of treatment on nose poking behavior in any of the other analyzed sessions and no interaction effects.

2.4.6 Baclofen/muscimol infusions into the NAc disrupted spontaneous locomotion

Because mPFC inactivation results were unexpected compared to previous studies, we verified the effect of our BM infusions by inactivating NAc during spontaneous locomotion - a reliable behavioral assay that is sensitive to BM inactivation of NAc (Fuchs et al., 2004; Stopper and Floresco, 2011). We infused BM or aCSF bilaterally in NAc (Figure 2.6A) and measured locomotor activity in 10 minute bins (Figure 2.6B). As expected, there was a statistically significant interaction between the effects of drug and time on locomotion, (Figure 2.6B; $F(5, 24) = 3.35$, $p = 0.020$; two-way ANOVA). Locomotion was initially elevated and decreased over time in aCSF-infused rats ($F(5,2)=6.99$, $p=0.005$; one-way ANOVA). BM-infused rats showed decreased locomotion during the initial stages of testing relative to aCSF and did not show a significant difference in locomotion over time ($F(5,2)=0.22$, $p=0.947$; one-way ANOVA). These results are consistent with previous findings (Fuchs et al., 2004; Stopper & Floresco, 2011), and confirmed that differences observed between our mPFC inactivation effects and those described in previous studies were not due to lack of efficacy of our BM infusions.

2.4.7 Baclofen/Muscimol decreased sPSCs in rat prefrontal neurons

To further validate the inhibitory influence of our BM infusions at the specific concentrations given, we measured the effects of BM application on mPFC neuronal activity *in vitro*. BM bath application significantly decreased spontaneous activity in prefrontal neurons (Figure 2.6C, n = 7 neurons from 2 rats), as demonstrated by a statistically significant suppressive effect of BM on sPSCs (5b; $F(2,6)=5.6$, $p=0.0189$; one-way ANOVA). Post hoc analyses revealed a significant decrease in number of sPSCs during BM and during washout (Figure 2.6D; $p<0.05$; Tukey's Multiple Comparison Test). These results confirm the reliably inhibitory effect on mPFC neurons of the BM cocktail concentration used in our behavioral studies.

2.5 Discussion

Previous work has led to the hypothesis that dorsal and ventral mPFC play opposing roles in driving behavior, particularly in the context of action execution vs. suppression (Barker et al., 2014; Gass & Chandler, 2013; Gourley & Taylor, 2016; Muller Ewald & LaLumiere, 2018; Peters et al., 2009). The reasons for this distinction are relatively clear, as described in multiple studies referenced in detail in (Gourley & Taylor, 2016; Moorman et al., 2015; Muller Ewald & LaLumiere, 2018; Peters et al., 2009). For example, manipulation of dorsal mPFC frequently disrupts behavioral execution such as drug/reward seeking or expression of conditioned fear (Eddy et al., 2015; Ishikawa et al., 2008b; McFarland et al., 2004; Sierra-Mercado et al., 2011; Trask et al., 2017). In contrast, ventral mPFC manipulation has been shown to regulate behavioral inhibition in certain circumstances, such as during extinction (Augur et al.,

2016; Ishikawa et al., 2008b; Muller Ewald & LaLumiere, 2018; Peters, Vallone, et al., 2008; Peters & De Vries, 2013; Sierra-Mercado et al., 2011). However, a number of studies have called the ubiquity of this dichotomy into question (Bossert et al., 2011; Chen et al., 2013; Gutman, Ewald, et al., 2017; Jonkman et al., 2009; Martín-García et al., 2014; McFarland et al., 2003; McGlinchey et al., 2016; Moorman et al., 2015; Moorman & Aston-Jones, 2015; Willcocks & McNally, 2013), prompting us to perform the experiments described here.

Our results do not support a clear dichotomy for dorsal vs. ventral mPFC during natural reward seeking. Based on the studies described above, we expected that inactivation of dorsal mPFC would decrease sucrose seeking and have no effect on extinction, and that ventral mPFC inactivation would increase sucrose seeking and induce cue-induced reinstatement during extinction. Instead, dorsal mPFC inactivation increased sucrose seeking during FR1 self-administration and had no effect during cue-induced reinstatement. Ventral mPFC inactivation decreased sucrose seeking during cue-induced reinstatement and had no effect during FR1. Inactivation of both subregions decreased responding during late extinction, as shown by significantly reduced nose pokes and well-entries after dorsal mPFC inactivation and significantly reduced well entries after ventral mPFC inactivation. Inhibition of neither region influenced reward seeking under a progressive ratio schedule, again in line with a lack of general regulation of action execution or suppression. Together these results make a strong case against a universal dichotomous role for dorsal vs. ventral mPFC in action execution vs. inhibition.

Because our results were somewhat surprising, we performed controls to verify that our inactivations were effective. NAc inactivation with BM decreased spontaneous

locomotion, in line with previous work (Fuchs et al., 2004; Stopper & Floresco, 2011), and bath application of BM inhibited spontaneous activity in rat mPFC neurons. Both findings support the efficacy of our BM treatments. We conclude that the effects observed did in fact result from mPFC inactivation during behavior.

The absence of absolute differences is in line with some previous work examining dorsal vs. ventral mPFC in execution vs. suppression of reward seeking, as described above. However, in many of these studies, the tasks employed used slightly more complex rules to guide behavior such as the use of a discriminative stimulus (Gutman, Ewald, et al., 2017; Ishikawa et al., 2008b; Moorman & Aston-Jones, 2015). The goal of this study was to attempt to isolate self-initiated action execution or inhibition to identify mPFC subregion contributions, in line with those seen in studies of drug seeking. If, in fact, dorsal and ventral mPFC play opposing roles in the regulation of action execution and inhibition, this should have been clearly demonstrable under the behavioral conditions in the current study. Instead, our data argue for an influence of context, in this case the behavioral task performed, on mPFC regulation of behavior, as reported previously (McGlinchey et al., 2016; Moorman & Aston-Jones, 2015). Similarly complex results have been observed in Pavlovian contexts (Mendoza et al., 2015; Sangha et al., 2014).

An additional finding was an overall lack of effect of unilateral inactivation on sucrose seeking. Previous studies have shown differential contributions of left vs. right mPFC in stress-related paradigms (Sullivan & Gratton, 2002b), leading us to consider the possibility that left or right mPFC may play a disproportionate role in reward seeking. Although the only significant effect during FR1 was seen after bilateral dorsal mPFC

inactivation, right hemisphere dorsal mPFC inhibition produced qualitatively similar results in some cases, though the effects were not significant in planned comparisons. Accordingly, we did not pursue unilateral inactivations in cue-induced reinstatement or progressive ratio. Despite our overall lack of lateralization findings, a study more directly designed to explore this question may be worth undertaking in future work.

One possible distinction between our results and some previous studies is the type of behavior used to evaluate mPFC control. It might not be surprising that studies using different behaviors may result in different effects of mPFC inactivation. This is most obvious for fear conditioning studies, where the behavioral readout is actually freezing – a combination of both an emitted behavior (based on a decision to freeze) and a lack of action (freezing), in some cases combined with a suppression of food self-administration (Giustino & Maren, 2015; Sierra-Mercado et al., 2011). A more subtle distinction is between the use of nose poke operanda, as employed here and in some studies (Willcocks & McNally, 2013), and the use of lever presses in other previous studies (Ishikawa et al., 2008b; Peters, Vallone, et al., 2008). Although this may not be a critical determinant, there are differential learning rates between nose poke and lever presses (Schindler et al., 1993), and different neural substrates underlying the two behaviors (Bassareo et al., 2015). This influence of action type on mPFC contributions to behavior is currently under investigation in our laboratory.

The most salient differences exist between our findings and previous studies of cocaine self-administration, extinction, and reinstatement. Multiple studies have shown a prominent role for dorsal mPFC in driving cue-induced reinstatement of cocaine seeking as well as a critical role for ventral mPFC suppressing cocaine seeking after extinction

learning (Fuchs et al., 2005; Gass & Chandler, 2013; Gourley & Taylor, 2016; LaLumiere & Kalivas, 2008; McFarland & Kalivas, 2001; McLaughlin & See, 2003; Moorman et al., 2015; Muller Ewald & LaLumiere, 2018; Peters et al., 2009; Peters, Vallone, et al., 2008), though see counterexamples such as (Chen et al., 2013) and others described in (Moorman et al., 2015). A fundamental and yet-unanswered question is why these reliable roles for dorsal and ventral mPFC in regulation of cocaine-associated actions are not observed in sucrose seeking, as described here, or in other types of reward seeking (Gutman, Ewald, et al., 2017; McFarland & Kalivas, 2001; McGlinchey et al., 2016). One possibility might be the nature of the reinforcer. Cocaine may be a more salient reinforcer than sucrose, thereby differentially engaging mPFC subregions based on some motivational intensity gradient, though see (Lenoir et al., 2007). Another possible explanation is that repeated cocaine induces neuroplastic changes in the mPFC that results in differential regulation of seeking behavior relative to natural rewards (McFarland et al., 2003; Muñoz-Cuevas et al., 2013; Radley et al., 2015; Robinson et al., 2001; Robinson & Kolb, 1999; Siemsen et al., 2019). Cocaine also induces both appetitive and aversive behaviors (Ettenberg, 2004), whereas there are fewer aversive components to sucrose. mPFC subregions may regulate behaviors associated with a mixed-valence pharmacological stimulus differently than a purely appetitive reinforcer. Another potential explanation may be the way that reward is delivered: cocaine is typically self-administered intravenously whereas sucrose must be collected following a correct operant response. These and other potential explanations are currently under investigation in our laboratory, motivated by the very clear differences in mPFC contributions to ostensibly the same behavior related to different outcomes.

Rodent mPFC subregions play a host of functions instead of or in addition to action expression vs. inhibition (Cassaday et al., 2014; Dalley et al., 2004; Euston et al., 2012; Kesner & Churchwell, 2011). In some cases, dorsal and ventral mPFC functions have been shown to be dichotomous. For example, when comparing goal-directed (outcome sensitive) vs. habitual (outcome insensitive) reward seeking, there appear to be differences whereby dorsal mPFC preferentially regulates goal-directed and ventral mPFC controls habitual behaviors (Barker et al., 2014, 2015; Killcross & Coutureau, 2003; K. S. Smith et al., 2012; R. J. Smith & Laiks, 2018). Because we did not explicitly test goal-directed vs. habitual behavior using, e.g., reward devaluation, we cannot make strong claims about our effects in this framework, though this might be a useful avenue for future studies integrating mPFC functions across behavioral paradigms.

Despite not observing clear dichotomous dorsal and ventral mPFC functions, we did see selective effects of inactivation. Bilateral dorsal mPFC inactivation increased FR1 sucrose seeking. This finding is aligned with those demonstrating a response-suppression role for dorsal mPFC, such as is observed during punishment-associated cocaine seeking (Chen et al., 2013). It is also in line with previous work demonstrating increased operant behavior following dorsal mPFC inactivation (Jonkman et al., 2009) and other studies showing dorsal mPFC involvement in response inhibition in other tasks (Bari & Robbins, 2013; Hardung et al., 2017; MacLeod & Bucci, 2010; Meyer & Bucci, 2014; Narayanan et al., 2006; Ragozzino, 2007). Although in our study there was no need for dorsal mPFC to suppress behavior, reward-associated decisions, even without challenges such as punishment, may require balance between response inhibition driven by the effort associated with reward seeking vs. the excitatory drive to acquire a reward.

Here dorsal mPFC inactivation increased both rewarded and non-rewarded nose pokes. On the one hand, this suggests that dorsal mPFC inactivation resulted in a general release on any inhibition of behavior, or "taking the brakes off." However, it is worth noting that these increases were not seen for inactive nose pokes, during other non-rewarded tasks (extinction, reinstatement), or even during progressive ratio testing, in which rewards were available. In fact, dorsal mPFC inactivation decreased nose pokes in late extinction, when reward was not available. These results underscore the fact that behavioral context and task details influence contributions of mPFC to behavior – in some cases dorsal mPFC plays a response-invigorating role whereas in others it is suppressive.

Similarly, ventral mPFC is frequently associated with behavior suppression, particularly during extinction (Gourley & Taylor, 2016; Maren & Quirk, 2004; Muller Ewald & LaLumiere, 2018; Peters et al., 2009, 2009; Sierra-Mercado et al., 2011). In our study, ventral mPFC inactivation decreased cue-induced reinstatement, in line with previous studies of reinstatement for heroin (Bossert et al., 2011, 2012; Rogers et al., 2008) and methamphetamine (Rocha & Kalivas, 2010) seeking, but in contrast with previous studies of cocaine seeking and fear conditioning (LaLumiere & Kalivas, 2008; Muller & LaLumiere, 2018; Peters et al., 2008). Ventral mPFC inactivation also had little inhibitory effect on alcohol seeking and did not counteract extinction (Willcocks & McNally, 2013). It is unclear what differentiates ventral mPFC contributions to sucrose, alcohol, methamphetamine, and heroin reinstatement vs. extinction of cocaine and fear conditioning, though there are obviously substantial differences in neural encoding of different drugs/rewards/punishment, type of reinstatement (e.g., cue vs. context), or other as-yet undefined factors (Badiani et al., 2011; Peters et al., 2013).

In summary, our results make it clear that dorsal and ventral mPFC do not universally exhibit opposing control over behavior. Instead our findings should be integrated with previous work in which dichotomies were observed, along with other studies involving, e.g., response inhibition, in order to identify how different behavioral tasks differentially engage mPFC subregions. We also note that an emphasis on neuronal ensembles and networks should be emphasized in future work (Bossert et al., 2011; Gabbott et al., 2005; George & Hope, 2017; Kim et al., 2017; Moorman et al., 2015; Pfarr et al., 2015; Warren et al., 2016). It is possible that different findings across studies may result from differentially targeting subregional circuits (e.g., mPFC projections to NAc or amygdala). The use of circuit specific techniques and other precision-enhancing technologies, combined with a rigorous assessment of behavioral details, has the potential to significantly advance our understanding of mPFC function, including its contributions to complex behavior and mental diseases.

