Lever Pressing and Nose Poking

in the Rat Medial Prefrontal Cortex

by

Changtian Ye

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TABLE OF CONTENTS

LIST OF FIGURES

Page

ABSTRACT

The rodent medial prefrontal cortex (mPFC) is thought to be associated with a wide range of cognitive functions such as memory and goal-directed behavior. It can be further separated into two subregions, the prelimbic (PL) and infralimbic (IL) cortices. Common techniques employed to investigate functions of the mPFC, such as learning to nose poke or lever press for reward, are assumed to be regulated by the same brain regions and thus utilized interchangeably in research. This study questions that assumption and hypothesizes that nose poking and lever pressing are different behaviors regulated by different subregions of the mPFC. By examining and comparing acquisition of the two operant behaviors in rats trained on a fixed-ratio 1 schedule to respond to sucrose delivery by nose poking and lever pressing, respectively, the study considers the innate learning differences between the two behaviors. The study also considers effects of PL DREADD inactivation on acquisition, extinction, and reinstatement of both actions. Though receptor expression was confirmed with immunohistochemistry, PL inactivation yielded no significant behavioral effects. Inherent learning differences between the two behaviors, however, were indicated by the disparity in the number of days rats spent acquiring the respective behaviors. Although preliminary, the results of the study suggest that a larger sample size and analysis of both subregions of the mPFC may help to elucidate the role of the mPFC in different learning behaviors.

Introduction

The rodent medial prefrontal cortex, or mPFC, is thought to be associated with a wide range of cognitive functions such as decision-making, short-term and long-term memory, and goal-directed behavior. It may have a specialized and even lateralized role in evaluating and balancing costs of actions (Croxson *et al*., 2014, Zeeb *et al*., 2015). Considered to be part of the mesocorticolimbic system involved in reward and learning behaviors, the mPFC receives input from the amygdala, ventral hippocampus, and other limbic structures, allowing it to integrate and consolidate information such as contextual cues and environmental factors (Gourley and Taylor, 2016). Additionally, the mPFC projects to various other structures such as the amygdala, thalamus, and nucleus accumbens (NAc) (Vertes, 2004), which are involved in emotion regulation and sensory perception. That the mPFC is so complex and deeply involved in cognitive processes compounds the problem in inspecting functions and dynamics of the mPFC. In this study, I am interested in elucidating the role of the dorsal mPFC, or the prelimbic cortex (PL), in regulating two different behaviors acquired during reward-seeking; nose poking and lever pressing. These two behaviors are commonly employed and have been used interchangeably in research targeting the mPFC, but it is unknown whether the same regions of the mPFC are responsible for acquisition, extinction, and reinstatement of both actions. Results of this study would contribute to a better understanding of the functions of the mPFC in rewarding-seeking behavior and inform future research of effective experimental design.

Subregions of the mPFC

Although the rodent mPFC is a highly advanced and well-integrated cortical structure, it can be further separated into two main regions; the prelimbic cortex (PL) and the infralimbic cortex (IL). These two anatomically and functionally disparate subdivisions of the rodent mPFC form the ventral mPFC, with IL positioning ventral to PL. To note one particular difference in connectivity, PL projects to both NAc shell and core, and IL projects more selectively to the NAc shell (Vertes, 2004).

Physiological activity of the mPFC is dictated, in part, by the structural and functional interactions between afferent projections to the cortex, intracortical circuits, and efferent projections to other brain regions. As mentioned before, the mPFC receives input from various limbic structures, gathering and consolidating information, and provides output to various structures such as the thalamus and nucleus accumbens. Intracortical circuits within the mPFC consist of mainly pyramidal cells, which have glutamate-mediated excitatory projections, and interneurons, which have GABA-mediated inhibitory axonal arbors to the pyramidal neurons. Excitatory pyramidal neurons represent the vast majority (85%) of rodent mPFC neurons, whereas inhibitory interneurons compose the other 15% of cells (Gabbot *et al*., 1997). In particular interest to the current study, glutamatergic inputs to the nucleus accumbens (NAc) are primarily originated from the mPFC. These inputs facilitate conditioned drug and reward seeking behaviors (McGlinchey *et al*., 2017).

It is also important to mention that the rodent mPFC is comparable to the human mPFC, which makes it an excellent model from which to study the human brain. Based on the common input from the thalamus, the rodent PL is roughly analogous to the human Brodmann area 32, whereas IL is analogous to Brodmann 25 (Figure 1) (Gabbot *et al*., 1997).

Because of the small size of the rodent brain, many investigators divide the rodent mPFC into simply dorsal and ventral regions (Gass and Chandler, 2013).

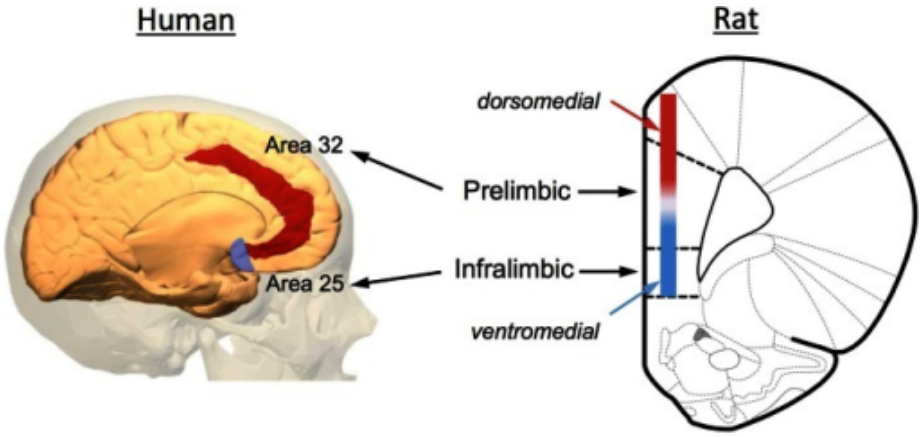

Figure 1: Anatomical depiction showing the location of the prelimbic (PL) and infralimbic (IL) subregions of the medial PFC of the rat and their equivalent regions of the human brain. (Adapted from Gass and Chandler, 2013)

Different roles of PL and IL in literature

Many results from various studies support a distinct dichotomy of roles between PL and IL. These studies utilize a multitude of research methods to examine motivation, learning, fear response, and substance studies in the context of manipulating the mPFC.

Conditioned Place Preference A common way to measure the motivational effects of objects or experiences is using conditioned place preference (CPP). By measuring the amount of time an animal spends in an experimental area that has been associated with or reinforced by a stimulus, researchers can infer the animal's preference for the stimulus. In rats trained to simultaneously acquire conditioned cue and place preference in a radial maze, short-term PL inactivation through blocking dopamine neurotransmission mitigated place conditioning learning, and IL dopamine receptor blockade attenuated conditioned cue preference (Hayen *et al.,* 2014). The findings indicate that PL is critical for the acquisition of Pavlovian place learning, while IL opposes the effects of PL during the learning processes.

Drug-seeking Studies looking at addiction behavior often target the mPFC and its associated areas. Limpens *et al*. (2014) found that pharmacological inactivation of PL reduced the effect of conditioned cocaine and sucrose-seeking suppression in self-administrating animals who were trained to lever press in response to cocaine reward. This experimental design utilized cocaine or sucrose self-administration in rats by lever pressing, and cocaine or sucrose seeking is suppressed by presentation of a foot shock. Naïve rats will stop pressing a lever for sucrose once they realize that they get shocked every time they do so, but PL inactivated rats would continue to seek sucrose even if they are being shocked. Therefore, the study suggests that reduced activity in PL could promote compulsive reward seeking, and that PL regulates compulsive behaviors. Martin-Garcia *et al*. (2014) obtained comparable results that optical inactivation of PL strongly increased cocaine self-administration via nose

poking, but inactivation of IL yielded no significant effects. However, in rats that self-administered cocaine at a high frequency, the same inactivation to PL decreased cocaine-reduced reinstatement of cocaine seeking. Cocaine-reduced reinstatement occurs after a rat has been completely weaned off of cocaine and then receives cocaine to reinstate their memory of self-administration. This is slightly different from context-induced reinstatement in that rats are given exposure to relevant contextual cues also present at the time of their earlier self-administration periods. The results from this study indicate that the PL may only exert control over non-adapted operant responses, or responses that have not yet become a habit. An earlier study had obtained similar results, though utilizing a lever press apparatus; TTX infusions into the PL blocked both stress and cocaine induced reinstatement, but IL inactivation had no effect (Capriles *et al*., 2002). Overall, data from these studies strongly suggest that PL activity is critical for regulating compulsive reward-seeking and preventing reinstatement in substance-of-abuse studies.