2.6 Figures

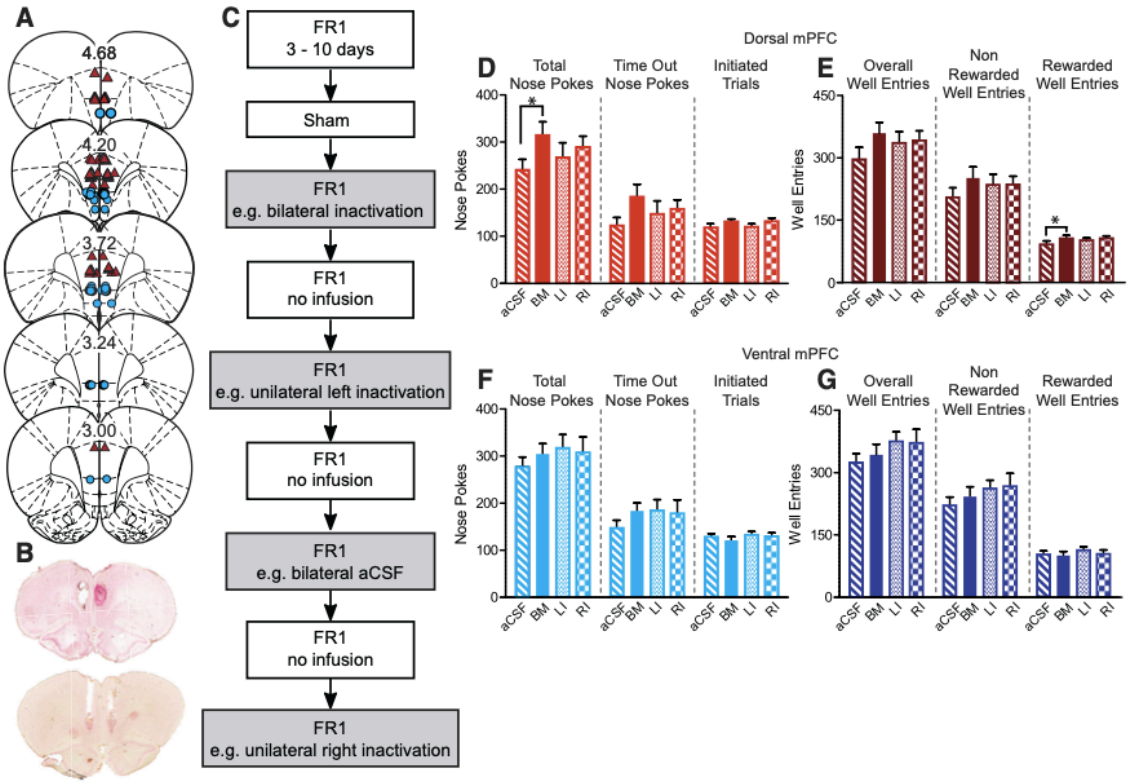


Figure 2.1. Cannula placements, test design, and FR1 data.

(A) Cannula placements for FR1 cohort. Dorsal mPFC cannula placements (triangles) and ventral mPFC cannula placements (circles). Numbers are A/P distance from bregma. (B) Histology of coronal slices stained with neutral red showing cannula tracks for dorsal (top) and ventral (bottom) mPFC. (C) Timeline for FR1 testing. Rats were retrained for 3 to 10 days after surgery. They then received sham infusions followed by 8 days of FR1 tests. Rats received one of four infusions every other day of testing: bilateral inactivation, bilateral aCSF, unilateral left, or right inactivation, counterbalanced across rats. All rats received all four conditions. aCSF (stripes) = control infusion, BI (solid) = bilateral inactivation, LI (dots) = inactivation of left hemisphere, RI (checkers) = inactivation of right hemisphere. (D, F) total number of nose pokes, time-out nose pokes, and initiated trials. (E, G) total number of well entries, non-rewarded well entries, and rewarded well entries. (D, E) There was a significant increase in total number of nose pokes and total number of rewarded well entries when the dorsal mPFC was bilaterally inactivated (*). (F, G) Ventral mPFC inactivation did not affect nose poking or well entries. *$p<0.05$, Dunnett's test for planned multiple comparison.

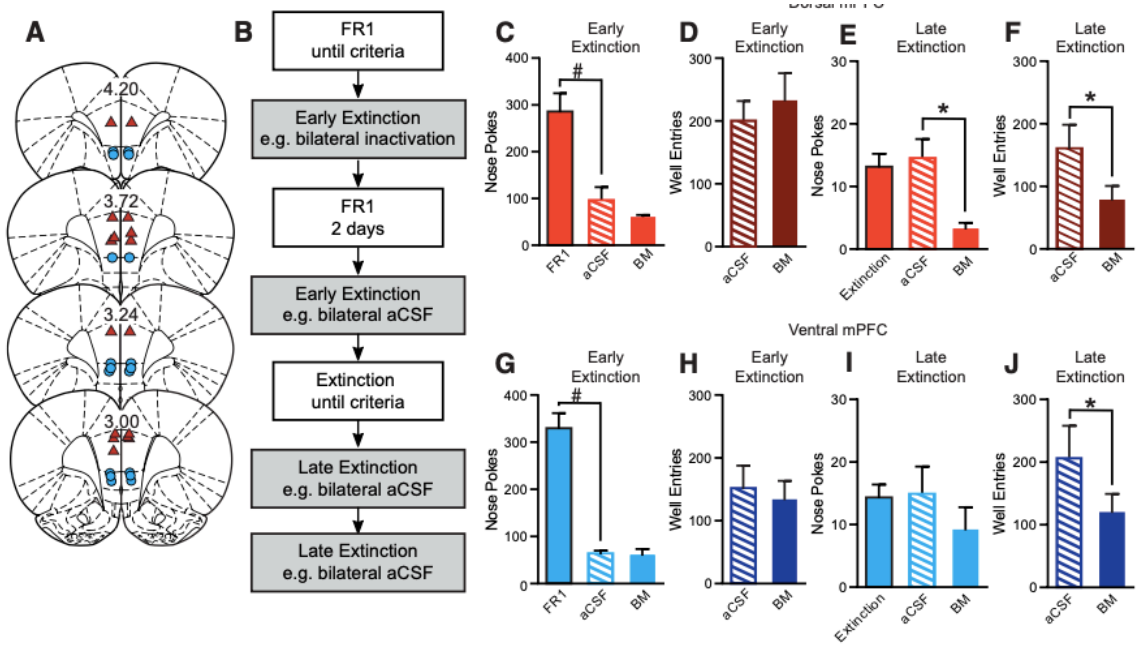


Figure 2.2 Cannula placements, test design, and extinction data for extinction cohort.

(A) Dorsal mPFC cannula placements (triangles) and ventral mPFC cannula placements (circles). (B) Timeline for extinction task. Extinction rats were trained on FR1 but only received bilateral infusions during early and late extinction. (C, G) There was a significant decrease in number of nose pokes between last day of FR1 and aCSF treatment during extinction (#). (C, D; G, H) Bilateral inactivation of dorsal or ventral mPFC did not significantly affect nose pokes or well entries during early extinction. (E, F) Inactivation of dorsal mPFC during late extinction decreased nose pokes and well entries (*). (I) There was no effect of ventral mPFC inactivation for number of nose pokes during late extinction. (J) However, there was a decrease in number of well entries during ventral mPFC inactivation during late extinction (*). # and *$p<0.05$, paired t-test.

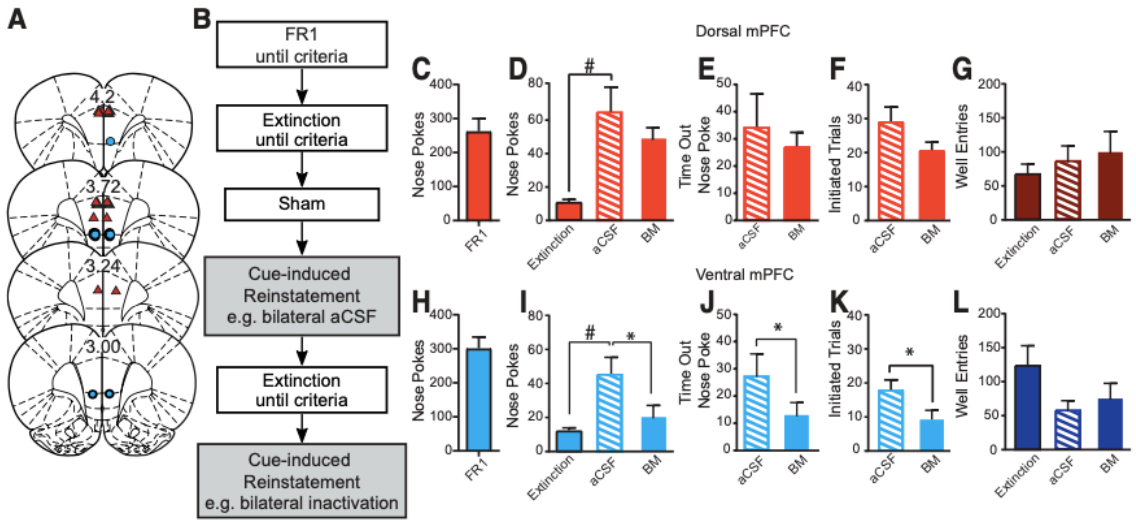


Figure 2.3. Cannula placements, test design, and reinstatement data for reinstatement cohort.

(A) Dorsal mPFC cannula placements (triangles) and ventral mPFC cannula placements (circles). (B) Timeline for reinstatement task. Reinstatement rats were trained on FR1 and extinction but only received bilateral infusion during reinstatement. (C, H) Number of nose pokes during FR1 session the day before extinction training. (D, I) There was a significant increase in nose pokes on aCSF reinstatement infusion day compared to last day of extinction (#). (D-G) Bilateral inactivation of dorsal mPFC did not significantly affect nose pokes, time-out nose pokes, initiated trials, or well entries. (I-L) Bilateral ventral mPFC inactivation significantly decreased total number of nose pokes, time out nose pokes, and initiated trials (*), but not rewarded well entries. # and *$p<0.05$, paired t-test.

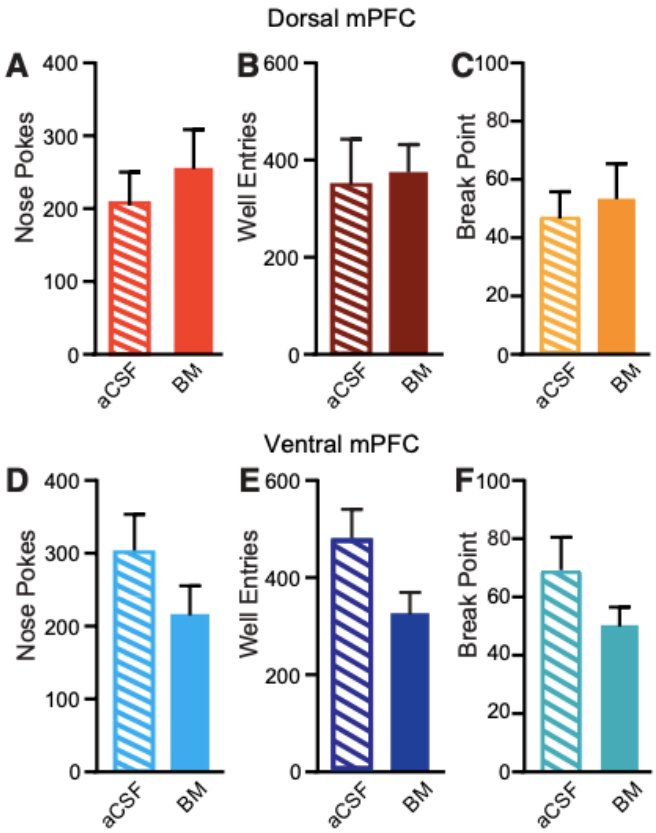


Figure 2.4. Progressive ratio data.

No significant effects of dorsal mPFC (A-C) or ventral mPFC (D-F) inactivation on nose pokes, well entries, or break point.

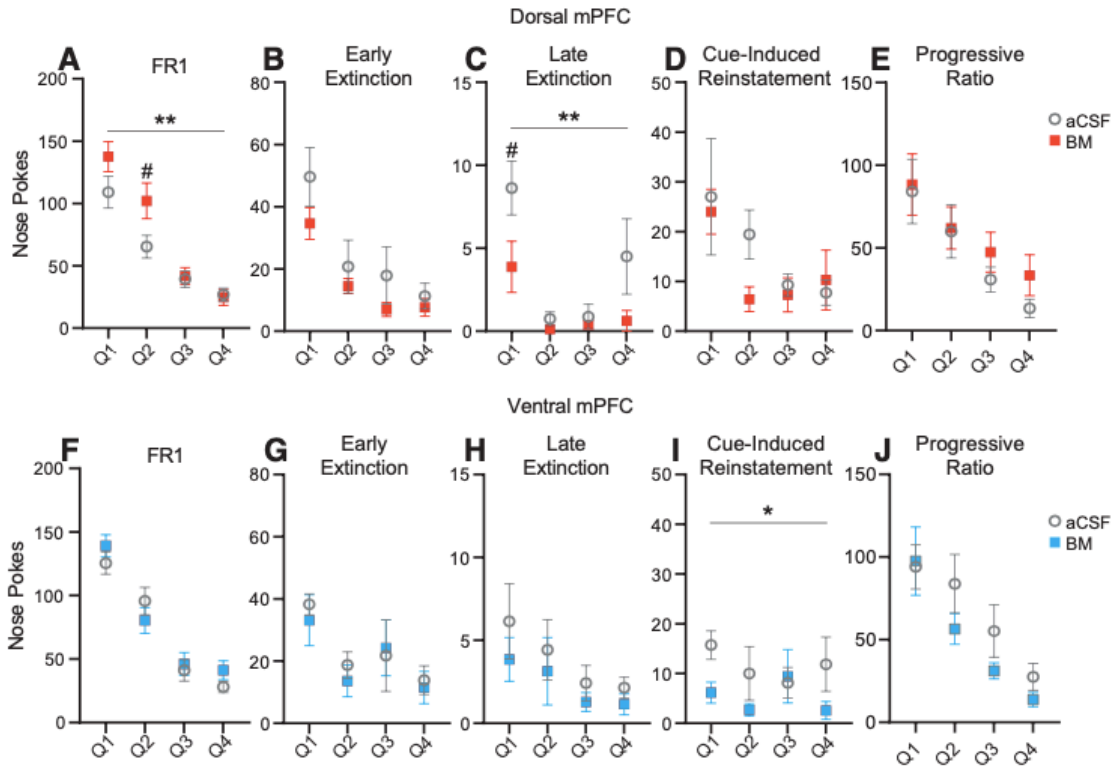


Figure 2.5. Average number of nose pokes per quartile for FR1, early extinction, late extinction, cue-induced reinstatement, and progressive ratio.

Dorsal mPFC inactivation increased FR1 nose pokes, notably in the first half of the session. Dorsal mPFC inactivation decreased late extinction nose pokes, primarily early in the session. Ventral mPFC inactivation decreased cue-induced reinstatement nose pokes, but the effect was distributed across the session. *p<0.05, **p<0.01, two factor ANOVA (treatment x quartile); #=p<0.05, Sidak's MCT.

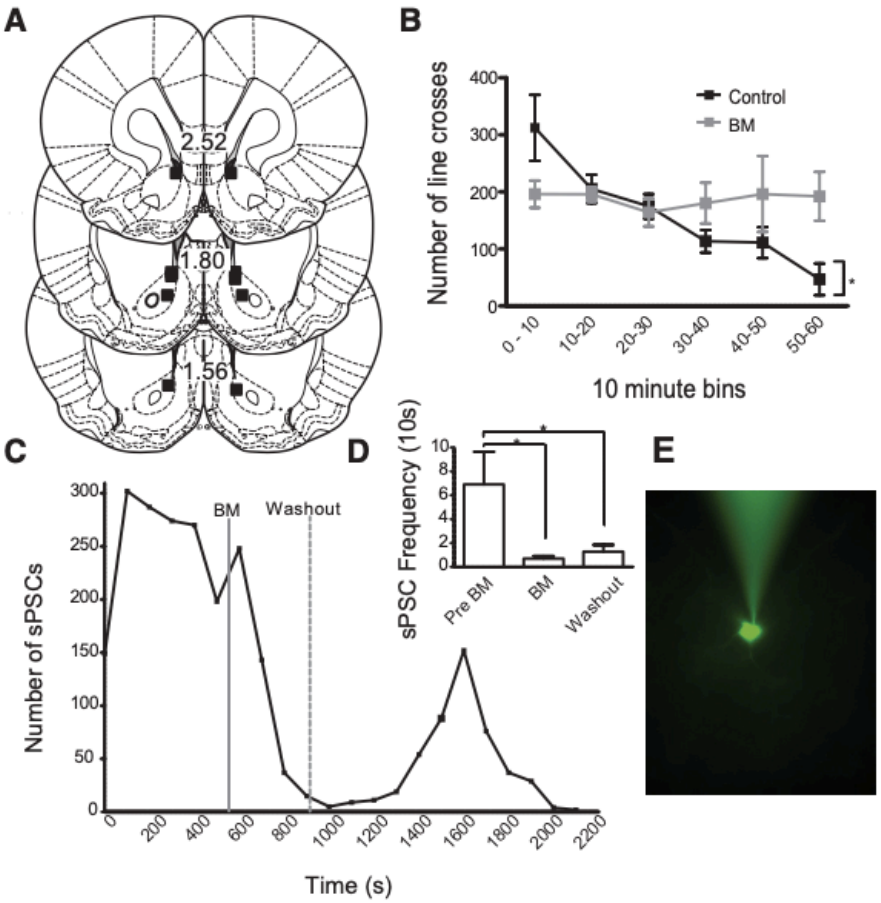


Figure 2.6. Behavioral and physiological verification of BM efficacy.
BM infusion in NAc disrupted spontaneous locomotion, and in vitro BM infusion decreased sPSCs in mPFC neurons. (A) Cannula placements for locomotion study. (B) aCSF-infused rats decreased locomotion over time, but this effect was not observed for rats receiving BM infusions *p<0.05, RM ANOVA. (C) sPSCs of one representative neuron. (D) Mean sPSC frequency before BM, after BM, and after washout. *p<0.05, Tukey's Multiple Comparison Test. (E) Example recorded rat mPFC neuron stained with Alexa Fluor 488.

CHAPTER 3

EFFECTS OF INACTIVATION OF PRELIMBIC EFFERENT PROJECTIONS TO ACCUMBENS DIFFER ON MOTIVATED SUCROSE SEEKING BEHAVIORS IN FOOD RESTRICTED AND FREE FED RATS

3.1 Abstract

The prelimbic (PL) subregion of the medial prefrontal cortex in the rodent is involved in highly-motivated behaviors such as reward-seeking for drugs. However, the role of PL in mediating the seeking behavior to natural rewards, like sucrose, is unclear. Similarly, the nucleus accumbens (NAc), mediates reward-seeking behaviors of drugs, but little is known about its role in sucrose reward seeking. Thus, it is likely that PL and NAc interact to mediate highly-motivated behaviors. For this reason, we hypothesized that PL-NAc circuit controls motivated behaviors to sucrose rewards. Furthermore, we hypothesized that the level of motivation is dependent on state of satiation. To test this hypothesis, we used chemogentic inactivation of PL-NAc neurons during two hunger states: 1) low-motivation (free fed animals); 2) high motivation (food-restricted animals). We trained and tested them using sucrose seeking operant fixed ratio 1 (FR1), extinction, cue-induced reinstatement, and fixed ratio 5 (FR5). Consistent with previous findings, we found that food restricted rats performed more reward seeking behaviors compared to free fed rats. We saw an increase in extinction and cue-induced reinstatement inactive lever presses when inactivating PL-NAc in hungry rats. When inactivating PL-NAc in free fed rats, we found an increase in number of trials for cue-induced reinstatement. Our

data reveals that hunger or satiation impacts the role that the PL-NAc circuit plays in motivated behaviors. Our findings suggest that PL-NAc mediate the ability to carry out goal-directed reward seeking behaviors when rats are hungry, specifically by suppressing behaviors that are non-conducive to reward. In free fed rats, our data suggest that PL-NAc facilitates cue-induced reinstatement learning.

3.2 Introduction

Previous research has demonstrated that the prelimbic (PL) medial prefrontal cortex (mPFC) drives reward seeking, as observed during cocaine and heroin seeking (Euston et al., 2012; Peters, LaLumiere, et al., 2008). Prior studies have shown that the nucleus accumbens (NAc) mediates the appropriate behavioral responses to reward predictive cues and plays an important role in mediating drug seeking reinstatement behaviors (Augur et al., 2016; Mogenson et al., 1980; Stefanik et al., 2016). It is important to note that the PL projects to several areas throughout the brain, heavily projecting to nucleus accumbens core (NAcC) (Vertes, 2004).

However, the role PL and NAc plays in natural reward seeking behaviors is not as clearly described and often reveals contradicting evidence. PL inactivation decreases sucrose reward seeking during FR1 and facilitates extinction recall (Caballero et al., 2019). In a PL-NAcC pharmacological disconnection study, blocking dopamine signaling in the PL and glutamate signaling in the contralateral NAcC in free-fed rats reduced cue-induced reinstatement of cocaine, but not sucrose (McGlinchey et al., 2016). Both the PL and the NAc play a critical role in the acquisition of response-outcome associations for instrumental learning and goal-directed action (Hart et al., 2014). The NAcC is an

important area regarding goal-directed behaviors, and is often framed as the 'limbic-motor interface' because it receives projections from limbic structures and projects heavily to motor structures (Hart et al., 2014). When NAcC lesioned rats were trained on a devaluation task using levers, they reduced overall responding (i.e. both active and inactive levers), instead of only to the active lever which delivered food or sucrose reward (Corbit et al., 2001). Additionally, ablating PL to NAcC projecting neurons prevented cue-induced reinstatement of alcohol (Keistler et al., 2017).

One possible explanation for the differential function of PL and PL-NAcC in guiding reward seeking behaviors for drug and natural reward could be the motivational and/or hedonic value of the reward. In a Pavlovian-to-instrumental transfer task, rats that had been food restricted for 18 hours lever pressed more, approached a food port more, and had a higher concentration of dopamine in the NAcC in response to a sucrose cue, compared to rats that were not food restricted (Aitken et al., 2016). To date, little is known about how PL-NAc regulates sucrose reward seeking behaviors in food restricted, highly motivated rats. The present study aims to test if PL-NAc controls reward seeking behaviors differently in food restricted versus free fed rats. We hypothesize that PL-NAc drives motivated sucrose seeking behaviors differently, depending on the state of hunger or satiation. Therefore, we predicted that if we silence PL-NAc in food restricted rats, we would see a decrease in sucrose seeking behaviors, similar to the effects seen in the drug seeking literature. However, an alternative hypothesis is that silencing the PL-NAc in both food restricted and free fed rats would increase sucrose seeking behaviors by potentially blocking the action-outcome association during cue-induced reinstatement. To test our hypothesis, we used retrograde Gi-DREADDs to target and silence PL

projections to NAc while rats were trained and tested on a sucrose seeking fixed ratio 1 (FR1) task, extinction, cue-induced reinstatement, and fixed ratio 5 (FR5). As expected, we found that food restricted rats sought the reward more, and faster compared to free fed rats. We also found that our alternate hypothesis was true; when inactivating PL-NAc there was an increase in inactive lever presses for both extinction and cue-induced reinstatement testing in food restricted rats; which is a seeking behavior. However, on the first day of cue-induced reinstatement testing, we observed that there was an increase in number of trials for free fed rats, and not food restricted rats. Additionally, we found that DREADD viral spread positively correlated with extinction, cue-induced reinstatement, and FR5 reward seeking behaviors. Our findings suggest that PL-NAc is on-line when rats are hungry and the sucrose reward is uncertain, allowing the rats to inhibit the behavior that least results in the reward. The PL-NAc also seems to play a role in making the new association between the previously learned sucrose-cue and the new outcome of not receiving a reward. Specifically, in rats that are motivated to obtain a reward because of its appetitive nature and not motivated by the need associated with hunger.

3.3 Methods

3.3.1 Animals

58 male Long-Evans rats (~9 weeks old and 275-300g upon arrival; Charles River) were used in behavioral studies. Of these 58, 16 were used as food restricted PL-NAcC inactivation studies, 8 were used as food restricted td-Tomato viral controls, and 14 were used in free fed PL-NAcC inactivation studies. The remaining 20 rats were used

in food restricted vs free fed behavioral studies. All rats were single-housed on a reversed light cycle (7:00 am on and 7:00pm off). Free fed rats were allowed free access to food and water and food restricted rats were fed for one hour a day. Experiments were conducted during active cycle (lights off). All animal procedures were performed in accordance with the University of Massachusetts Amherst local ethics committee (IACUC) and National Institute of Health guidelines.

3.3.2 Surgery

Rats were anesthetized with isoflurane in a closed container (5%) and transferred to a stereotaxic frame where they received isoflurane through a nose cone (1.5%-2%). Rats were given systemic antibiotic (0.1 mL cefazolin) and analgesic (1mg/kg meloxicam), and incisions were treated with local anesthetic (0.3mL, 2% lidocane). Coordinates for bilateral craniotomies in PL were +3.0 mm AP; +/- 0.6 mm ML; -3.5 mm DV and for NAc were +1.4 AP, +-1.4 ML, -7.5 DV. For experimental surgeries, .3 µL of AAV8-hSyn-DIO-hM4Di(Gi)-mCherry was infused into the PL and .5 µL of retrograde AAV-pm-Syn1-EBFP-Cre or pENN.AAV.hSyn.Cre.WPRE.hGH was infused into the NAc. For control surgeries, .3 µL of AAV9-CAG-FLEX-tdTomato-WPRE or AAV9-FLEX-tdTomato was infused into the PL. Both viruses were infused at a rate of .1 µL/min and allowed an extra 5 minutes for the virus to diffuse. Rats were allowed 1 week to recover following surgery and waited 4 weeks in order for the virus to express in the targeted neurons, which will allow us to later infuse a Clozapine-N-Oxide (CNO) ligand that binds to the receptors the virus expressed. The DREADD viruses we infused express

mCherry or tdTomatoe, which are red fluorescent proteins that we later amplified with immunohistochemistry to confirm virus expression.

3.3. 3 Apparatus

All operant testing was conducted in Med Associates chambers housed in sound attenuation cubicles (Med Associates, Fairfax, VT). Two levers were placed on one wall of the operant chamber (one on the left side and one on the right side) with lights above each lever. In between the two levers was a receptacle where reward (0.12 ml of 12% sucrose solution) was dispensed. Each chamber was illuminated by a house light, and a fan provided approximately 60 dBA background noise. The same boxes were used for extinction, cue-induced reinstatement, and FR-5 experiments.

3.3.4 Food restriction

Half of the rats were freely fed water and food. The other half had free access to water and were food restricted for 18 hours before the behavioral tasks. Rats were fed two hours after completing the behavioral tasks and had one hour to eat. Left-over food was removed after the hour was done (Aitken, 2016).

3.3. 5 Fixed-ratio 1: reward seeking requiring low motivation

Rats were trained to seek sucrose reward that requires low motivation. To achieve this, rats were trained to self-administer sucrose on a fixed-ratio 1 schedule 4 weeks after the surgery was performed. A 20 sec house light illumination signaled the time-out, during which pressing the left or right lever (one active and one inactive) were recorded

but did not result in any consequence. Upon house light offset, pressing the active lever elicited a tone (15 kHz, 74 dBA), a yellow light above the active lever, and the delivery of .12mL of 12% sucrose in a receptacle between the active and inactive lever. Rats were randomly assigned to a box where either the left or right lever was assigned as active and the opposite lever (right or left) was assigned as inactive. Rats were trained and tested in the same box with the same active lever throughout the paradigm. The first active lever press after the time-out (when the house light turned turned off) was counted as a "trial initiation". Trials in which the rat entered the receptacle in less than 1 sec after sucrose was dispensed were counted as "rewarded well-entries". In order to compare our results more closely to the cocaine literature, we followed a commonly used operant conditioning training and testing parading (McGlinchey et al., 2016). Rats were trained for 10 days of meeting criteria of over 100 trials in two hours (Figure 3.1). Once criteria were met, rats were tested 20 minutes after intraperitoneal injections of 1 ml/kg of body weight with either CNO (3mg/ml) or vehicle (DMSO in saline). There was one day of washout in between testing days, as well as after their last testing day, where rats did not receive any infusion in order to avoid potential rebound effects from CNO or vehicle infusion (Figure 3.1).