Fear Response In fear response experiments, rats are often trained to associate a negative stimulus of some sort, i.e. an electric foot shock, with a neutral stimulus, i.e. a tone. During a trial, a shock is administered simultaneous to a tone being played. Immediately following that, the rats will display a common fear response called freezing, which involves them crouching and pausing all movement for a period of time. This is a common biological response to the foot shock. Once the acquisition of the fear response is complete, rats will freeze upon hearing the tone and in anticipation of the foot shock, even if the shock is absent. During the extinction phase of an experiment, a rat will receive just a tone and no foot shock in order to disassociate the two behaviors. Extinction of conditioned fear is an active learning process involving inhibition of fear expression, therefore, it is similar to the learning or acquisition stage in that they both require active recruitment of cognitive regions of the brain. In one fear response experiment, the researchers found that PL inactivation did not

impair acquisition of freezing behavior during both training and reinstatement, but decreased the number of fear responses, i.e. freezing, during the extinction period. On the other hand, IL inactivation disrupted long-term retention and recall of fear extinction (Laurent and Westbrook, 2009). Results from this study suggests that IL activity is strongly implicated in inhibitory learning, whereas PL activity enhances fear responding. Sangha *et al*. (2014) found that rats who received pharmacological IL inactivation were unable to discriminate between the pairing of a foot with a cue and the presentation of a safety cue which was not paired with a shock, whereas rats who received PL inactivation exhibited reduced freezing during the shock-associated cue as compared to saline control. These results support the role of IL in inhibitory learning, and the researchers further considered that the imbalance of activities between PL and IL could be involved in anxiety and addiction disorders. Other groups have reported corroborating data. While looking at neuronal activity in PL and IL, Santini *et al*. (2008) found that in comparison to naïve animals who have yet to receive fear conditioning, well-trained rats experienced increased burst spiking in IL neurons during extinction, whereas PL neurons of the same rats were not observed to exhibit the same changes. This also supports the role of IL in retaining extinction of a learned behavior. Various other studies have also shown that inactivation of PL impaired extinction learning and fear expression after extinction (Kim *et al*., 2015, Sierra-Mercado *et al*., 2010). In general, results from fear-conditioning studies consistently support the notion that IL activity is crucial in the learning of inhibition of fear. In contrast, PL is more involved in the expression of fear responses.

Appetitive conditioning Many studies utilizing appetitive conditioning found results supporting distinct roles of different regions of the mPFC. Appetitive conditioning is different from aversive conditioning, i.e. fear conditioning, in that the rats are presented with a desirable unconditioned stimulus, such as candy, and a neutral stimulus, such as a tone.

The rats learn to associate the unconditioned stimulus with the neutral stimulus (NS), turning the NS into a conditioned stimulus. In a study done by Burgos-Robles *et al*. (2013), researchers found that different regions of the mPFC regulate different aspects of appetitive behavior. In the study, rats were trained under a variable interval schedule to lever press for sucrose reward. The variable interval schedule varies from the fixed ratio schedule in that rewards are given over variable intervals of time instead of given after a fixed number of lever pressing behaviors. Recordings from both PL and IL neurons differed in that even though both signals were prominently excitatory during rewarded lever presses, PL signals were faster and more transient, whereas IL neurons displayed delayed and prolonged responses. In addition, the group performed local pharmacological inactivation of PL and IL, respectively, and found that inactivation of PL had no effect in changing reward-seeking behavior, but inactivation of IL significantly delayed the collection of earned reward. This suggests that IL, not PL, is responsible for the rat physically going to collect earned reward. Similarly, Killcross and Coutureau (2003) also trained rats to lever press for sucrose and food pellet rewards. This group found that naïve rats whose brains have not suffered lesions and have not undergone extended training will usually show sensitivity to changes in the value of reward, such as a change in the number of reward, but this sensitivity will generally be lost after overtraining. PL-lesioned rats showed no sensitivity to changes in goal value in neither limited nor extended training, but IL-lesioned rats showed increased sensitivity to goal value even after the behavior has become a habit. This suggests that PL is responsible for maintaining goal value and corresponding action, and IL mediates the tendency of extended training to override goal-directed responses. In contrast to Killcross and Coutureau's findings, Dalton *et al.* (2016) found that in rats trained to complete a probabilistic reversal learning task involving lever presses (80% reward on correct responses, 20% on incorrect responses), PL inactivation enhanced sensitivity to recently rewarded actions and diminished

sensitivity to negative feedback, but IL inactivation produced no significant effect. Another study found supporting evidence that in vivo photostimulation of PL inhibiting neurons will accelerate extinction of a well-learned cue-reward association in which rats will exhibit high anticipatory and reward licking after cue presentation (Sparta *et al*., 2014). The above studies all suggest a distinct role for PL and IL in learning associations.

Interactions within the mPFC

Though much literature supports the strict dichotomy of PL and IL functions, some research has found indications that the case might not be as straightforward. Recordings from acute rodent brain slices provide support for PL and IL interaction during cognitive processes. During fast network oscillations, which are linked to cognitive processing and working memory capacities, PL and IL can maintain fast oscillations independent of each other when they are not connected. But when they were joined, the difference in frequency disappeared (Van Aerde *et al*., 2008). Richard and Berridge (2012) found that intense motivations such as addiction, which are often generated by NAc shell glutamate disruptions, were powerfully inhibited by IL activation. This finding, opposing that of Limpens et al. (2014), suggests that IL regulates compulsive behaviors instead of PL and possibly indicates PL and IL interaction. In a separate study, Willcocks and McNally (2013) noticed that PL activity is important for context-induced reinstatement of extinguished alcohol seeking, but inactivation of IL didn't prevent expression of extinction. This study employed nose poking as an operant response. PL also seemed to potentiate responding to alcohol during rapid acquisition reinstatement. Overall, the data do not support a strict dichotomy of PL and IL in promoting and prevent relapse. Cue directed inhibition experiments also provided insight into the complication. A 2014 study found that rats that received PL lesions before training were not able to discriminate between cues that indicated availability of food distribution, similar to the findings of Sangha *et al*. (2014). However, after overtraining, PL-lesioned rats exhibited marginally significant decrease in discrimination, whereas IL-lesioned rats saw a significant decrease. However, IL lesion effects were transient (Meyer & Bucci, 2014). This supports a PL and IL interaction during Pavlovian conditioned inhibition, as well as complex interactions between IL and other cortical regions, which seemed to compensate for loss of IL activity. In one appetitive Pavlovian conditioning experiment, inactivating IL facilitates

extinction of learning, but inactivating PL had no effect. In comparison, there was no impact of IL or PL inactivation on acquiring or recalling extinction under instrumental conditioning involving lever presses (Mendoza *et al*., 2014), contrary to converging evidence in the literature that IL suppresses conditioned responding during extinction and PL promotes responding. Finally, Moorman and Aston Jones (2015) recorded PL and IL neurons during discriminative stimulus sucrose seeking via lever pressing and found rapid cue-evoked activity in both PL and IL during extinction of behavior. Neurons in either regions can contribute to response inhibition; PL neuronal responses were equally divided between excitation and inhibition, whereas IL signals were mostly inhibitive. It is currently unknown whether these contradicting evidences are due to difference in the nature of used experimental behaviors, i.e. lever pressing and nose poking, or other complications. Most studies utilize lever pressing as the operant behavior, whereas certain studies trained rats to nose poke.

Differences between nose poking and lever pressing in rats

Lever pressing seems to demand a higher cognitive function involving advanced motor skills, while nose poking is a species-specific behavior in rats with evolutionary significance. Schindler *et al*. (1993) compared nose poking and lever pressing in Long Evans rats in a fixed-ratio 5 task and concluded that the nose poking response would be particularly useful in studying the acquisition of operant responses due to the quick acquisition period, as compared to lever pressing. Nose poking was also sensitive to manipulations in experimental design, such as delay of reinforcement and food deprivation levels. Rats quickly learn how to perform tasks involving nose pokes, but experience difficulty when acquiring a new motor behavior, lever pressing in this case. Support for the inherent biological difference between nose poking and lever pressing is found in a 2015 study measuring dialysate dopamine levels in the NAc. The NAc consists of two structurally and functionally disparate subdivions: NAc shell and core. As mentioned before, PL projects to both NAc shell and core, and IL projects more selectively to the NAc shell (Vertes, 2004). Bassareo *et al.* (2015) found that rats trained on an FR1 schedule to lever press for sucrose pellets exhibited an increase of dialysate dopamine concentration in both NAc shell and core, whereas rats trained on an FR1 nose poke schedule only exhibited dopamine increase in the shell. This finding is particularly salient in that it suggests that the NAc shell and core are responsible for different actions. Similarly, for rats trained on an FR5 schedule, immunoreactivity in the accumbens shell significantly increased on the first day, and core activity only increased on the second day. This suggested that for lever pressing, different subregions of the striatal complex are differentially activated (Segovia *et al*., 2012). Therefore, it is possible that PL and IL are differentially responsible for lever pressing and nose poking.

Concerning different learning behaviors, aforementioned studies indicate a functional disparity between PL and IL, which are closely connected to different regions of the NAc. In addition, further research concerning the differences and advantages between separate types of operant behaviors may inform future researchers in devising their experimental design. The proposed study will compare the length of time needed for acquiring the behavioral tasks, to confirm the results of Schindler *et al*. (1993) on an FR1 schedule. I hypothesize that rats trained on a FR1 task would acquire the nose poking task much faster than those trained with lever pressing. If the acquisition time for the two behaviors are different, it is worth discussing why these differences exist.

Finally, in order to deconstruct functions of the mPFC, investigations must be done to understand the type of responses or contextual cues for which each region of the mPFC—in this case, PL and IL—is responsible. That PL inactivation seemed to have the most potent effects and that IL effects seemed to be transient leads the current study to focus on investigating whether PL plays a different role in behaviors such as lever pressing and nose poking. In other words, does PL inactivation differentially effect lever pressing and nose poking during acquisition, extinction, and reinstatement of reward seeking behavior during an FR1 task? The study hypothesizes that PL inactivation will decrease the number of lever presses during acquisition, extinction, and reinstatement testing in rats who have learned the lever press but will not affect the number of responses in nose poking rats.

Methods

Animals

All experiments were conducted in accordance to University of Massachusetts Institutional Animal Care and Use Committee protocols and in compliance with the National Institute of Health guidelines. Six Long-Evans rats (~9 weeks old and 275-300g upon arrival; Charles River) were housed individually and maintained on a 12hr light/ dark cycle (7:00am lights on and 7:00pm off) at University of Massachusetts. To eliminate sex differences in the cohort, all rats were male. Each rat was given a wooden block with which to play in their home cage. All rats were allowed *ad lib* food and water before surgery and were maintained on a food-restricted diet (20g of food per day) starting from 7 days after surgery and lasting until the end of the experiment.