3.3.6 Extinction of lever presses as an index of motivation levels

After FR1 testing, rats were placed in the same context as FR1, but now lever pressing the active or inactive lever did not elicit any cue. Rats were tested once criteria of two consecutive days of less than 25 lever presses in two hours was met (Figure 3.1). Seven rats (four food restricted, three free fed) did not meet criteria and were tested after

four to 19 days of training. Training was halted for those rats in order to complete the remaining experiments before UMass COVID lockdown and data for those rats was excluded from extinction training analysis. Rats were injected with CNO or vehicle on their first day of testing, then they had another washout day where rats did not receive any injection, and then another day of testing where they were injected with the opposite Vehicle or CNO from the first day (Figure 3.1). Then rats were run on an extra extinction session as a washout, before advancing to cue-induced reinstatement testing (Figure 3.1).

3.3.7 Cue-Induced Reinstatement by the cue to assess motivation levels

After extinction, rats were placed in the same context as in both FR1 and extinction. However, lever pressing on the active lever elicited a tone cue and a light above the well for 2 sec but no reward was delivered into the well. Rats were tested on cue-induced reinstatement with two days of washout in between (Figure 3.1). Rats received either CNO or vehicle on their first day of testing, then they received two days of extinction and then followed by a second day of cue-induced reinstatement testing with the opposite injection they received on the first day of testing (Figure 3.1).

3.3.8 Fixed-ratio 5: reward seeking requiring high motivation

Rats were trained to seek sucrose reward that requires high motivation. Once rats were done with cue-induced reinstatement testing, rats were re-trained on FR1. Once meeting criteria of at least 100 trials in 2 hours, they were trained on FR2 on the following day, once they met criteria for FR2, they were trained on FR3 and so on until reaching FR5 (Figure 3.1). Rats were run on FR5 for two days of meeting criteria and

then received CNO or vehicle on their first day of testing, then were run on a day of FR5 with no infusion, and then tested on the opposite injection on the next day of FR5 testing (Figure 3.1).

3.3.9 Immunohistochemistry to confirm chemogenetic control and evaluate activity levels in brain regions involved in motivation.

On their second and final day of FR5 testing, rats were deeply anesthetized with Ketamine/Xylazine (80 mg/kg: 10 mg/kg i.p.), and perfused transcardially with 0.9% saline and 10% formalin. Brains were extracted, stored in 10% formalin overnight, and transferred to 20% (wt/vol) solution of sucrose/0.1% sodium azide in phosphate buffer at 4 °C. Brains were frozen with isopentane and sliced into 40 micron sections with a cryostat (Leica CM3050 S) and kept in PBS azide. Slices were washed in PBS three times for five minutes and then blocked in immuno buffer: 3% Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA) in PBS for 60 minutes. Sections were then incubated overnight in primary antibodies for two nights at 4ºC: rabbit anti c-fos (1:1000; Synaptic Systems, Goettingen, Germany) and chicken anti red fluorescent protein (1:500; Rockland, Limerick, PA) diluted in immuno buffer. Sections were re-washed in PBST three times for five minutes and then incubated in the dark for two hours in secondary antibody: biotinylated donkey anti rabbit (1:500; Jackson ImmunoResearch, West Grove, PA) and donkey anti chicken 594 (1:250; Jackson ImmunoResearch, West Grove, PA) diluted in immuno buffer. Sections were re-washed in PBST three times for five minutes and incubated in tertiary antibody: streptavidin 488 (1:500; Jackson ImmunoResearch, West Grove, PA) diluted in PBST. Sections are then washed once in PBST, once in PBS,

once in PB and then mounted and cover slipped onto microscope slides (Fisherbrand Superfrost Plus). 13 of the 37 brains that were immunohistochemically analyzed were stained only for RFP which followed the same protocol but excluded c-fos antibodies.

3.3.10 Analysis

Data were analyzed using Prism 9 (GraphPad Software, La Jolla, CA). Using a counterbalanced and within-subject design, rats were first delivered vehicle (or CNO) on Day 1 of testing, and subsequently delivered CNO (or vehicle) on Day 2 of testing. Differences between the total numbers of trials, active and inactive lever presses, rewarded well entries and total number of well entries were assessed using a paired t-test for within-subject analysis. Latency to collect reward and latency to initiate trial were calculated by using a paired t-test on the mean latency per condition for individual rats. Paired t-test was also used to assess differences between last extinction session and vehicle condition for cue-induced reinstatement. Unpaired t-test was used to assess differences between food restricted and free fed rats, days to meet criteria for FR1 and extinction training, and to assess differences between vehicle and CNO conditions using only the first day of cue-induced reinstatement testing. 2-way ANOVA were used to analyze differences between food restricted and free fed rats and Sidak's MCT was used to assess main effect of vehicle and CNO for each group. Sidak's MCT was also used to analyze main effect of day of extinction training. Pearson correlation analysis was used to analyze relationship between DREADD viral spread and total numbers of trials, active and inactive lever presses, rewarded well entries and total number of well entries. Imaging and cell counting were performed using NIS Elements software (Nikon,

Melville, NY) and virus area spread was quantified using FIJI. Two rats from the free fed group were excluded from inactivation analysis because there was no virus expression.

3.4 Results

3.4.1 Food restricted rats performed more trials compared to free fed rats

In order to assess differences in reward seeking behaviors while learning, food restricted and free fed rats were trained for 10 days on FR1 after meeting criteria of over 100 trials in 2 hours. Food restricted rats lever pressed more compared to free fed rats ($t(18)=5.064$, $p<0.0001$, unpaired t-test; Fig. 3.2).

3.4.2 No effect of PL-NAc inactivation on FR1 or FR5 reward seeking behaviors

We observed no effect of PL-NAc inactivation in either food restricted or free fed rats during FR1 on number of trials, active or inactive lever presses, well entries, or rewarded well entries (all p's>0.05, paired t-test; Fig. 3.3A-F). There was also no effect on latency to initiate trial or latency to collect reward for both food restricted or free fed rats for FR1 inactivation (all p's >0.5, paired t-test, Fig. 3.3F-G). A subset of rats was trained and tested on FR5 sucrose seeking in addition to FR1, extinction, and cue-induced reinstatement. Inactivation of PL-NAc had no effect on trials, active lever presses, inactive lever presses, rewarded well entries, total number of well entries, or latency to collect reward for both food restricted (N=5) or free fed rats (N=4) (all p's 0.5, paired t-test; Figure 3.4 A-F).

3.4.3 Food restricted rats were more motivated to seek reward compared to free fed, regardless of treatment for both FR1 and FR5.

In order to assess differences in motivation between food restricted and free fed rats, we performed a 2-way ANOVA to assess main effect of food restricted and free fed rats for FR1 reward seeking behaviors. 2-way ANOVA revealed a significant main effect of food restricted vs free fed in number of trials ($F(1, 52) = 81.96$, $p<0.0001$; Figure 3.3A), active lever presses ($F(1, 26) = 12.73$, $p=0.0014$; Figure 3.3B), rewarded well entries ($F(1, 26) = 25.65$, $p<0.0001$; Figure 3.3D). 2-way ANOVA also revealed main effect of food restricted vs free fed for latency to initiate trials ($F(1, 25) = 27.44$, $p<0.0001$; Figure 3.3G-H). Post-hoc multiple comparisons test (Sidak's MCT) revealed that food restricted rats performed more trials than free fed rats in both vehicle ($t=6.814$, $p<0.0001$) and CNO ($t=5.989$, $p<0.0001$) conditions. Food restricted rats also pressed the active lever more in both vehicle ($t=3.301$, $p=0.0035$; Figure 3.3B) and CNO ($t=3.7$, $p=0.0010$; Figure 3.3B) conditions compared to free fed rats. Food restricted rats also had a higher number of rewarded well entries in both vehicle ($t=4.973$, $p<0.0001$; Figure 3.3D) and CNO ($t=4.907$, $p<0.0001$; Figure 3.3D) conditions. Post-hoc analysis also revealed that free fed rats have a shorter latency to initiate trials in both vehicle ($t=4.585$, $p<0.0001$; Figure 3.3H) and CNO ($t=2.905$, $p=0.0109$ Figure 3.3H) conditions. 2-way ANOVA did not reveal main effect of satiety status in number of inactive lever presses, well entries, or latency to collect reward (all p's > 0.5; Figure 3.3 C, E-F).

We also analyzed FR5 reward seeking behaviors between food restricted and free fed rats. 2-way ANOVA revealed significant main effect of trials ($F(1,7)=25.74$, $p=0.0014$; Figure 3.4A); active lever presses ($F(1, 7) = 45.15$, $p=0.003$; Figure 3.4B);

and rewarded well entries (F (1, 7) = 22.93, p=0.002; Figure 3.4D) for food restricted vs free fed rats. Post-hoc multiple comparisons test (Sidak's MCT) revealed that food restricted rats performed more trials than free fed for both vehicle (t=5.055, p= 0.0004; Figure 3.4A) and CNO (t=4.760, p=0.0006; Figure 3.4A) infusions. Food restricted rats also performed more active lever presses than free fed in both vehicle (t=5.179, p=0.0003; Figure 3.4B) and CNO (t=5.621, p=0.0001; Figure 3.4B) conditions. And food restricted rats also performed more rewarded well entries compared to free fed rats for both vehicle (t=4.737, p=0.0006; Figure 3.4D) and CNO (t=4.513, p=0.001; Figure 3.4D) conditions. 2-way ANOVA did not reveal any significant main effects or interactions for total number of well entries or inactive nose pokes (all p's > 0.05; Figure 3.4C, E-F). A possible explanation for the differences in inactivation effects on number of well entries or inactive nose pokes we see in this chapter, compared to the previous chapter could be because in the previous chapter, we were inactivating the PL as a whole. Versus in this chapter, where we selectively inactivated PL projections to NAc. When inactivating PL as a whole, we are also silencing the vast number of neurons PL is projecting to, which can include areas that are responsible for vast behaviors ranging from emotion to motor movements (Dalley et al., 2004; Vertes, 2004, 2006). In turn, by selectively inactivating PL-NAc neurons, we are targeting a circuit that is thought to control the execution and inhibition of motivated behaviors, specifically. Here, we see that hunger drives more reward seeking behavior, regardless of the involvement of PL-NAc, suggesting that it is the PL projections to another area which drives this behavior. A circuit that could possibly be involved in mediating these hunger driven behaviors is the PL-IL. There is

evidence that shows that the IL controls food seeking behaviors related to hunger (Riveros et al., 2014, 2019).

3.4.4. Food restricted rats pressed the lever more compared to free fed rats during the first 3 days of extinction training.

After FR1 testing, rats were trained on extinction until meeting criteria of two consecutive days of less than 20 lever presses. Four food restricted rats and three free fed rats did not meet criteria and were excluded from extinction training analysis (Figure 3.5A, bars outlined in yellow). These rats were excluded from training analysis because the reasons they were advanced to the testing stage was not random and could affect the analysis and interpretation of the extinction testing results (see methods section). Rats that met criteria took between 3 to 19 days to reach criteria. We compared number of lever presses between food restricted and free fed throughout extinction training and did not find significant differences in number of lever presses throughout the 19 days of training ($p>0.05$, unpaired t-test; Figure 3.5B). We also did not find any significant differences in number of days to reach criteria between food restricted and free fed rats ($t(48)=1.255$, $p=0.2157$, unpaired t-test; Figure 3.5D). Therefore, we decided to use a 2-way Mixed effects ANOVA to compare number of lever presses for food restricted and free fed for the first three days of extinction training. 2-way Mixed effects ANOVA revealed significant main effects of food restricted vs free fed ($F(1, 56) = 23.77$, $p<0.0001$; Figure 3.5B-C). Sidak's multiple comparisons revealed food restricted rats pressed the lever more during the first ($t=5.999$, $p<0.000$; Figure 3.5B-C), second

(t=3.4.71, p=0.0032; Figure 3.5B-C), and third (t=2.999, p=0.00128; Figure 3.5B-C) day of extinction training.

3.4.5 PL-NAc inactivation during extinction testing increased inactive lever pressing in food restricted rats, but had no effect on free fed rats.

After extinction training, all of the experimental rats regardless of meeting criteria of two consecutive days of <20 trials were tested during extinction. One food restricted and one free fed rat did not meet criteria but their data was included in the analysis because their data did not affect the outcome of the results (data not shown). Inactivation of PL-NAc did not affect active lever presses or total number of well entries for both food restricted and free fed rats during extinction testing (all p's>0.05, paired t-test; Figure 3.6A-B). PL-NAc inactivation resulted in an increase in inactive lever presses in food restricted rats (t(13)=2.164, p=0.0496, paired t-test; Figure 3.6C, black dots) but not in food restricted tdTomatoe rats or free fed rats (all p's>0.05, paired t-test; Figure 3.6 C, gray and maroon dots). We also performed 2-way ANOVA analysis to assess differences between food restricted and free fed rats for extinction behaviors. However, our analysis did not reveal any significant differences among each group (all p's>0.05, 2-wayANOVA; Figure 3.6A-C).

3.4.6 PL-NAc Inactivation in food restricted rats increased inactive lever pressing during cue-induced reinstatement.

In order to assess if rats reinstated, we performed a paired t-test comparing the extinction session preceding cue-induced reinstatement testing and the cue-induced

reinstatement vehicle condition (Figure 3.7.A). All three groups had higher number of lever presses during vehicle cue-induced reinstatement compared to the last extinction session before cue-induced reinstatement testing: food restricted ($t(14)=4.385$, $p<0.0006$, paired t-test), food restricted tdTomatoe DREADD ($t(7)=3.941$, $p=0.0056$, paired t-test), free fed ($t(11)=2.310$, $p=0.0413$, paired t-test) (Figure 3.7A). Paired t-test did not reveal any effect of inactivation for number of trials, active lever presses, rewarded well entries, well entries, or latencies to collect reward or initiate trial (all p's >0.05, paired t-test; Figure 3.7B, D-I). However, we decided to use unpaired t-test to analyze effects of inactivation using data from their first day of cue-induced reinstatement testing (Figure 3.7B). We did not find any significant effects of inactivation for number of active or inactive lever presses, rewarded well entries, well entries, or latency to collect reward (data not shown). But we did find that PL-NAc inactivated rats had higher number of trials compared to vehicle infused rats ($t(10)=2.731$, $p=0.0212$, unpaired t-test; Figure 3.7C).

We were interested in assessing the differences between food restricted and free fed rats for number of trials, active and inactive lever presses, rewarded well entries, well entries, and latencies to collect reward and initiate trial (Figure 3.7B, D-I). We found a main effect of food restricted vs free fed for number of trials ($F(1,52)=8.94$, $p=0.0043$; 2-way ANOVA; Figure 3.7B). Food restricted rats had higher number of trials compared to free fed for the vehicle condition ($t=2.432$, $p=0.0368$; Sidak's MCT), but not CNO condition ($t=1.798$, $p=0.1499$; Sidak's MCT). We also found a main effect of food restricted vs free fed for number of lever presses ($F(1,52)=7.163$, $p=0.0099$; 2-way ANOVA; Figure 3.7D). However, we did not find any significant differences for vehicle

or CNO conditions (all p's >0.05; Sidak's MCT; Figure 3.7D). We also did not find any significant effects for active and inactive lever presses, rewarded well entries, well entries, and latencies to collect reward and initiate trial (all p's >0.05; 2-way ANOVA; Figure 3.7D-I).

2.4.6 Larger viral spread correlated with increase in extinction, reinstatement, and reward seeking behaviors.

In order to assess if there was a correlation between DREADD viral spread and reward seeking behavior during the CNO condition (inhibition of neurons), we performed individual Pearson Correlations between DREADD viral spread and number of trials, active lever presses, inactive lever presses, well entries, rewarded well entries, and latency to collect reward and to initiate trials for FR1, extinction, cue-induced reinstatement, and FR5. See Figure 3.1 for representation of viral spread. Our analysis revealed positive correlations for only food restricted rats, and not free fed rats (Figure 3.9). For extinction reward seeking behaviors, our analysis revealed a positive correlation between DREADD viral spread and active lever presses ($r(12)=0.6574$, $p=0.0106$, Pearson correlation; Figure 3.9A). Pearson correlation also revealed a positive correlation between DREADD viral spread and number of well entries ($r(12)=0.6730$, $p=0.0083$; Figure 3.9C) during extinction. For cue-induced reinstatement, we found a positive correlation between DREADD viral spread and rewarded well entries ($r(15)=0.5268$, $p=0.0436$, Pearson correlation; Figure 3.9E), and also a positive correlation between DREADD viral spread and overall number of well entries ($r(13)=0.5285$, $p=0.0428$; Pearson correlation; Figure 3.9G). For FR5 reward seeking behaviors, we found a

positive correlation between DREADD viral spread and number of trials ($r(3)=0.9335$, $p=0.0204$, Pearson correlation; Figure 3.9I), and a positive correlation between DREADD viral spread and rewarded well entries ($r(3)=0.9830$, $p=0.0026$, Pearson correlation; Figure 3.9K).

We performed further Pearson Correlation analysis between DREADD viral spread and behavior after vehicle infusion in order to assess if the correlation effects we found were specific to PL-NAc inactivation from CNO infusion. For extinction reward seeking behaviors, our analysis did not reveal correlation between DREADD viral spread and active lever presses ($p>0.05$, Pearson correlation; Figure 3.9B). Pearson correlation did reveal a positive correlation between DREADD viral spread and number of well entries ($r(12)=0.7433$, $p=0.0015$; Figure 3.9D) during extinction. For cue-induced reinstatement, we did not find a correlation between DREADD viral spread and rewarded well entries ($p>0.05$, Pearson correlation; Figure 3.9F). We did find a positive correlation between DREADD viral spread and overall number of well entries ($r(13)=0.7376$, $p=0.0017$; Pearson correlation; Figure 3.9H). For FR5 reward seeking behaviors, we found a positive correlation between DREADD viral spread and number of trials ($r(3)=0.8924$, $p=0.0417$, Pearson correlation; Figure 3.9J), and a positive correlation between DREADD viral spread and rewarded well entries ($r(3)=0.9238$, $p=0.0250$, Pearson correlation; Figure 3.9L).

We did not find correlations between viral DREADD spread and FR1 reward seeking behaviors (all p's >0.05, Pearson correlation, data not shown). We also did not find any correlation between viral DREADD spread and number of inactive lever presses

during extinction testing ($p>0.05$, Pearson correlation, data not shown). There was also no correlation between viral DREADD spread and trials, active or inactive lever presses, or latencies to collect reward or initiate trial (all p's >0.05, Pearson correlation, data not shown). For FR5, we did not find correlations between viral DREADD spread and number of active or inactive lever presses, or latency to collect reward (all p's >0.05, Pearson correlation, data not shown).

3.5 Discussion

The objective of this study was to test if PL projections to NAc control motivated goal-directed behaviors differently depending on satiety state (i.e. food restricted or satiated rats). This research is important because although the role the PL and the NAc play in regulating drug seeking behaviors has been extensively studied, results are still unclear in terms of natural reward seeking behaviors, and often contradict the cocaine literature (Augur et al., 2016; Caballero et al., 2019; Corbit et al., 2001; Euston et al., 2012; McGlinchey et al., 2016; Mogenson et al., 1980; Peters, LaLumiere, et al., 2008; Stefanik et al., 2016). In order to fill this gap in the literature, we used retrograde inhibitory DREADDs to inactivate PL projections to NAc in food restricted and free fed male Long-Evans rats. We also included a third group of food restricted rats with a retrograde td-Tomato DREAD to label projections from PL to NAc. This group serves as a measure that the inactivation effects we see in the food restricted retrograde inhibitory DREADDs group was a result of PL-NAc inactivation and not due to extraneous factors (K. S. Smith et al., 2016). We trained and tested rats on a sucrose seeking FR1, extinction, cue-induced reinstatement, and FR5. We chose these behavioral paradigms

and food restriction protocol because they are classic behavioral tests commonly used in the cocaine seeking literature (Aitken et al., 2016; McGlinchey et al., 2016). Our findings have implications for further understanding the complex and dynamic role the prefrontal to accumbens projections play in guiding reward seeking behaviors.

As expected, we found that food restricted rats were more motivated to seek the sucrose reward (Figure 3.2 & Figure 3.5). In line with our alternate hypothesis and contrary to effects seen in cocaine literature, both food restricted and free fed rats demonstrated an increase in seeking behavior when inactivating PL-NAc. However, not specifically sucrose seeking behaviors. Food restricted rats only demonstrated an increase in inactive lever presses during extinction and cue-induced reinstatement. These results suggest that the PL-NAc plays a different role depending on hunger state. For food restricted rats, PL-NAc seems to play a role in negative reinforcement by suppressing behaviors that are not conducive to obtaining a reward. Free fed rats demonstrated an increase in initiated trials when inactivating PL-NAc during cue-induced reinstatement. Because we only saw an increase in initiated trials, but not well entries, this potentially suggests that for free fed rats, PL-NAc plays a role in inhibiting the reward seeking behaviors when they are not as motivated to obtain the sucrose outcome.

A possible explanation for these results is that because these rats have a limited availability of food, and therefore calories, this shifts their motivation to a state of "need" (i.e. wanted and needed the sucrose). Contrary to free fed rats which are less motivated, but still desire and seek the sucrose (i.e. wanted but did not need the sucrose) (Figure 3.7A). By inactivating the PL-NAc in food restricted rats, this "need" motivation potentially influences their reward seeking behaviors and leads them to press the inactive

lever more. Perhaps, because it increases their exploratory behavior to find an alternate route and inactivating the PL-NAc liberates the inhibitory effect this circuit was exerting towards pressing the inactive lever. We demonstrated that the PL-NAc inactivation effects we see in food restricted rats are indeed a result of Gi DREADD inhibition because we do not see these effects in the td-Tomato food restricted group (Figure 3.6C & 3.7E). However, another approach that would strengthen our claims is to perform a c-fos analysis in both food restricted Gi DREADD rats and food restricted td-Tomato rats in the tissues we collected when perfusing the rats 90 minutes after their last cue-induced reinstatement testing day. C-Fos is an immediately early gene present commonly used as a marker for neuronal activity (Cruz et al., 2015). By performing a c-fos quantification and localization analysis we could assess if there was neuronal activation in the PL-NAc in rats perfused after vehicle session, demonstrating that indeed this circuit was being employed during this task. Additionally, by quantifying c-Fos in the food restricted Gi DREADD PL-NAc rats that received CNO before perfusion, we could confirm that we inhibited this circuit. A further step that would strengthen our extinction results is to train, test and perfuse additional cohort of food restricted rats: half receiving Gi DREADD in the PL-NAc and the other half receiving td-Tomato in the PL-NAc. But, instead of perfusing and analyzing c-fos in these rats after cue-induced reinstatement testing, we would perfuse them after extinction testing in order to assess that indeed the PL-NAc was active during extinction.

As mentioned before, we also saw an increase in reward seeking behaviors for free fed rats during cue-induced reinstatement. Specifically, we saw an increase in number of trials when we inactivated the PL-NAc in free fed rats. In our cue-induced

reinstatement task, trials are self-initiated, and rats can initiate a trial after the house light that signals a "time-out" is turned off. Interestingly, we only saw this inactivation effect of increase in trials in free fed rats when analyzing the data from their first cue-induced reinstatement testing (i.e. first day they were re-exposed to the house light and tone cue). A possible explanation is that for free fed rats, PL-NAc inhibits the behaviors that are not conducive to a reward by facilitating the cue-outcome association. In this case, it is possible that re-introducing the house-light and tone cues learned during FR1 triggered that previously formed memory, but because PL-NAc was offline, rats were unable to override that previously established memory with a new memory that initiating a trial will not trigger a reward delivery. Also, because the free fed rats were satiated, and therefore did not "need" the reward, motivation was manifested differently. Free fed rats were probably were not motivated enough to find alternate ways of seeking the reward, as we saw with food restricted rats, but were indeed more motivated to continue pressing the lever to obtain the sucrose reward. This is important because we can observe a difference between the "need" and the "want" a natural reward can exert over behavior. One limitation to our study is that we did not have a free fed group of rats with the control td-Tomato virus. This control group would allow us to compare if the inactivation effects we see were a product of the inhibitory Gi DREADDs silencing the PL-NAc, or if it was due to extraneous factors. An alternate approach would be to analyze c-fos expression levels between vehicle and CNO conditions in the PL-NAc for free fed rats that were perfused 90 minutes after the start of cue-induced reinstatement testing.

The fact that we did not see any effect of PL-NAc inactivation for FR1 and FR5 provides more evidence towards the claim that PL-NAc is important for the formation of

action-outcome associations (Hart et al., 2014). Testing for FR1 and FR5 was performed after substantial training, and because it was a fixed-ratio schedule of reward delivery, there was no ambiguity towards the delivery of the reward after the reward seeking behavior. Probably, the behavior we were testing was a goal directed behavior, but it was also habitual, which could mean that another circuit was in charge of mediating this behavior, and not the PL-NAc.

There are two outstanding limitations that our study needs to address. The first is the finding that higher DREADD viral spread for both CNO and vehicle infusions positively correlates with sucrose reward seeking behaviors in food restricted rats during extinction, cue-induced reinstatement, and FR5 CNO infusions (Figure 3.9). This raises the question as to why we are seeing these positive correlations in both CNO and vehicle infusions, instead of just CNO or vehicle. One possible explanation is that because our study was not specifically designed to answer the question of the effects that DREADD viral spread has on reward seeking behaviors, we did not specifically target different areas or layers within the prefrontal cortex which could mean our results are an artifact. In other words, our virus did not only stay within the layers of the PL. Some rats had DREADD viral spread into the anterior cingulate cortex (ACC), the rats had DREADD viral spread into the infralimbic (IL), and some rats had DREADD viral spread into ACC and IL. Therefore, we did not control the specific regions where the DREADD viral spread into, which means that with our design, we are not considering the function of each area and instead are grouping areas that have different functions. For instance, it has been shown that layers within the PL and IL differentially control reward seeking behaviors (Hardung et al., 2017). Specifically, when using a liquid reward, more ventral

layers of the PL and the IL are responsible for inhibiting reward seeking behaviors (Hardung et al., 2017). Furthermore, studies in both rodents and humans have found that the ACC assesses and integrates different dimensions of motivational values in order to mediate goal-directed behaviors (Devinsky et al., 1995; Yee et al., 2021). These are all different functions and behaviors that we are not considering with our analysis. An approach that we could take in order to uncover if these results are an artifact, is to include more rats in the study and further divide the analysis into 3 groups: 1) rats that received DREADD viral spread in exclusively the PL; 2) rats that received DREADD viral spread in the ACC and PL; and 3) rats that received DREADD viral spread in PL and IL. This way, we can how DREADD viral spread into the various layers of PL, and also layers of ACC, and layers of IL, differentially control reward seeking behaviors.

Another limitation to our study that we need to address is the lack of analysis of axons in the NAc. Because our tissue was collected at 20x, we could clearly identify neuronal cell bodies, but we were not confident that all axons were visible which made quantification difficult. Additionally, because of reasons outside of our control which led to malfunction of 4^{o}C refrigerator, some of the tissue presented difficulties to confidently quantify. As a next step, the NAc tissue should be quantified at a higher resolution (i.e. 40x) and perform the necessary analysis.

Our study identifies differences in prefrontal to accumbens behavioral control of goal-directed actions depending on if the animal is hungry or satiated which will give further insight needed to develop specialized treatments for disorders like binge-eating. Our project also allows us to compare and contrast our results with previous literature

that generally describes the PL as an area solely responsible for the execution of reward seeking behaviors.