DREADDS

Designer Receptors Exclusively Activated by Designer Drugs, or DREADDs (Armbruster *et al*., 2007), is a common chemogenetic technology used for intervention of cellular activity in lab animals (Roth, 2016). To achieve this end, the technology posits the use of mutated human muscarinic receptors, i.e. hM3Dq and hM4Di, which are activated exclusively by an inert compound clozapine N-Oxide (CNO). CNO seems to have a long *in vivo* residence in rodents following administration, and the effects of CNO-mediated inactivation is robust and prolonged (Bender *et al*., 1994). A virus carrying the gene expressing the receptors is first engineered and then injected into the area of interest, thus infecting the host cell and integrating the gene for the receptors into the genome of the host cell. The host cell will begin to express the receptors, which can be activated or inactivated by attaching to CNO. These receptors are G-protein coupled receptors and have the ability to induce membrane potentials or inhibit neuronal firing (Roth, 2016). Therefore, by injecting

CNO into a lab animal who has received DDREADDs, researchers can manipulate the activity of the targeted cells. In the present study, I injected a virus locally into PL neurons in rats, and once the cells were given enough time to express the receptors, I followed with injections of CNO into the body cavity (intraperitoneal, IP) in order to inactivate PL neurons (Figure 2).

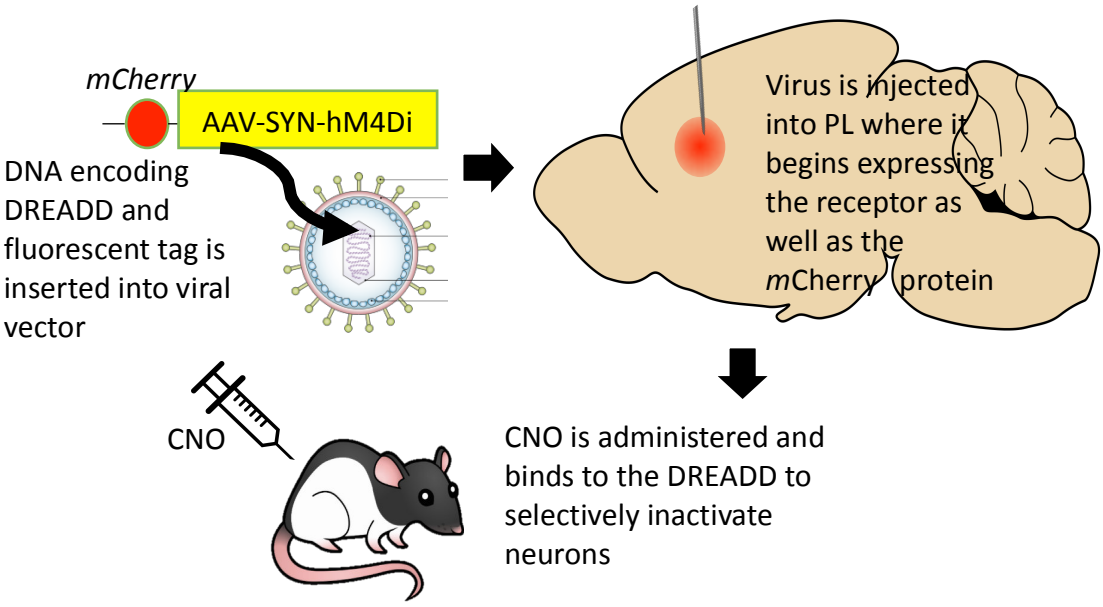

Figure 2. Diagram of CNO inactivation of DREADDS.

Stereotaxic Surgery

Rats were anesthetized with 5% isoflurane in a closed container and then quickly transferred to a stereotaxic frame, where they received 1.5%-2% isoflurane through a nosecone for the duration of the surgery. Ear bars attached to the stereotaxic frame provided stability to the skull. Following that, rats received injections of systemic antibiotics (0.1 mL cefazolin) and analgesics (1mg/kg meloxicam). After clearing the surgical region of excess hair, I made incisions on their scalps to provide access to the skull. These incisions were treated with a local anesthetic (0.3mL, 2% Lidocaine). Bilateral intracranial holes were drilled directly above the mPFC to enable injector access to the brain. 500 nL of a virus carrying designer receptor genes (Addgene AAV-SYN-hM4Di-*mCherry*) were delivered to

PL (+3mm AP; ±0.6mm ML; -3.5mm DV) of each hemisphere through house-made glass injectors using a micro-infusion pump (Micro 4, World Precision Instruments, Sarasota, FL). The injections took 10 minutes each. When injections were complete, holes in the skull were repaired by bone wax and incisions were sutured. More local anesthetic was applied to minimize pain upon waking up. Rats had one week to recover following surgery before any behavioral testing began. Consequently, rats were given free access to water but were restricted to 20g per day of standard laboratory rat chow.

Apparatus

All operant testing sessions were conducted in Med Associates chambers housed in sound attenuation cubicles (Med Associates, Fairfax, VT, USA). Each rat had their own operant chamber for the duration of the study, and there were six chambers for six rats. Two nose poke wells (Med Associates, Fairfax, VT, USA) or two lever press boxes (Coulbourn Instruments, Langhorn, PA) were attached to one wall of each chamber (Figure 3). Three chambers had two nose poke wells, and the other three had two lever boxes. In each chamber, only one nose poke or lever press apparatus was active, and the other remained inactive, meaning a nose poke or lever press did not evoke a reward. A sucrose-delivery well lay in the middle of the same wall, between the two nose pokes or lever presses. It dispensed a sucrose reward (0.1 ml of 15% sucrose solution) during the experiment. Each of the nose poke apparatuses and delivery well housed a laser, and the number of nose pokes or well entries were calculated by the number of photobeam breaks. Each chamber also accommodated a house light and a fan that provides a 61dBA background noise.

Figure 3. Example of the behavior chamber set up for nose poking and lever pressing rats. Each behavioral chamber has either two lever presses and nose poke apparatuses, a house light, a fan, and a sucrose delivery well. The height of the nose poking cone was adjusted to be equal to that of the levers.

Behavioral training

Animals were trained to either nose poke or lever press for sucrose on a fixed-ratio 1 (FR1) schedule until criteria (75% correct trials, >50 rewarded nose pokes for two days straight). Trials in which the rat exited the nose poke or stopped pressing a lever to enter the well in less than a second following sucrose dispensing constituted correct trials. Each nose poke or lever press resulted in 0.1 ml 15% sucrose delivery in the well. Each training session lasted 2 hours, and each rat underwent one training session per day. A 15 second house light illumination signaled session initiation, during which nose poking and lever pressing behavior did not elicit reward. From then on, each nose poke and lever press, respectively, evoked a tone (15 kHz, 68 dBA, 1 second) and simultaneous delivery of sucrose in the well. A computer program (MedPC IV) is used to calculate the number of nose pokes or lever presses, number of well entries, and number of rewards elicited in each chamber. During extinction, nose poking or lever pressing elicits no response. In reinstatement, nose poking or lever pressing elicits a tone but no sucrose delivery (Figure 4).

CNO/Saline Injections

Rats were given intraperitoneal injections of either CNO (3g/kg weight, in 0.17%DMSO and 0.9% saline, 1mL/ mg of weight) or 0.9% saline before each testing session and were placed into operant boxes 30 minutes after injections. All rats received one injection of 0.9% saline (1 mL/kg of weight) before any CNO injections to habituate them to the injection process. Testing began after the animals reached criteria.

Testing

Acquisition All testing sessions were 2 hours long, and each rat performed one session per day. Both lever press and nose poke rats followed the same schedule for acquisition

testing. On the first day, all rats received a sham injection with 0.9% saline. On the second day, rats were run on an FR1 schedule and received one injection, either saline or CNO. On the third day, the rats received no injections and continued on the FR1 schedule. On the fourth day, they received either CNO or saline injections in counterbalanced order. Whichever injection they did not receive on the second day, they received on the fourth. The exact testing and injection schedule is included at the end of the methods section (figure 5).