3.6 Figures

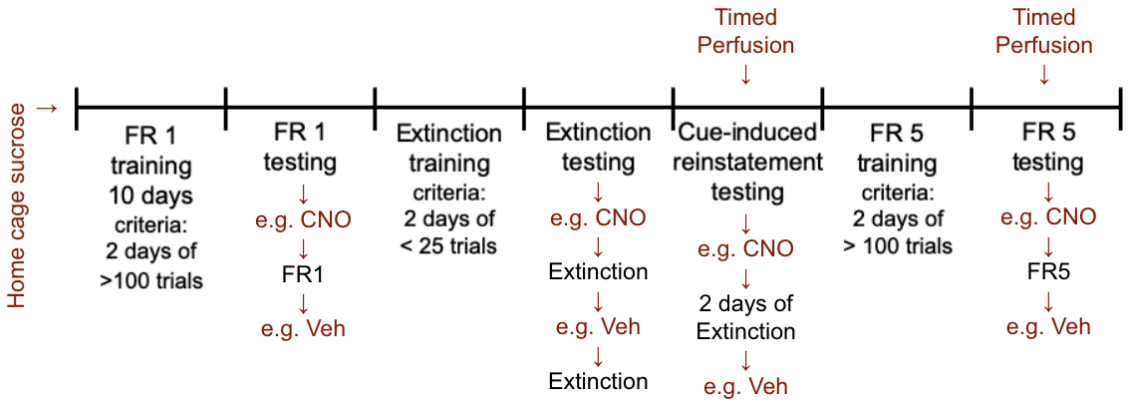


Figure 3.1. Timeline for behavioral training and experiments.
Rats were first trained on FR1 for 10 days of meeting criteria and then tested on CNO and Vehicle (Veh) with one day an FR1 washout day in between. Then they were trained on extinction until meeting criteria of less than 25 trials for 2 days straight and then tested on CNO and Vehicle (Veh) with one day an extinction washout day in between. Then they were tested on cue-induced reinstatement with 2 days of extinction in between. A subset of rats was perfused on their second day of testing, 90 minutes after testing started. Another subset of rats was trained and tested on FR5 with a washout FR5 in between testing days. Rats that were tested on FR5 were perfused 90 minutes after the start of their last testing day.

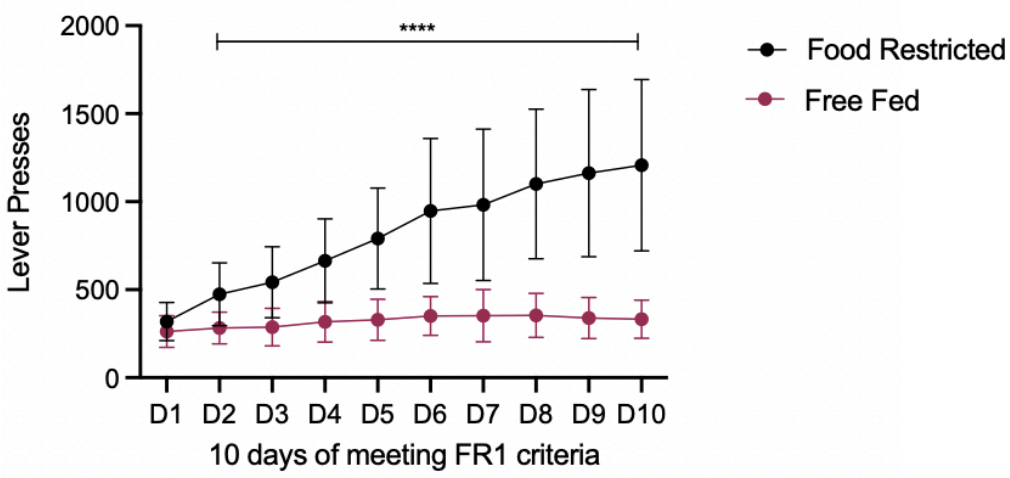


Figure 3.2. Lever pressing during FR1 training.

Food restricted rats pressed the active lever more during training, compared to free fed rats ****= $p<0.0001$, unpaired t-test.

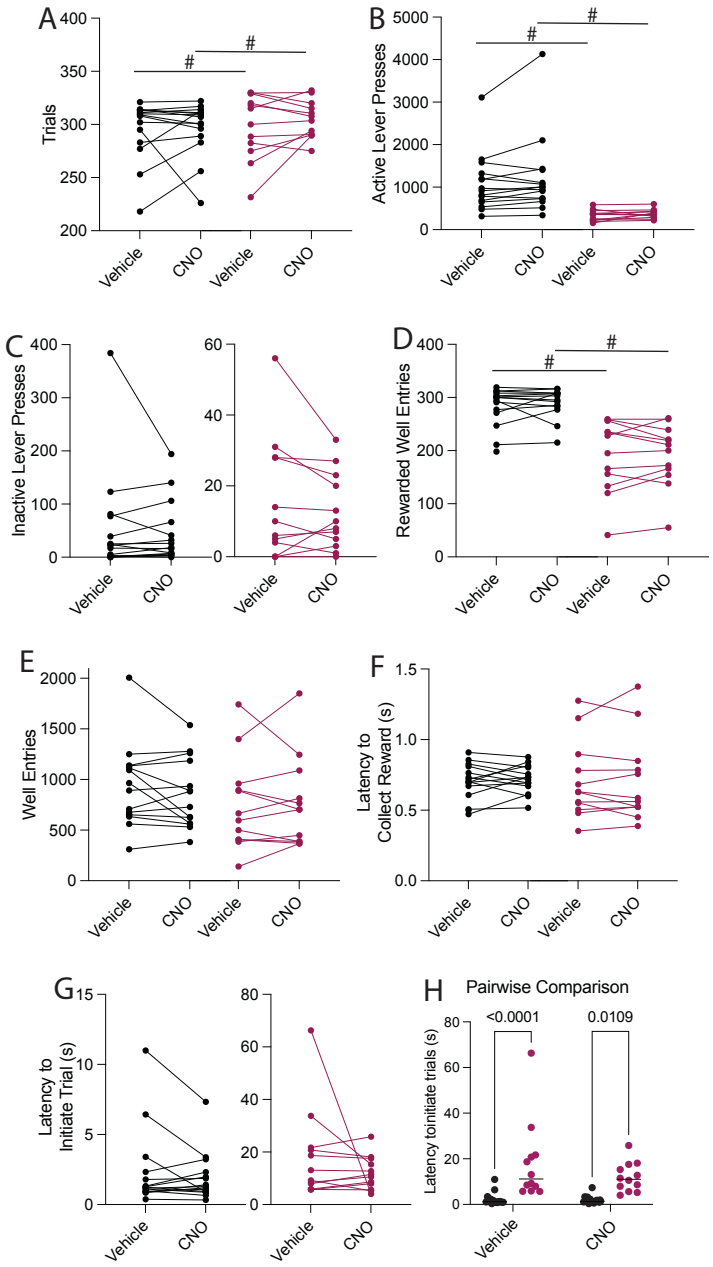


Figure 3.3. FR1 reward seeking behavior for food restricted and free fed *rats.* Food restricted rats are represented in black (●) and free fed rats are represented in maroon (●). (A-G) There was no effects of PL-Nac inactivation on number of trials, active or inactive lever presses, rewarded or overall number of well entries, and latencies to collect reward or initiate trial. (A-B, D) Food restricted rats has a higher number of trials, active lever presses, and rewarded well entries. (G-H) Food restricted rats also has shorter latency to initiate trials compared to free fed rats. (H) Pairwise comparison for latency to initiate trial #=$p<0.05$, Sidak's MCT.

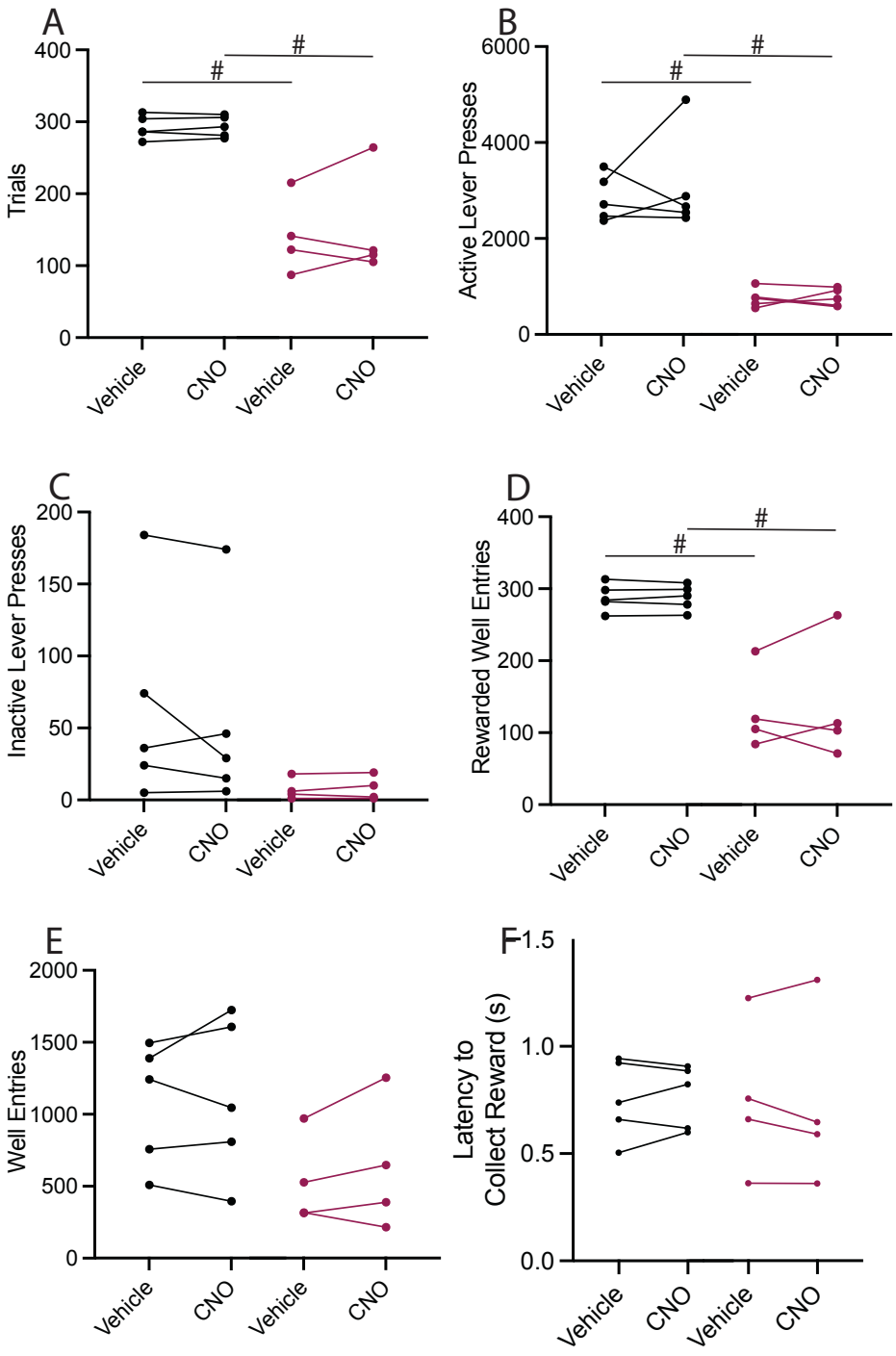


Figure 3.4. FR 5 reward seeking behaviors for food restricted and free fed rats. Food restricted rats are represented in black (●) and free fed rats are represented in maroon (●). (A-F) There was no effect of PL-Nac inactivation for number of trials, active or inactive lever presses, rewarded and overall number of well entries, and latency to collect reward. (A-B, D) Food restricted rats has a higher number of trials, active lever presses, and rewarded well entries compared to free fed. #=$p<0.05$, Sidak's MCT.

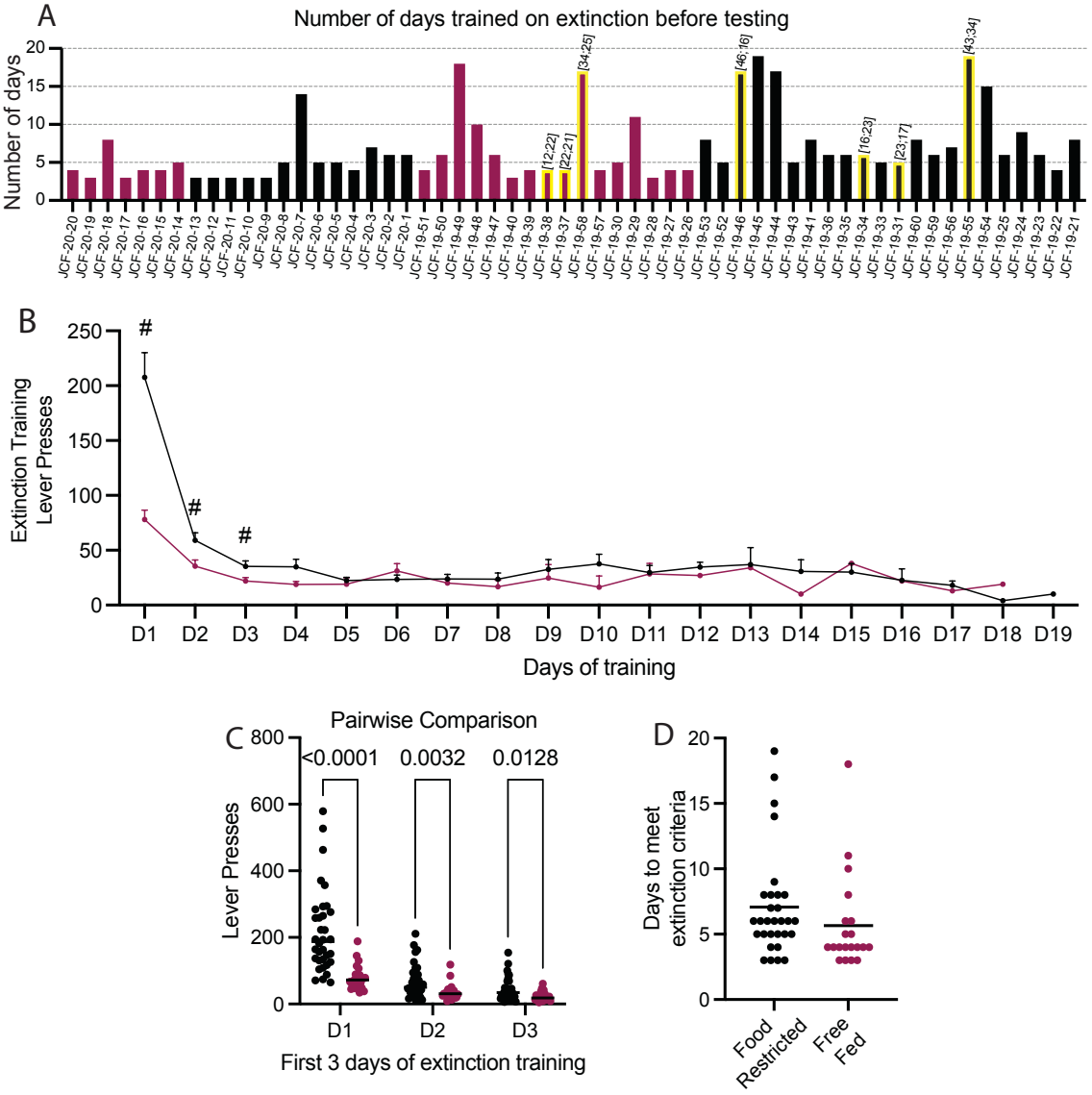


Figure 3.5. Extinction training.

Food restricted rats are represented in black (●) and black bars. Free fed rats are represented in maroon (●) and maroon bars. (A) Number of days each rat took to reach criteria for extinction testing. Bars with yellow outline are rats that did not meet criteria for extinction and the numbers included at the top of each yellow outlined bar are the number of lever presses for the two days that preceded testing. (B) Number of lever presses across the extinction training days. (C) Individual values per rat for first three days of extinction training. (B-C) Food restricted rats pressed more compared to free fed rats during the first three days of extinction training #=$p<0.05$, Sidak's MCT. (D) Days to meet extinction criteria for food restricted and free fed rats.

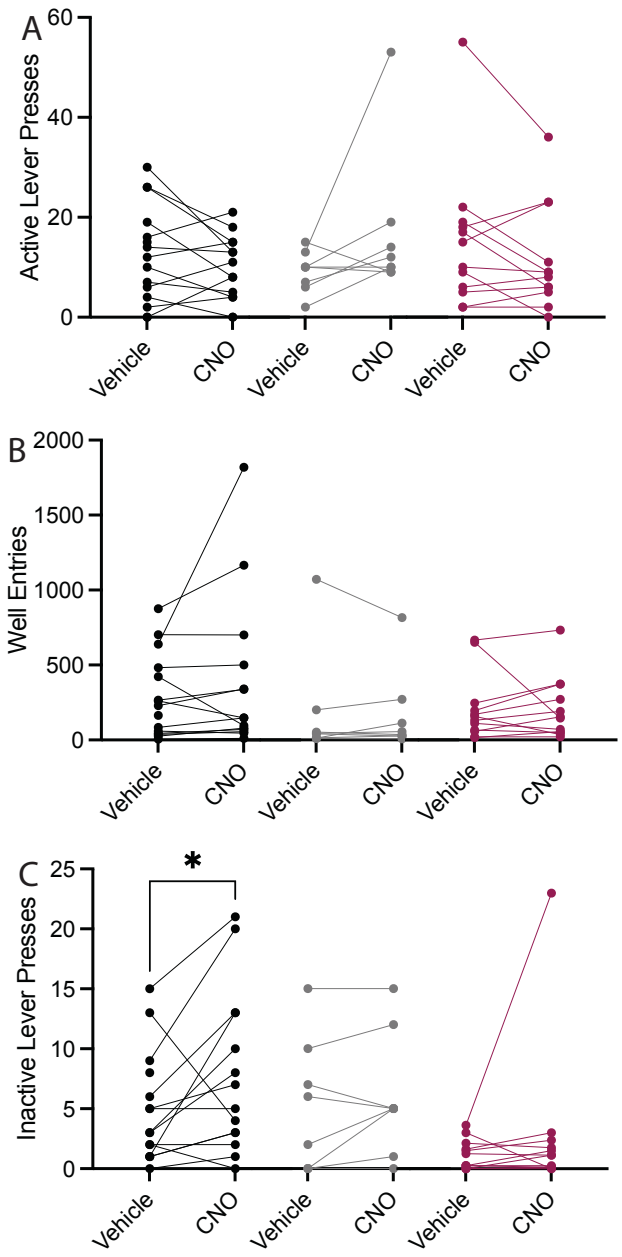
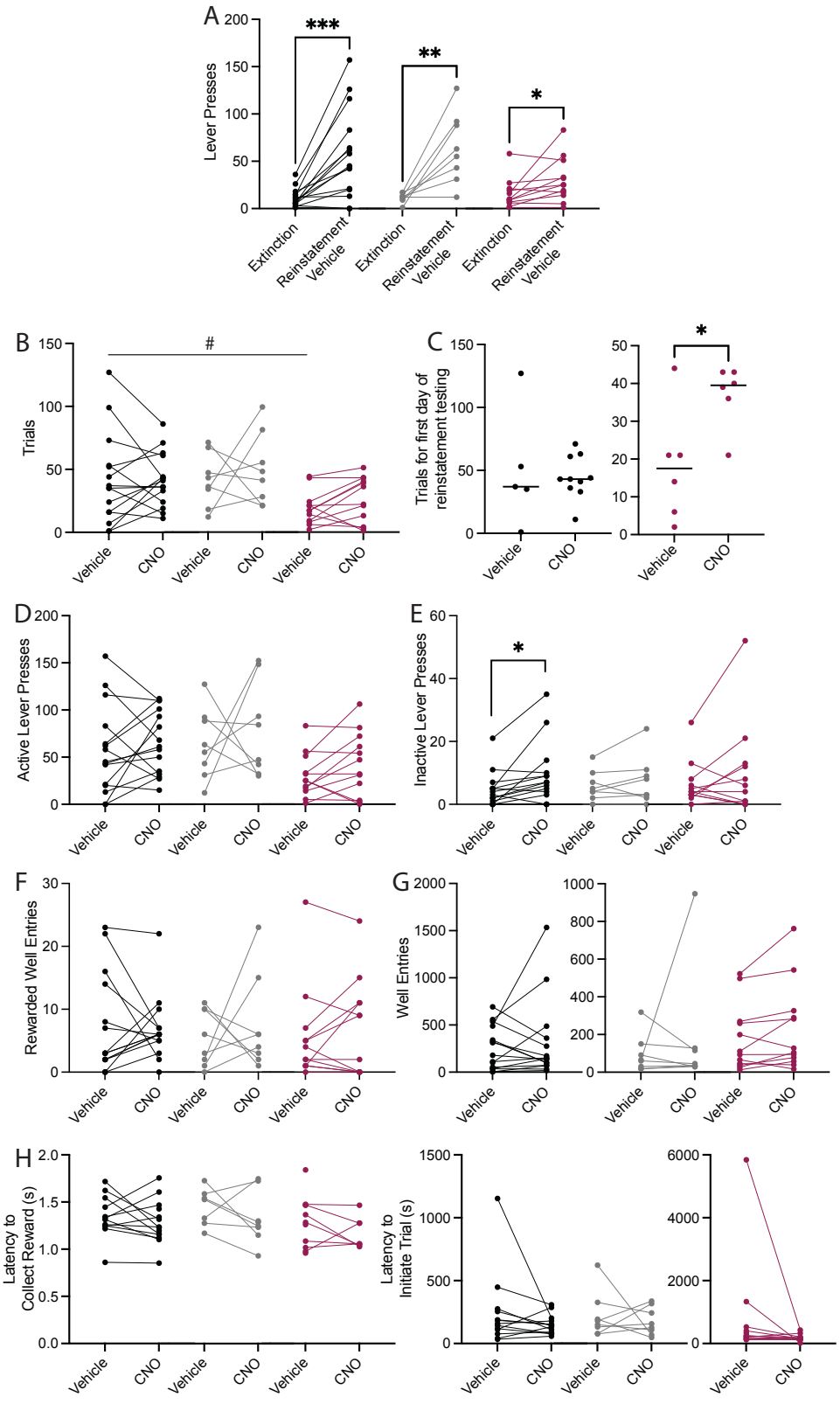


Figure 3.6. Extinction reward seeking behavior for food restricted and free fed rats. Food restricted rats are represented in black (●), food restricted rats with tdTomatoe DREADD are represented in gray (●) and free fed rats are represented in maroon (●). (A-B) No effect of PL-NAc inactivation on total number of active lever presses or well entries. (C) Inactivation of PL-NAc increased inactive lever presses in food restricted rats *p=0.0496, paired t-test.


A
Lever Presses
200
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Extinction
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Active Lever Presses
200
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0
E
Inactive Lever Presses
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40
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Rewarded Well Entries
30
20
10
0
G
Well Entries
2000
1500
1000
500
0
1000
800
600
400
200
0
H
Latency to Collect Reward (s)
2.0
1.5
1.0
0.5
0.0
Latency to Initiate Trial (s)
1500
1000
500
0
6000
4000
2000
0

Figure 3.7. Cue-induced reinstatement reward seeking behavior for food restricted and free fed rats.

Food restricted rats are represented in black (●), food restricted rats with tdTomatoe DREADD are represented in gray (●), and free fed rats are represented in maroon (●). (A) Number of lever presses was significantly higher during the vehicle reinstatement condition compared to the last extinction session before reinstatement testing for all three groups ***, **, *p's <0.05, paired t-test. (B, D-I) No effect of inactivation on number of trials, active and inactive lever presses, rewarded and overall number of well entries, or latency to collect reward and initiate trial. (B) Food restricted rats had higher number of trials compared to free fed for vehicle condition #p=0.0368, Sidak's multiple comparison. (C) Number of trials only including data for first day of reinstatement testing for free fed rats. Inactivation of PL-NAc increased number of trials. *p=0.0212, unpaired t-test. (E) Number of inactive lever presses. Inactivation of PL-NAc increased number of inactive lever presses in food restricted rats *p=0.0182, paired t-test.

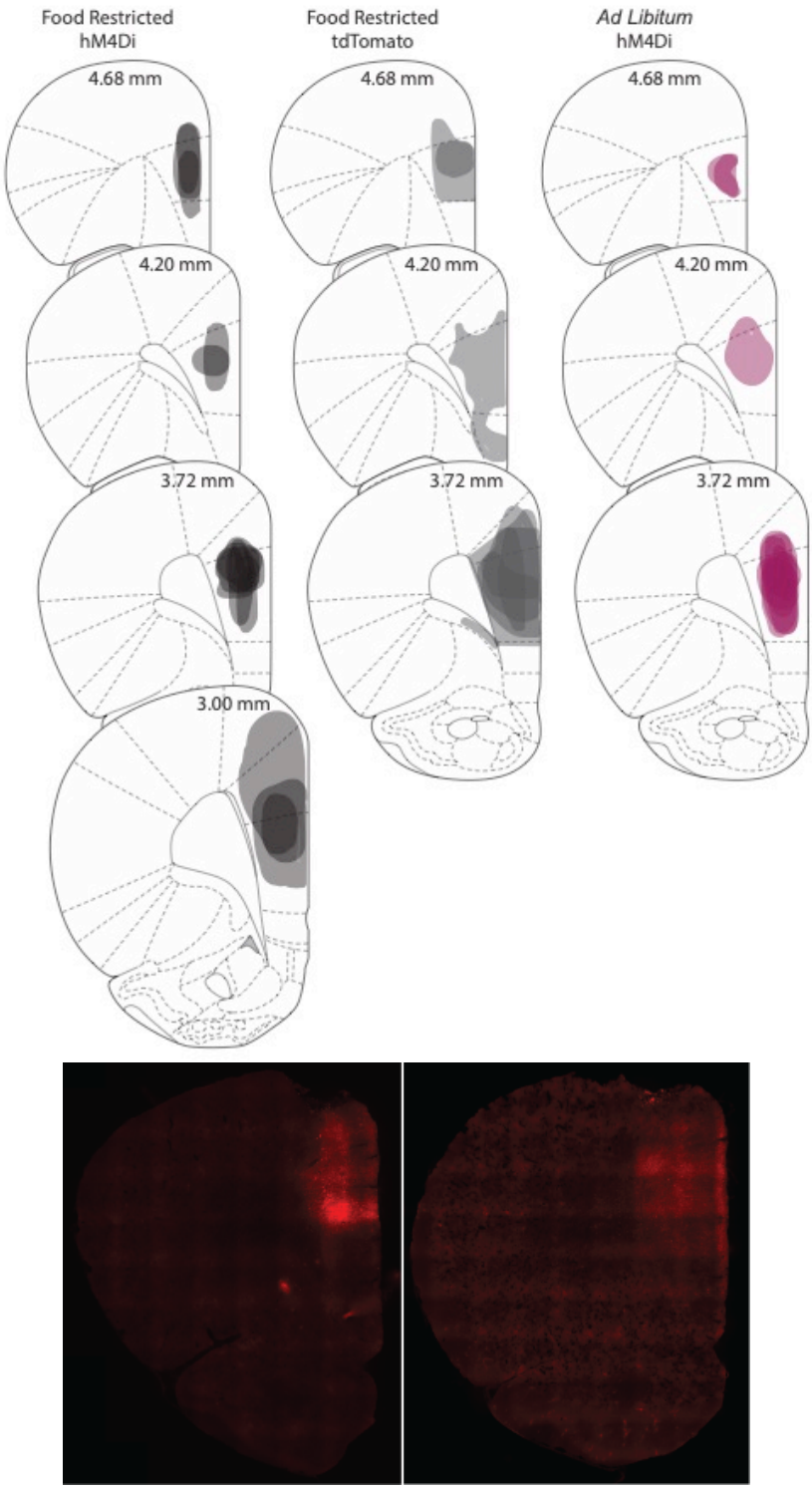

Figure 3.8. DREADD viral spread.
Food restricted hM4Di rats (left black shading), food restricted td-Tomato rats (middle gray shading), and free fed rats (right maroon shading). Bottom images are example of Gi DREADD expressing PFC slices taken at 20x resolution.