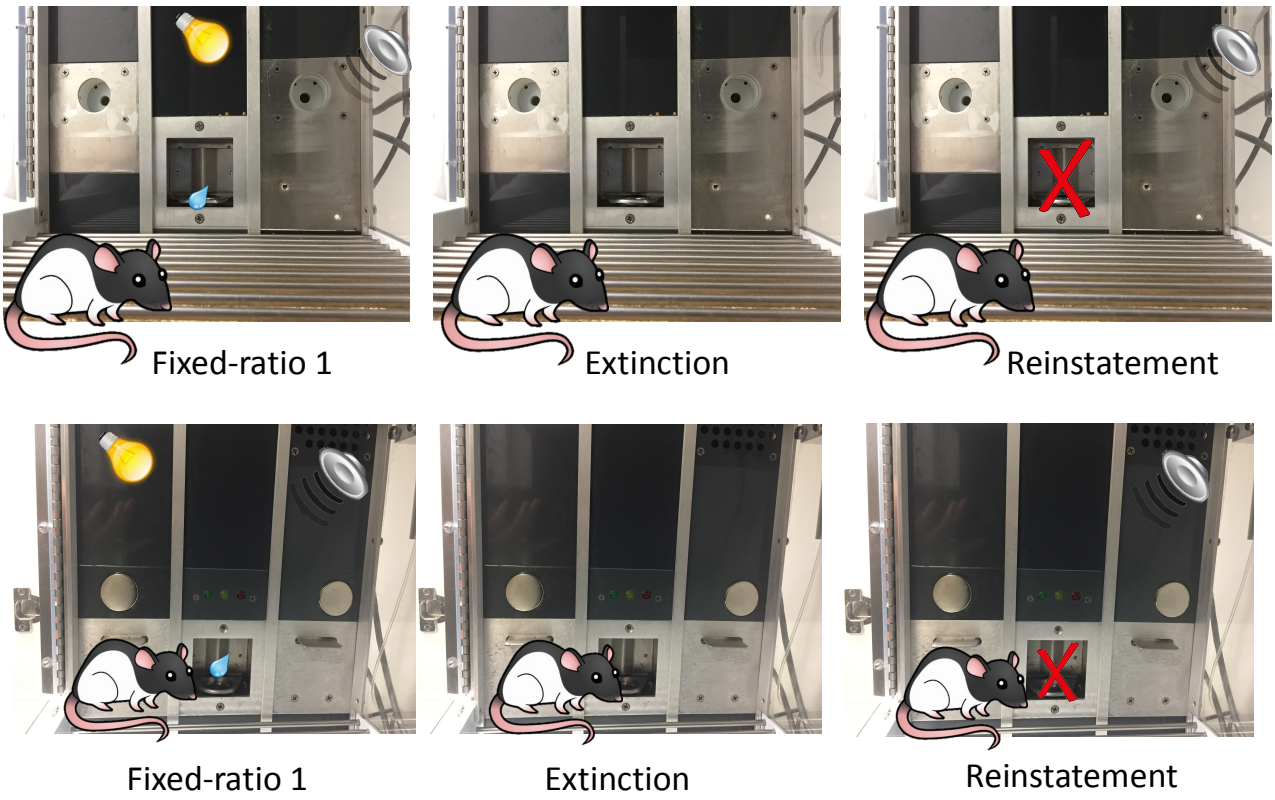

Figure 4. Schematic of FR1 task, extinction training, and reinstatement of behavior for nose poking and lever pressing rats. During FR1, the house light illumination signals the start of the session. Nose poking or lever pressing elicits a tone and simultaneous delivery of sucrose in the well. During extinction, nose poking or lever pressing elicits no response. In reinstatement, nose poking or lever pressing elicits a tone but no sucrose delivery.

Extinction The purpose of extinction is for rats to forget the association they made between the learned behavior and the reward, as the behavior will no longer be reinforced with a desirable reward. Both lever press and nose poke rats followed the same schedule for

extinction training and testing. During extinction training, rat nose poking and lever pressing elicited no sucrose reward or tone, but all nose pokes, lever presses, and well entries were recorded in the program. Extinction testing will be separated into two segments; early extinction and late extinction. Early extinction examines the ability of rat to learn to forget a behavior-reward association, whereas late extinction examines the ability of a rat to retain the forgotten association. On the first day of extinction training, rats received either a CNO or a saline injection. After two more days of FR1 training, I administered a counterbalanced injection to each rat on the fourth day of extinction. This is the early extinction segment of extinction testing. Starting from day 5 of extinction, rats continued to extinguish until they have reached criteria (less than 20 active nose pokes in two consecutive days). This is the late extinction segment of extinction testing. Immediately thereafter they received one day of either CNO or saline injections, one day without injections, and one day of a counterbalanced injection, all while running on the extinction schedule. Finally, rats ran on one last day of extinction before reinstatement.

Reinstatement Reinstatement often models relapse in alcohol and drug abuse studies. In this study, I am using it to model relapse to food seeking. Nose pokes and lever presses during reinstatement sessions elicited a tone but not sucrose reward. All nose pokes, lever presses, and well entries were recorded during the sessions. On the first day, rats received either one CNO or saline injection. Following that, they ran on extinction until criteria (< 20 nose pokes for two consecutive days). After reaching criteria, the rats received the counterbalanced injection on the last day of reinstatement testing.

Progressive Ratio After all reinstatement sessions were completed, rats were run on a progressive ratio task for two days, with 2 FR1 days in between. The progressive ratio task

differs from the FR1 schedule in that it requires the rat to complete twice the number of behaviors as the they did for the previous reward in order to obtain the next reward (0.1 ml 15% sucrose delivery). For example, a rat will first lever press once and receive a sucrose reward. To get the next reward, he would need to press the lever two more times. To get the n^{th} reward, he would need to press 2^n more times. Rats also received either a CNO or a vehicle injection 30 minutes before testing began on each of the progressive ratio days.

Immunohistochemistry

After the final reinstatement session, rats were euthanized with a high dose of ketamine/Xylazine (1.4 ml/kg IP) and transcardially perfused using 0.9% saline and 4% paraformaldehyde. They received one shot of either CNO or saline before they were euthanized. I removed the brains and fixed them in 4% paraformaldehyde overnight and transferred them the following day to 20% (weight/volume) sucrose solution and 0.1% sodium azide in phosphate buffer to be stored at 4°C. 40 μm thick coronal slices of the brains were obtained using a cryostat and kept in PBS-azide (phosphate buffered saline w/ azide) in individual well plates until immunohistochemistry (IHC). To check for receptor expression as well as activation of neurons, I employed parallel fluorescent double labelling of mCherry and *c-fos*.

Earlier in the study, I infused a virus into the brains of the rats to be able to inactivate specific neurons in the PL by giving rats CNO injections. The virus, which carries the designer receptor gene that expresses a G-protein coupled receptor in the cell, also carries a gene that will express a fluorescent protein, mCherry. Derived from the protein DsRed, which is isolated from Discosoma sea anemones, mCherry is commonly used as a cellular marker to tag molecules and cell components. In the case of DREADDS, if the virus had

successfully integrated its genes into the genome of a neuron, mCherry should be present throughout the entire cell.

C-fos is a proto-oncogene expressed in some neurons consequent to depolarization (Bullitt 1990). Similar to mCherry, the product of this expression, *c-fos* protein, can also be used as a cellular marker for neuronal activity. In neurons that have been recently activated, *c-fos* protein can be identified using immunohistochemical techniques. In cells that have been inactivated, no *c-fos* should be present.

Brain slices stored in PBS-azide were first washed three times in PBS. All washes are five minutes in length. In between washes, I moved the slices to different well plates with a brush. Slices were then washed three times in PBST (phosphate buffered saline w/ Triton, pH 7.4) and blocked in PBST and 3% NDS (normal donkey serum) for 60 minutes. After blocking, the brain slices were incubated overnight with primary antibodies diluted in PBST and 3% NDS. All incubations were done on a shaker at room temperature. The primary antibodies will recognize the aforementioned protein markers, mCherry and *c-fos*, and bind to the proteins. Millipore Anti-*c-Fos* (Ab-1) Mouse mAb (2G9C3) and Clontech Living Colors DsRed Polyclonal Rabbit Antibody were used, both at a concentration of 1:500, or 5 μg/mL. The following day, I washed the brain slices in PBST for three times and transferred all of the slices into a dark room. Slices received a second incubation with fluorescent dyes conjugated to secondary antibodies diluted in PBST + 3% NDS for two hours at room temperature. The secondary antibodies used were Invitrogen Alexa-Fluor Donkey anti-mouse 488 and Invitrogen Alexa-Fluor Donkey anti-rabbit 594, both at 1:500 concentration. The functions of secondary antibodies are to bind to the primary antibodies and amplify cellular signal. After incubation, the brain slices received one PBST wash, one PBS wash, and one 0.1M PB (phosphate buffer) wash. Sections were directly mounted from 0.1M PB onto non-coated glass slides, protected with Citifluor and coverslips, and sealed with nail polish at the

coverslip edges. Using a fluorescent microscope, I qualitatively assessed the slides for receptor expression and location in reference to a rat brain atlas (Paxinos and Watson 2007), to confirm virus injection sites. If rats did not show receptor expression, their data would be excluded from analysis. In addition, I checked for colocalization of mCherry and *c-fos*.

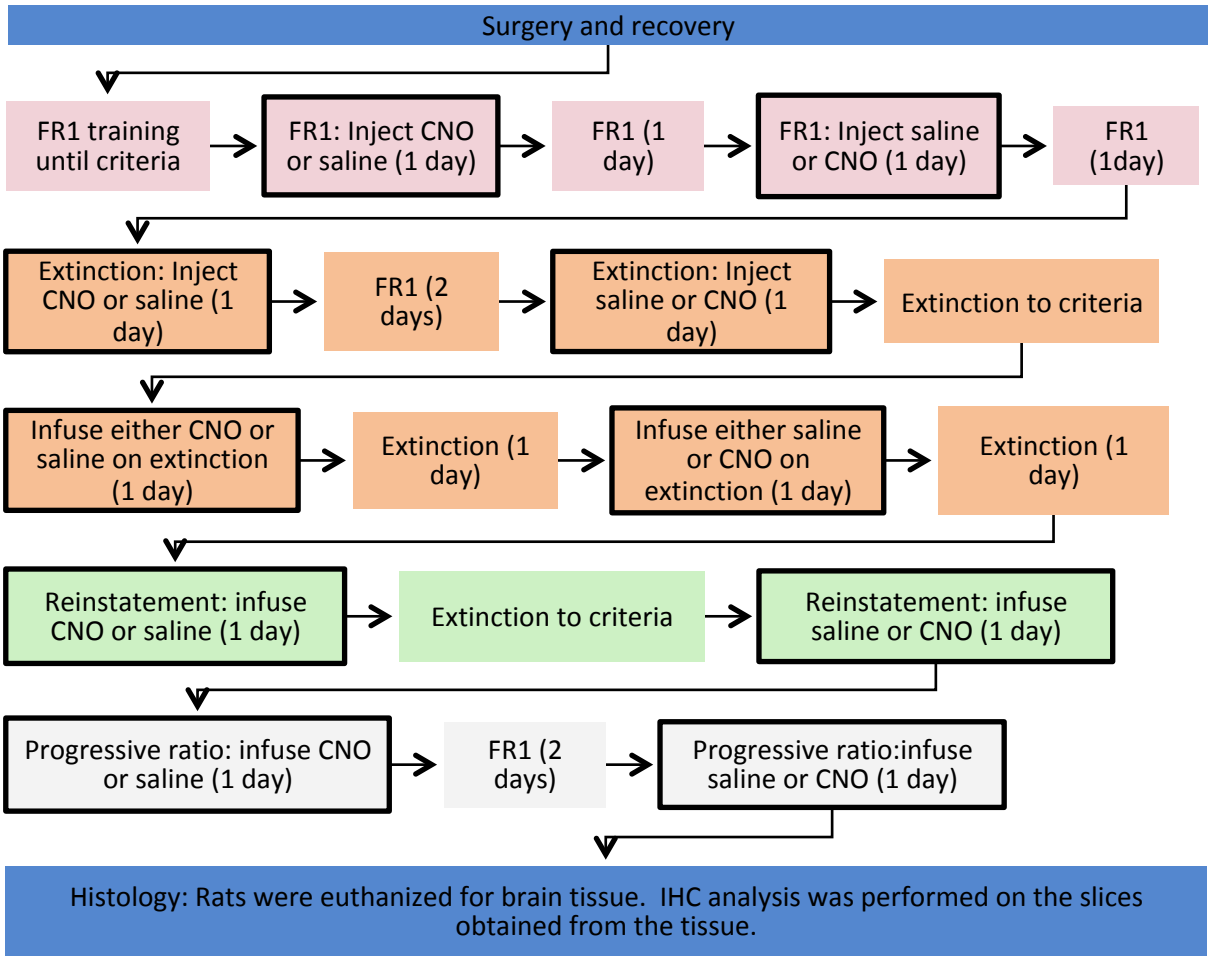

Figure 5. Flow chart of methodology for both lever pressing and nose poking rats. On each testing day (outlined in black), each rat received a counterbalanced shot of either CNO or saline vehicle 30 minutes before entering the behavioral chamber.

Data Analysis

I analyzed data collected from MedPC IV using statistical software Prism (GraphPad Software, La Jolla, CA, USA). For the FR1 task, I compared the number of training days completed for nose poking and lever pressing rats, respectively. The total number of nose pokes and lever presses for each testing day and injection type were calculated and assessed for differences using a paired t-test.

RESULTS

During the acquisition period for the FR1 task, I compared the number of training days for nose poking and lever pressing rats, respectively, using Prism. The average number of correct trials and the average percentage of correct trials were calculated and compared. Additionally, the total number of nose pokes or lever presses that each rat performed for each testing day were calculated and assessed for differences using a paired t-test in Prism. An alpha level of .05 was used for all statistical tests.

Acquisition of the FR1 task

Acquisition time was measured by examining the number of days rats were run on the FR1 program until they reached 75% correct trials and retained a performance of more than 50 behaviors for two days straight. A correct trial is defined as a trial in which the rat exited the nose poke or stopped pressing a lever to enter the sucrose delivery well in less than a second following sucrose dispensing.

As shown in Figure 6, the mean number of learning days for lever pressing on an FR1 schedule ($M = 15$, $SD = 3.606$) was significantly higher than that of nose poking ($M = 5$, $SD = 0$, $t = 4.804$, $p = 0.0407$).

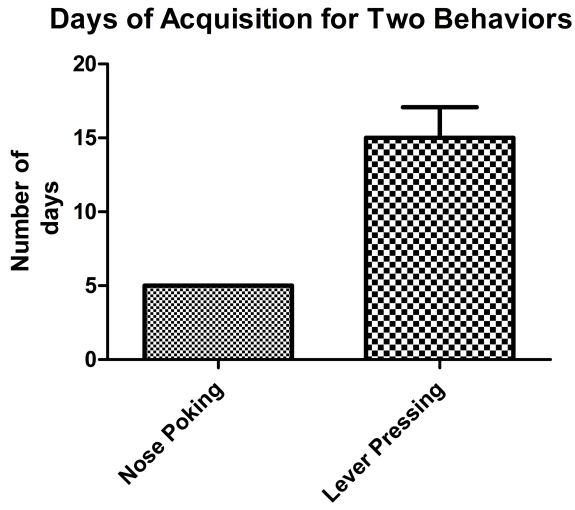

Figure 6. Acquisition time for each behavior during learning of the FR1 task

Initially, nose poking rats responded at a faster rate than lever pressing rats (Figure 7. a, b). On the first day, nose poking rats achieved a mean of 47.7 correct trials or 29.1% correct trials, whereas lever pressing rats took ten days to achieve the same level of response.

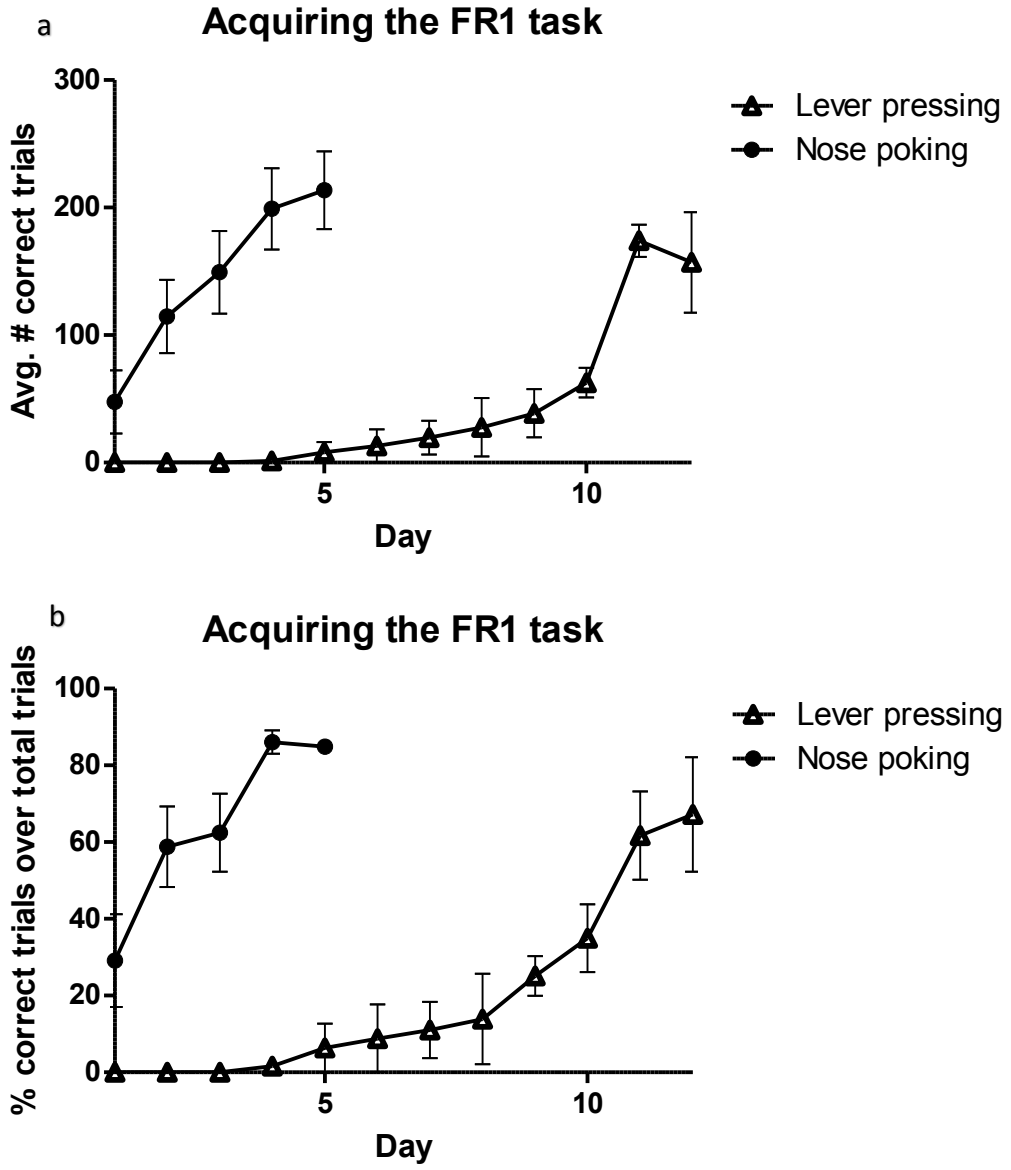

Figure 7. Initial response rates for each behavior during learning of the FR1 task. a. A comparison of the average number of correct responses for nose poking and lever pressing. All three rats in the nose poking group reached criteria on the fifth day, therefore no data points were entered in the graph after the fifth day. The first lever pressing rat to reach criteria did so on the 12th day, therefore lever pressing data shown here continued until the 12th day. b. A comparison of the percentage of correct responses over total trials for nose poking and lever pressing. Initial response rates of nose poking rats were much higher than that of lever pressing rats.

Testing during FR1

CNO injections did not yield significant effects for either nose poking or lever pressing behavior during FR1 (Figure 8). For nose poking rats, there was an insignificant difference between the number of nose pokes performed after receiving a CNO injection (M =344, SD= 141.1) and after receiving a vehicle injection (M = 369.7, SD = 75.94), t = 0.3372, p = 0.7681. For lever pressing rats, there was an insignificant difference between the number of lever presses after receiving a CNO injection (M = 685.3, SD = 402.7) and after receiving a vehicle injection (M = 454.3, SD = 135.9, t =1.157, p = 0.3666).