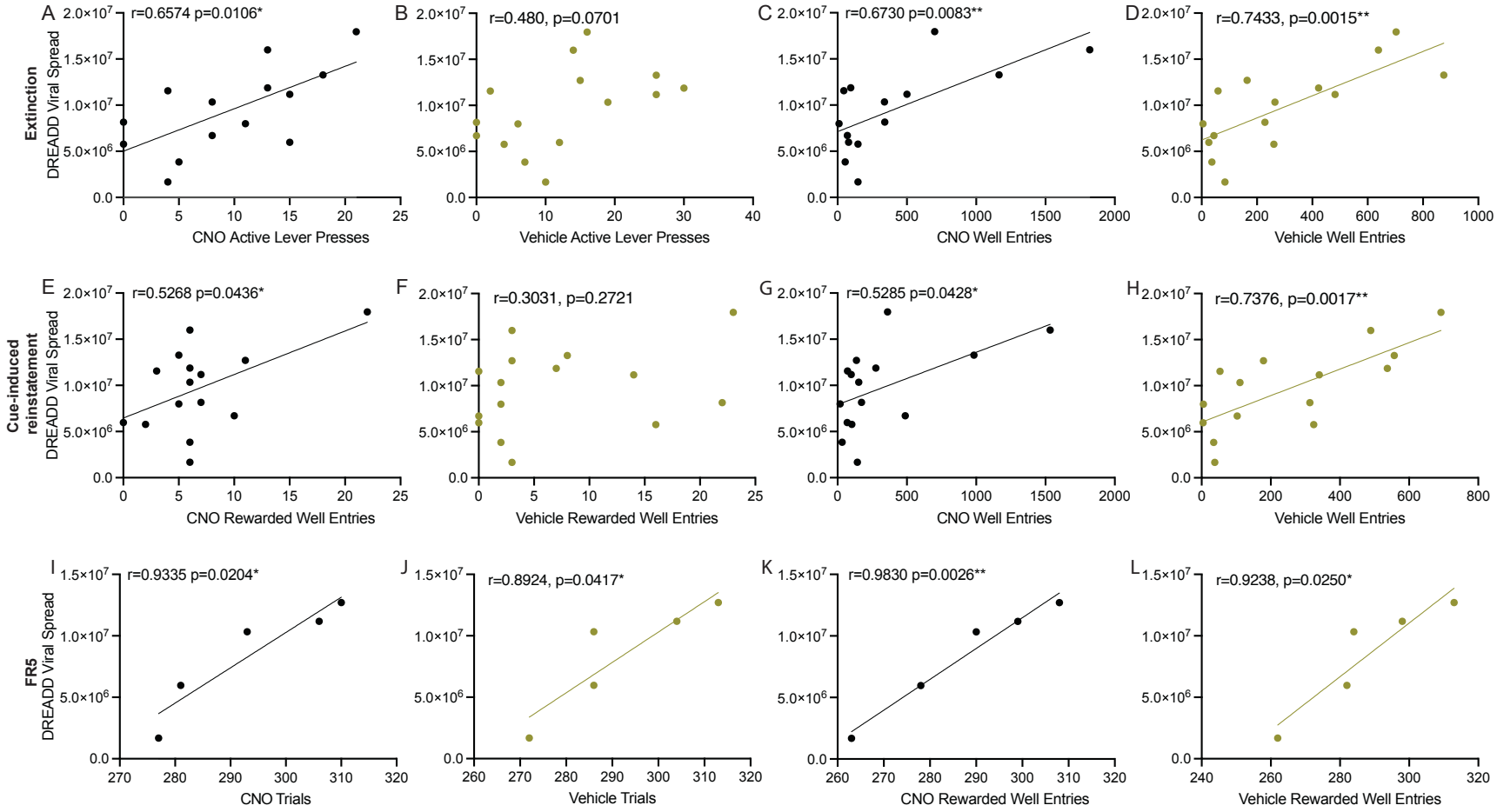

Figure 3.9. Correlation graphs for food restricted rats during extinction, cue induced reinstatement, and FR5 testing. (A,C,E,G,I,K) Black dots = correlations between DREADD viral spread and CNO behaviors; (B,D,F,H,J,L) Gold dots = correlations between DREADD viral spread and vehicle behaviors.

CHAPTER 4

DISCUSSION

4.1 Main Findings

This dissertation consisted of two main aims. Refer to Figure 4.1 for a schematic summarizing the main findings. In chapter 2, we describe our first aim where our goal was to understand the role of prelimbic (PL) and infralimbic (IL) medial prefrontal cortex (mPFC) in sucrose seeking behaviors. In chapter 3 we describe our second aim, which consisted of assessing differences in PL-NAc control of reward seeking behaviors for: 1) low-motivated/freed fed animals; 2) high motivated/food restricted animals. Results in chapter 3 show that PL-NAc differentially control sucrose seeking behaviors depending on how motivated they are to obtain the reward (i.e. level of satiety). We found that by controlling level of satiety, sucrose was a different type of reinforcer for free fed and food restricted rats. In free fed rats, sucrose was reward is pleasurable but not needed. In food restricted rats, sucrose turned into a need, because they had limited calories in the home-cage diet, rats needed the calories they could obtain from sucrose. With this in mind, the following is a summary of our main findings:

4.1.1 Infralimbic mPFC control of sucrose seeking behaviors

Results in chapter 2 showed that the IL plays a role in executing sucrose seeking behaviors during extinction (i.e. well entries) and cue-induced reinstatement (i.e. nose pokes, time-out nose pokes, and initiated trials). As described in detail in chapter 2, these rats were free fed and no sucrose was delivered during extinction and cue-induced

reinstatement. Extinction and cue-induced reinstatement testing was performed two to three weeks after their final Fixed-Ratio 1 (FR1) session, which is enough time to consider sucrose to have an effect of "craving" or "wanting" because of its palatable properties (Darling et al., 2016; Grimm, 2020). Thus, IL seems to play a role in mediating goal-directed behaviors of pleasurable rewards, which is why inactivating the IL decreased their motivation to continue seeking the sucrose.

4.1.2 Prelimbic mPFC control of sucrose seeking behaviors

Results in chapter 2 showed that the PL plays an important role in inhibiting sucrose seeking behaviors (i.e. nose pokes and rewarded well entries) during FR1 and executing sucrose seeking behaviors during extinction (i.e. nose pokes and well entries). These results show that the differential role the PL plays in sucrose seeking is dependent of the context where the reward is present (i.e. FR1) or not present (i.e. extinction). Specifically, when the animal is free fed, the PL seems to inhibit getting too much of a reward when the sucrose is available (i.e. FR1) and promotes sucrose seeking when the sucrose is not available (i.e. extinction). This could be because PL is in charge of "remembering the rules", or mediating the appropriate response to stimuli, given the internal state of the animal. Because they were free fed, the appropriate response during FR1 is to seek for sucrose until the desire is satiated, however, by inactivating the PL, the animals continued to seek the sucrose. Moreover, the free fed animals decreased sucrose seeking during extinction when PL was inactivated, facilitating extinction. Potentially, this context, the role of the PL was to seek the sucrose reward, but because there was no sucrose present and the PL was inactivated, they gave into their lack of motivation.

Because we only see this effect during FR1 and extinction recall, but not during cue-induced reinstatement, this suggests that PL might play a role in mediating the recall of sucrose seeking behaviors, but not the acquisition portion of cue-association.

4.1.3 PL-NAc control of sucrose seeking in low-motivated/ free fed rats

In low motivated/free fed rats, we saw an increase in sucrose seeking behaviors (i.e. initiated trials) when inactivating the PL-NAc during cue-induced reinstatement. Interestingly, we only see this effect on the first day of cue-induced reinstatement, which is the first day that rats are re-exposed to the cue after extinction training and testing. This finding provides more evidence that PL-NAc plays a role in establishing cue-outcome associations. Additionally, it seems to provide evidence towards the role PL-NAc plays in motivated behaviors. Sucrose is a reward for free fed rats since they do not have a calorie deficit, although sucrose is a highly pleasurable reward, it is not needed. When silencing the PL-NAc, these low-motivated/free fed rats seemed to have an increase in motivation to seek the sucrose which was triggered by the cue.

4.1.4 PL-NAc control of sucrose seeking in high-motivated/ food restricted rats

In highly motivated/ food restricted rats, PL-NAc inhibits sucrose seeking behavior that is not conducive to the reward during extinction and cue-induced reinstatement (i.e. inactive lever presses). A possible explanation for the inactivation effects we see could be driven by the fact that for food restricted rats, sucrose is a “need” in order to survive. Because they “needed” to find alternate ways of reaching their goal of obtaining the sucrose, they potentially engage in exploratory or alternate forms of the

previously learned behavior. And because we know that PL-NAc is important for forming cue-outcome associations, inactivating the PL-NAc potentially impaired the ability to form the association that inactive lever pressing does not result in sucrose delivery.

4.2 Proposed mPFC function for natural reward seeking

Based on the results of the studies mentioned above, in combination with the previous literature, I propose this model: the PL, IL, and PL-NAc circuit, work in combination to decide what the appropriate behavioral response is to the reward that the body needs or wants at that moment. The mPFC considers need vs want and also makes the cue-outcome association that inhibits reward seeking behavior in contexts where the reward is no longer present.

We can categorize rewards based on need vs want. For hungry rats, sucrose is a reward needed for survival, and for free fed rats, sucrose is a reward they find pleasurable, but not needed for survival. Under this classification of reward, cocaine and sucrose reward are both pleasurable, but when comparing cocaine and sucrose directly, we see that they have different properties in terms of biological mechanisms and value. Although we see craving and reinstatement of both cocaine and sucrose, cocaine is a reward often described as thought to relieve the negative state of withdrawal upon consumption (Koob, 2017). To date we do not have evidence that relief of negative state is also true when using sucrose reward in satiated rats. Therefore, it is imperative that we take into consideration the complexity of the mPFC and the vast neuroanatomical projections which contribute to the flexibility and dynamic function the mPFC plays. The

literature describes IL as an area that initiates behavioral and biological (i.e. body temperature) in response to appetitive stimuli (Lay et al., 2019; Quintana-Feliciano et al., 2021; Riveros et al., 2014; Valdés et al., 2006). These findings contribute to a better understanding of how and why we see differences between cocaine and sucrose reward literature. When looking into the specific roles PL and IL play in terms of sucrose seeking in satiated animals, PL seems to play a role in executing the previously established or "prepotent" behaviors in order to gain the sucrose reward and also regulating the intake (Capuzzo & Floresco, 2020), possibly in combination with the IL (Riaz et al., 2019). Hence, when inactivating PL we saw an increase in sucrose seeking during FR1, but a decrease in sucrose seeking during extinction. This is evidence towards the effects sucrose and sucrose cues exert over PL control of behavior. Potentially, the presence of sucrose and sucrose cues led the IL to increase sucrose seeking, and the role of the PL was supposed to stop the overconsumption of sucrose. But when PL was inactivated and there was no sucrose or cue to trigger this overconsumption response, neither PL or IL mediated a sucrose seeking role and therefore we see this decrease in sucrose seeking behaviors. Furthermore, when assessing the role PL-NAc play mediating food seeking behaviors, we see that sucrose cues trigger different responses depending on level of satiation. These findings provide further evidence that mPFC plays a dynamic role in assessing what the body needs at that moment, and responds differently according to the presence of the cue (Capuzzo & Floresco, 2020; Riaz et al., 2019; Stopper & Floresco, 2011; Valdés et al., 2006). A limitation that, if addressed, would further characterize the mPFC control of sucrose behavior would be to replicate the study

performed in chapter 3 by targeting IL-NAc projections. This way, we could understand the role of PL-NAc in sucrose cue-association.

Our findings can also give insight into other fields, like substance-use disorders. The behavioral control PL, IL and PL-NAc exert over reward seeking behaviors adapts depending on the internal needs of the animal and the contextual cues it receives from the environment. Although cocaine and sucrose are rewards that differ greatly in their chemical composition and biological effects, one could speculate that after repeated exposure of cocaine consumption- this reward could shift to a "need". Therefore, the mPFC could play a similar role for cocaine dependence such as what we see in food restricted rats seeking sucrose reward in order to fulfill their calorie deficit. This could potentially explain the various differences and discrepancies we see in studies using drugs of addiction vs food as rewards. It is possible that the mPFC plays different roles depending on the stage of drug use/abuse.

4.3 Concluding Remarks

This dissertation supports the idea that the simplistic PL "going" vs IL "stopping" model is not applicable to sucrose reward, instead, that the PL/IL mediates rewards differently depending on the reward and context being employed. Additionally, this dissertation supports an approach where we need to stop looking at whole mPFC sub regions, specifically at the PL and IL as separate, and instead further investigate cortical layers within the PL/IL and their concomitant projections to investigate their specific function. Specifically, because we provided further evidence that mPFC is a dynamic area that both assesses the needs of the body, performs the cue-outcome associations

needed to mediate the goal-directed response, and consequently triggers the appropriate behavioral response.

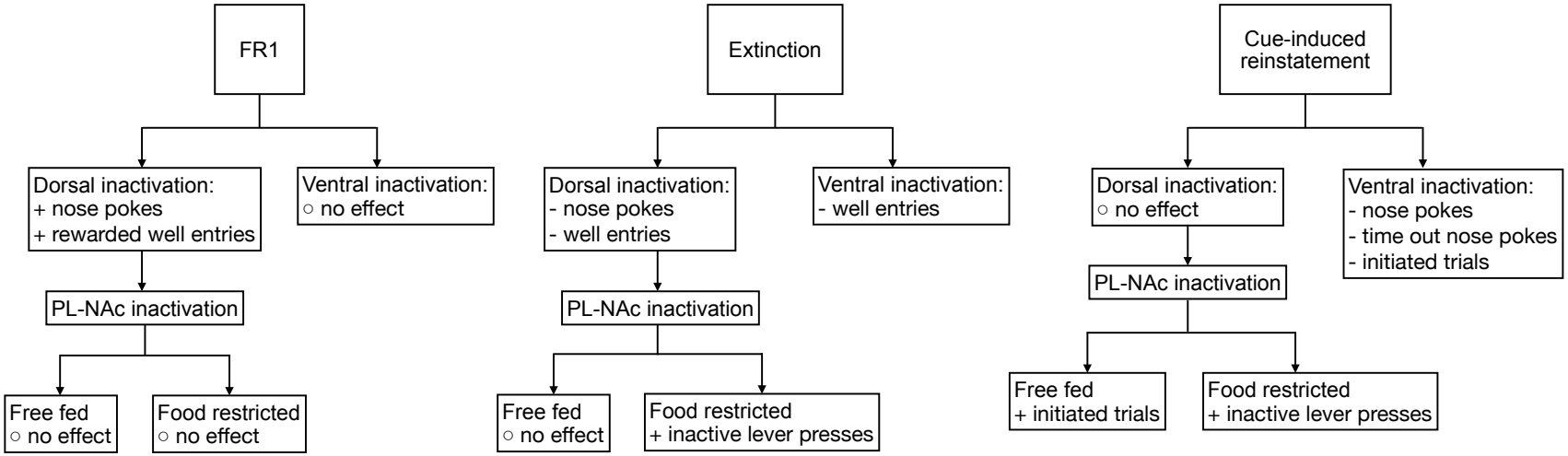

Figure 4.1 Summary of main findings.

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