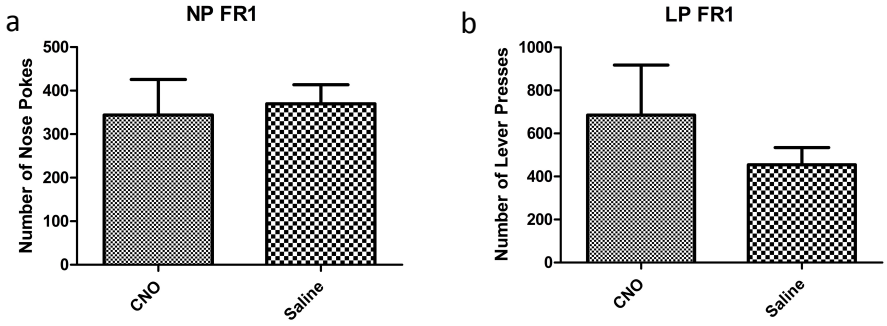

Figure 8. Effects of CNO vs. vehicle on number of nose pokes (a) and lever presses (b) during acquisition

Testing during extinction

The extinction phase is divided into segments; early extinction and late extinction. Early extinction examines the ability of rat to learn to forget a behavior-reward association, whereas late extinction examines the ability of a rat to retain the forgotten association. During each of the testing days of the late extinction phase, I ran the rats on FR1 instead of extinction due to manual error, thus analysis of the late extinction phase was excluded in the results. CNO injections did not yield significant effects for either nose poking or lever pressing behavior during early extinction (Figure 9). For nose poking rats, there was an insignificant difference between the number of nose pokes performed after receiving a CNO injection (M = 128, SD = 62.3) and after receiving a vehicle injection (M = 67, SD = 46.33, t = 0.9860, p= 0.4281). For lever pressing rats, there was an insignificant difference between the number of lever presses after receiving a CNO injection (M = 106.0, SD = 33.78) and after receiving a vehicle injection (M = 161.7, SD = 102.2, t = 0.8345, p = 0.4918).

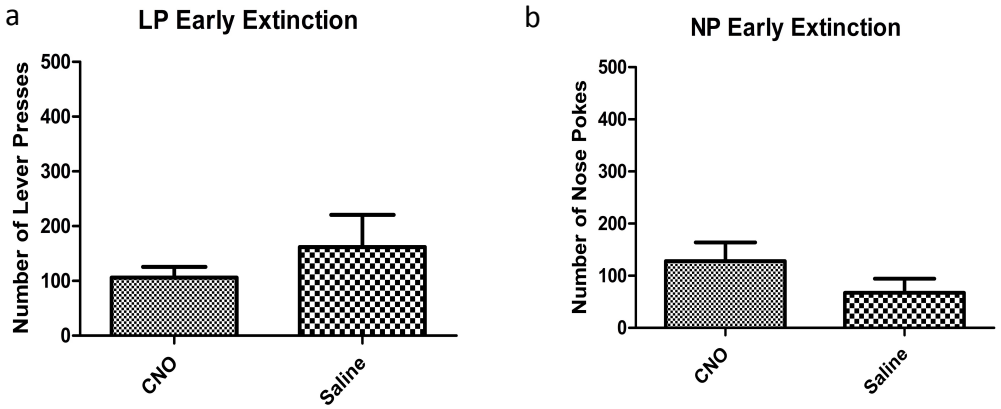

Figure 9. Effects of CNO vs. vehicle on number of nose pokes (a) and lever presses (b) during early extinction

Testing during reinstatement

CNO injections did not yield significant effects for either nose poking or lever pressing behavior during reinstatement (Figure 10). For nose poking rats, there was an insignificant difference between the number of nose pokes performed after receiving a CNO injection (M = 90.67, SD = 58.71) and after receiving a vehicle injection (M = 98.33, SD = 52.88, t=0.6986, p = 0.5571). For lever pressing rats, there was an insignificant difference between the number of lever presses after receiving a CNO injection (M = 194.7, SD = 15.95) and after receiving a vehicle injection (M = 159.7, SD = 97.99, t = 0.5864, p = 0.6170).

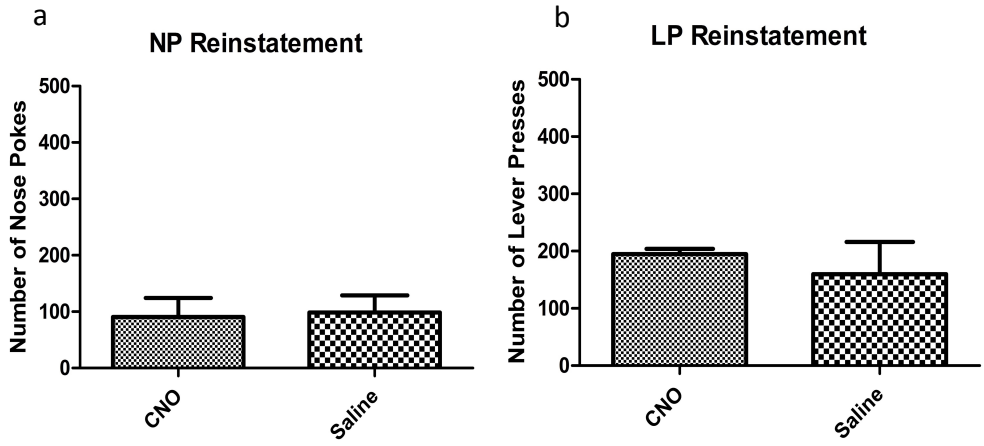

Figure 10. Effects of CNO vs. vehicle on number of nose pokes (a) and lever presses (b) during reinstatement

Testing during progressive ratio

CNO injections did not yield significant effects for either nose poking or lever pressing behavior during progressive ratio (Figure 11). For nose poking rats, there was an insignificant difference between the number of nose pokes performed after receiving a CNO injection (M = 192.0, SD = 218.0) and after receiving a vehicle injection (M = 98.33, SD = 52.88), t=1.322, p = 0.3172. For lever pressing rats, there was an insignificant difference between the number of lever presses after receiving a CNO injection (M = 194.7, SD = 15.95) and after receiving a vehicle injection (M = 159.7, SD = 97.99), t = 0.5864, p = 0.6170.

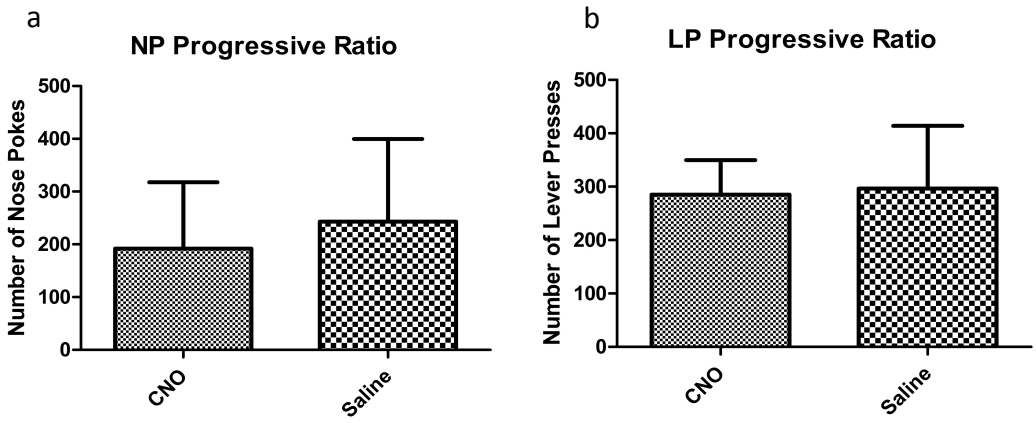

Figure 11. Effects of CNO vs. vehicle on number of nose pokes (a) and lever presses (b) during progressive ratio

Immunohistochemistry

Immunohistochemical analysis utilizing parallel fluorescent double labeling found DREADD receptor expression in the prelimbic area of all 6 rats, but no *c-fo*s expression in the PL. The fluorescent protein in the secondary antibody (Invitrogen Alexa-Fluor Donkey anti-rabbit 594) absorbs light at a wavelength of 594 nm and reflects red light. Thus, prelimbic neurons that expressed the designer receptor appeared red under green fluorescent light (Figure 12a, c, d). Neurons shown here are all PL pyramidal neurons. Examination of the slides also provided clear injector sites targeting the PL region (Figure 12b). Upon examination under blue light, green *c-fos* staining was not discovered in brain slices involving the prelimbic cortex. The secondary antibodies utilized here (Invitrogen Alexa-Fluor Donkey anti-mouse 488) absorb light at a wavelength of 488 nm and reflects green light, so any cells containing *c-fos* would appear bright green.

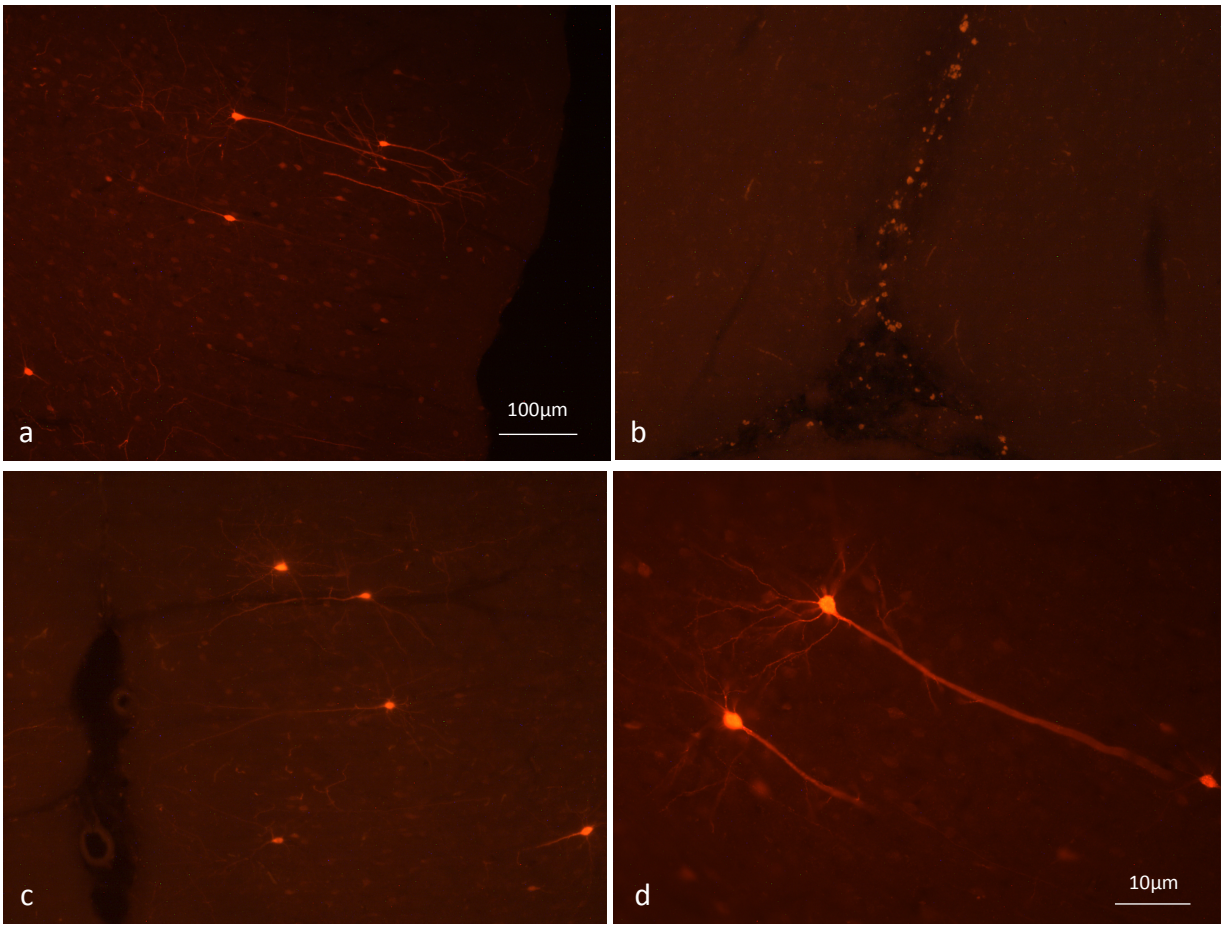

Figure 12. Immunofluorescent images of PL under green fluorescent light. a. 10x magnification of medial PL in rat 2. b. injector tracks under 10x magnification in rat 3. c. 10x magnification of medial PL in rat 3. d. 20x magnification of medial PL neurons in rat 2.

To understand why *c-fos* staining did not occur in tissue from all 6 animals, further *c-fos* immunohistochemistry using just the anti *c-fos* antibodies was performed on slices containing sections of areas posterior to PL, but staining was not discovered in any regions that would have been activated prior to sacrificing, such as the hippocampus or the motor cortex. To confirm whether the antibodies were effective or not, I performed a round of immunohistochemistry using a different set of antibodies (primary antibodies: Millipore Anti-*c-Fos* rabbit polyclonal ABE457, secondary antibodies: Jackson ImmunoResearch Biotin-SP-conjugated AffiniPure Donkey Anti-Rabbit IgG, tertiary antibodies: Alexa Fluor 488-conjugated Streptavidin) that has shown its efficacy in prior experiments in the laboratory. Using brain tissue harvested earlier in the lab that has proven to yield *c-fos* staining, I compared the performance of the two sets of antibodies. No *c-fos* staining was found in tissue that used the anti-mouse *c-fos* antibody, but *c-fos* staining was abundantly present in tissue that used the anti-rabbit *c-fos* antibody, particularly in the dentate gyrus of the hippocampus, paraventricular nucleus of the thalamus, and the piriform area (Figure 13. a, b, c, &d).

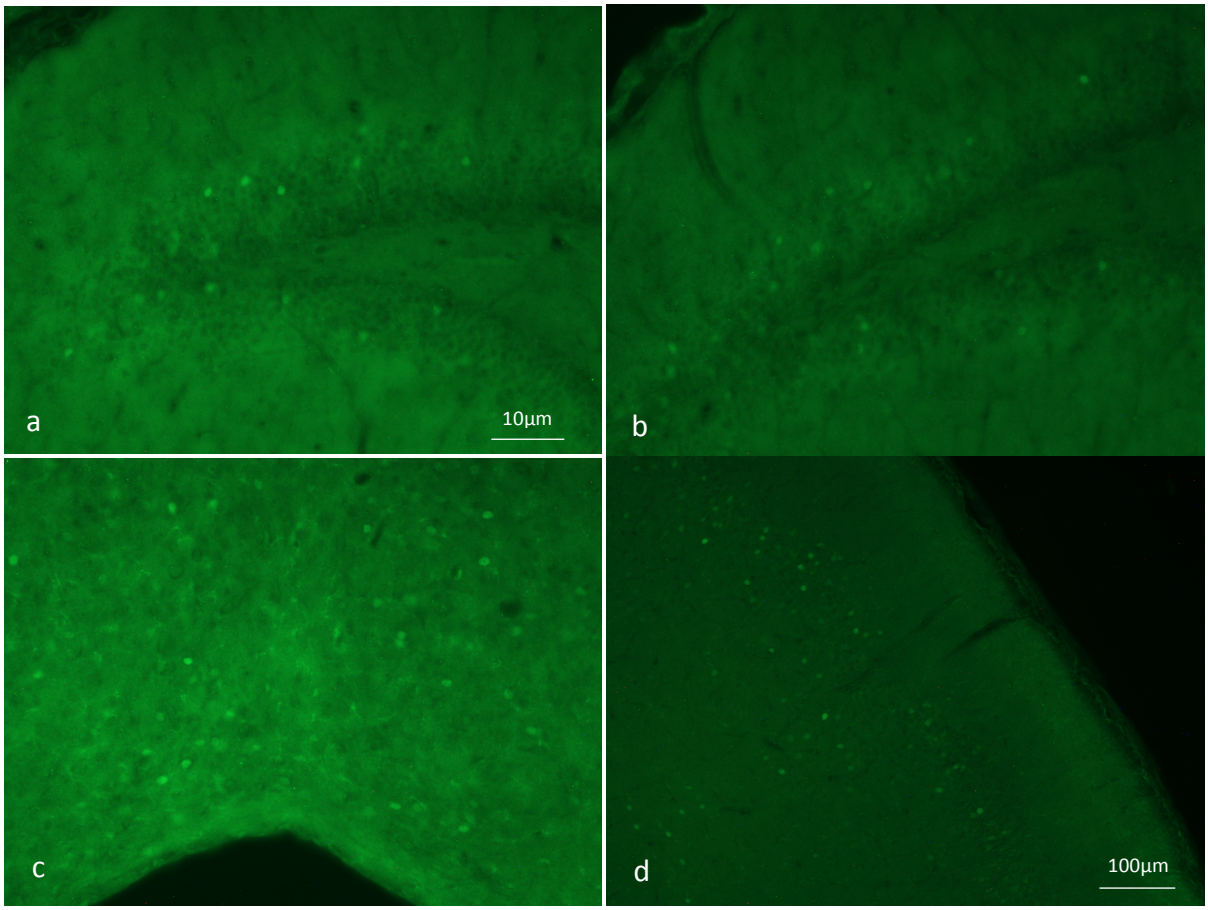

Figure 13. Immunofluorescent images of rat brain tissue under blue fluorescent light. a &b. 20x magnification of rat dentate gyrus with *c-fos* showing up as bright green spots. c. 20x magnification of paraventricular nucleus of the thalamus. d. 10x magnification of piriform area.

DISCUSSION

Summary of results

In support of the hypothesis, the results suggest some inherent difference between the two behaviors, lever pressing and nose poking. Firstly, rats who learned to nose poke for sucrose reward acquired their respective behavior significantly faster than rats who learned to lever press for reward. Also, nose poking and lever pressing rats differ in their approach to reward seeking. Initially, nose pokes responses occurred at a higher baseline level than lever presses. However, results did not support that the PL was responsible for acquiring, extinguishing, and reinstating lever press behaviors. Although receptor expression was confirmed by immunohistochemistry, CNO injections during testing days in acquisition, early extinction, late extinction, reinstatement, and progressive ratio did not yield significant behavioral effects in either nose poking or lever pressing rats, as compared to saline injections.

Implications of findings

1. Behavior acquisition

Results indicate that, in naïve rodents, nose poke responses are acquired at a higher rate than lever press responses. This is in agreement with previous research done by Schindler *et al*. (1993) as well as Mekarski (1989). They both found that an animal learning to nose poke for reward would quickly acquire the behavior without investigator intervention. Lever pressing, on the other hand, often requires investigators to shape the response by successive approximation (Pear & Legris, 1987). For example, the nose poking rats in my cohort performed an average of more than 47 correct trials during the first testing session, whereas lever pressing rats were unable to perform any correct trials for the first three training sessions. In particular, rat no.5 failed to complete a correct trial until the 9th training session. Although the initial experimental design was to limit manual involvement, it would take too long for the rat to eventually hit the lever by accident and consequently find sucrose in the well. Even though the appetitive effects of sucrose are prominent, without learning the association between reward and behavior, rats will remain naïve. Therefore, to encourage lever pressing behavior, sucrose solution was smeared on both levers before placing the rat inside the chamber and commencing a session. This provides positive reinforcement for rat contact, such as licking or scratching, with the lever. Because the levers are easily triggered upon impact, sucrose solution will then be dispensed in the sucrose delivery well for the rat to devour. In the next few days, rat no.5 successfully associated sucrose reward with hitting the levers.

My results also agree with those of Schindler *et al*. (1993) in that between-group variability was low for nose poking rats. All three rats reached criteria (>75% correct trials) on the 5th training session (std. dev.= 0). In addition, the number of incorrect responses, or inactive nose pokes, were consistently low, with a baseline rate of less than 10 behaviors per

2-hour testing session. In contrast, lever pressing rats had high between-group variability. Rat no. 4 and 5 took 14 and 12 days, respectively, to reach criteria, whereas rat no. 6 never reached criteria for the FR1 task. Upon examination of brain tissue, I found that the injectors may have damaged regions of rat no. 6's motor cortex while being driven into the brain, creating a tear in the motor areas of the right hemisphere. This may have contributed to the difficulty in learning the behavioral task and the abnormal movements the rat displayed post-surgery. However, rat no. 6 was not removed from consideration due to the fact that the PL was not damaged. These results seem to suggest that lever pressing demands a higher cognitive function involving advanced motor skills, while nose poking is a species-specific behavior with evolutionary significance, such as exploratory behavior beneficial to survival (Johansson & Hanson 2001, Abel 1995, Cloninger 1994). With highly developed sense of smell and a large olfactory bulb relative to their brain size, rats often sniff and use their whiskers to explore their surroundings. Because rats usually rely less on their front limbs in exploring, they might have a difficult time figuring out that hitting the lever gives them sugar in the well. This could explain why nose poking rats had a higher rate of response than lever pressing rats at the beginning; rats were biologically predisposed to nose poke instead of lever press. It is also curious to note that once they reached criteria, rats on a nose poke schedule will typically poke once, exit the nose poke well, and then immediately check the sucrose delivery well for reward. On the other hand, rats who learned to lever press will sometimes hit the lever multiple times in a row before entering the sucrose well for reward. The high number of lever presses could be a byproduct of the difficulty rats experienced in learning the response. There is corroborating evidence in the high percentage of number of active lever presses over correct trials for rat 6, relative to the number of active nose pokes over correct trials for nose poking rats. Suggestive of motor cortical involvement, the high number of lever presses also put into question the validity of the fixed ratio 1 task, in which

rats are rewarded once for every behavior they perform. Further considerations of the training process may include training rats to only lever press once as well as withholding reward if the rat presses more than once in a particular time frame.

2. Behavioral testing

Immunohistochemistry Fluorescent staining of DREADDs confirmed the expression of the virus-carried receptor gene in PL in all of the rats. Therefore, CNO injections should have inhibitory effects on the PL neurons expressing the receptor. Although the effects of CNO on DREADDs are usually robust in research, there has been some controversy regarding whether CNO is, in fact, an inert compound and does not take part in chemical reactions in the body. Gomez et al. (2017) suggested that CNO converts to clozapine which, unlike CNO, can enter the brain and readily occupy the nervous system. This means that CNO may not be the most efficient compound for DREADDS experiments. Others (Mahler & Aston-Jones, 2018) have countered the argument by arguing that CNO is relatively well-characterized and may be employed until a more selective agonist arises. Therefore, this study considers CNO highly effective in binding to the designer receptors and thus inactivating the neuron. On the other hand, fluorescent staining of *c-fos* during parallel double labeling yielded no staining in PL or the hippocampus. After running *c-fos* immunohistochemistry using a different set of antibodies and tissue that has proven to have *c-fos*, I found *c-fos* in the dentate gyrus of the hippocampus, paraventricular nucleus of the thalamus, and the piriform area. Using tissue from the same brain and the original set of antibodies, however, no *c-fos* was discovered. Therefore, the logical conclusion is that the mouse anti *c-fos* antibody is faulty. Due to the nature of the pilot study, I am using new antibodies that have not been used in the lab before. Further experiments may want to consider a different antibody combination for parallel fluorescent labeling.

Acquisition Even though receptor expression was confirmed, there is concern whether the virus was able to infect all of PL neurons. Judging by the mCherry staining, many of the infected neurons were located medially, towards the center of the brain, and in the posterior part of the prelimbic cortex. In addition, the number of stained cells is small as compared to previous staining of mCherry done in the lab. To understand this, I first considered the possible errors in the immunohistochemistry process. It is evident that the immunohistochemistry protocol was effective, due to the numerous successful staining done previously in lab. Assuming that the protocol was carried out correctly and assuming efficacy of the antibodies, it is likely that many PL neurons were not infected by the DREADD virus during the surgery and remained inactivated upon CNO injection. This may explain why behavioral results did not support that the PL was responsible for acquiring, extinguishing, and reinstating either behavior. CNO injections during testing days in acquisition, early extinction, late extinction, reinstatement, and progressive ratio did not yield significant behavioral effects in either nose poking or lever pressing rats, as compared to saline injections. Other possible explanations for the lack of effect of prelimbic inactivation on the respective learning of the two behaviors include that the prelimbic cortex may not be as involved in acquiring the behavior as hypothesized and that the sample size was simply too small for an effect to be significant. Considering the PL projections to the nucleus accumbens and the results of Bassareo *et al.* (2015), it is more probable that the second explanation stands true. Further analysis would benefit from immunohistochemical staining of *c-fos* to determine the activity of PL neurons during a task. If parallel fluorescent double labeling of PL neurons does not show colocation of *c-fos* and mCherry in a rat that has received a CNO injection, then I can conclude that PL was inactivated during the final testing session.

Extinction Despite the same lack of effect for extinction compared to acquisition, PL may not play an essential role in extinguishing the two behaviors. In fear conditioning experiments and appetitive Pavlovian conditioning, the infralimbic cortex seems to play a more important role during extinction (Santini *et al*., 2008, Kim *et al*., 2015, Sierra-Mercado *et al*., 2010, Mendoza *et al*., 2014). My results concerning early extinction also support that PL is not responsible for the extinction of behavior.

Reinstatement Reinstatement often models relapse in alcohol and drug abuse studies. In this study, I am using it to model relapse to food seeking. The specific biological mechanisms underlying the relapse to unhealthy eating habits, which has been a major problem in humans, are currently unknown. It would have been interesting to see if the neuronal basis for food relapse (in this case, cue-induced relapse of food seeking) is the same for substance-of-abuse relapse. Research has supported that drug-seeking neuronal pathways are actually separate from food and water reward pathways (Carelli *et al*., 2000) and often sucrose has a less robust effect on motivation as compared to alcohol or cocaine, However, reinstatement data in this study is difficult to interpret, and no concrete comparisons can be made. One major source of error is the fact that I mistakenly ran all of the rats on FR1 instead of extinction during late extinction testing. This means that the rats may not have learned to disassociate the response with sucrose delivery before I began late extinction testing, thus late extinction data is invalid. Besides not being able to evaluate late extinction data, this also raises questions about the reinstatement process. Although rats were given one extra day to extinguish between extinction testing and reinstatement, their behavior may have not been completely extinguished, and thus reinstatement would simply be another form of extinction learning, which undermines the purpose of studying reinstatement in this context. Since

studies have implicated PL in context-induced reinstatement of extinguished alcohol seeking (Willcocks & McNally, 2013) and cocaine seeking (Martin-Garcia *et al*., 2014), further examination of the reinstatement process is necessary to dissect the role of PL in lever pressing and nose poking.

Progressive ratio Similar to the FR1 task, the employed progressive ratio task examined learning of a new task and the effects of CNO on motivation to get reward. Though the motivational effects of sucrose are generally robust, CNO injections had no effects on rat behavior across both responses.

Limitations and Future Directions

As a pilot study, the current thesis project has many limitations. First and foremost, the small sample size made it difficult to compare results and draw significant conclusions from data. Future experiments should consider a larger sample size when designing the experimental process.

Secondly, the behavioral part of the study took a total of 49 days, which, in combination with surgery and recovery time as well as data analysis, prevented further experimentation with new rats. My original plan for the behavioral testing process did not take into consideration the length of time that lever pressing rats took to acquire the behavior, and therefore only estimated 25 days for training and testing. Because the current project is contingent on the rats making reward-behavior associations spontaneously, which is time consuming and inefficient, further studies should focus less on the initial acquisition of the two behaviors and more on the effects of prelimbic inactivation. Longer training sessions and automated training for lever pressing rats should be considered in further studies. For example, retractable levers could be employed to draw the rat's attention to the lever. Levers would be retracted at the start of the session, and when a lever would randomly extend, the rat would be surprised by the sudden movement and prompted to explore the lever, possibly triggering a trial. However, this means that each trial would be "gated" or controlled by the availability of the lever. Further experiments may also want to consider a similar way to gate the nose poking apparatus.

Thirdly, the pilot study lacked sufficient control. In particular, only rats who received DREADDS virus injections participated in the study. It would be of interest to examine rats who received vehicle injections in the prelimbic cortex, such as a virus carrying a gene for green fluorescent protein, or saline injections of the same volume. Similarly, due to the arbitrary selection of active and inactive behavior apparatuses, lateral effects of PL

inactivation have been overlooked. Further research may choose to see whether rats have an innate preference for the left or right nose poke or lever press, and whether PL inactivation changes the preference. Furthermore, further studies should also consider the effects of IL inactivation on acquisition, extinction, and reinstatement of nose poking and lever pressing for reward. This would provide a more comprehensive understanding of the two behaviors and the mPFC.

Lastly, the data analysis for this study was rudimentary. When analyzing results, further studies are suggested to correct for the high number of lever presses in each trial as compared to that of nose pokes, for example, looking at percentage of active lever presses over correct trials. Other possible analyses include comparing latencies, or the time interval between the stimulus and the response behavior, and response rate during reinstatement. reward.

In conclusion, the results of this thesis project revealed a difference between nose poking and lever pressing as operant responses, even though it was not successful in pinpointing the biological structures underlying these behaviors. Further researchers would have to consider the potential biological differences between the two behaviors when designing experiments targeting the mPFC.

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