Contribution of the Orbitofrontal Cortex to Delayed Punishment Discounting

Anna E. Liley

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CONTRIBUTION OF THE ORBITOFRONTAL CORTEX TO DELAYED PUNISHMENT DISCOUNTING

by

Anna Elizabeth Liley

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Major: Experimental Psychology

The University of Memphis
December 2022
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Preface

This work was written for the intended purpose of being distributed to the scientific community via publication to eNeuro. Therefore, the editing and referencing style have been formatted for this context.
Dedication Page

This dissertation is dedicated to Ilya Gofman, Lauren Schenck, and my dog, Thor.

Ilya, thank you for being my rock for the past three years. Thank you for celebrating the highs with flowers, dinner, and wine, and comforting me during the lows with words of encouragement, hugs, and reminders that I am in fact a great scientist. You have supported me emotionally, mentally, and physically throughout this journey and I will always be grateful. Please continue to make me take breaks before I drown when I insist on swimming in science.

Lauren, thank you for being a sister and best friend throughout graduate school. It has been nice to converse and vent with someone who has gone through the same trials and hardships, but also to celebrate our accomplishments as individuals who truly know the blood, sweat, and tears it takes to become a doctor. I owe much of my remaining sanity to you.

Thor, thank you for your unconditional love, keeping me company during the quarantine of the Covid-19 pandemic, reminding me to take breaks, and to always stop to smell the flowers. You may not be able to speak, but your companionship and thirst for adventure have helped me grow exponentially throughout my time in graduate school. I couldn’t have made it through the day to day without you and am so thankful to be your mom. I also consider you to be an honorary doctor since you have been with me from the start through studying, online classes, and all milestones of this degree.
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Thank you, Daniel Gabriel, for being my brother in science. I could not have asked for a better partner in crime/colleague. From long days of data collection and deep conversations about life to posting memes around the lab, my graduate school experience would not have been the same without you. I will miss working with you.

Finally, I would like to thank my parents, Tim and Jenne Liley. Your love and support have helped me so much throughout my life. You have both shown me the importance of education and hard work, and it has served me well in my endeavors. I am proud to be both the first master’s and PhD holder in our family. Hopefully Lydia and Lucas will continue to carry the torch.
Abstract

The ability to accumulate rewards while minimizing negative consequences is a valuable survival skill. Importantly, many psychiatric diseases such as substance use disorder (SUD; Bechara, 2005; Gowin et al., 2013), attention deficit hyperactivity (Magnus et al., 2021), anxiety (Hartley and Phelps, 2012), major depressive, bipolar, and schizophrenia disorders (Whitton et al., 2015) involve impaired decision-making that can lead to detrimental outcomes. One factor that causes maladaptive decision-making is insensitivity to negative consequences, especially those that occur later in time (Murphy et al., 2001; Bechara and Dolan, 2002; Field et al., 2019). These studies were among the first to investigate how the orbitofrontal cortex, a brain region implicated in cost/benefit decision-making (Floresco et al., 2008) and reward discounting (Zeeb et al., 2010), contributes to the discounting of delayed punishment.

Information gathered from the current work provided the first evidence that inactivation of LOFC reduced choice of delayed punishment compared to saline baselines, and LOFC inhibition occurred prior to different types of safe reward choices compared to immediate punishment. Preliminary optogenetics data also found that pre-choice inhibition reduced delayed punishment choice. In summation, LOFC drives the undervaluation of delayed punishment, and future therapeutic treatments aiming to improve discounting of delayed punishments during decision-making would benefit from selectively suppressing LOFC activity.
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Introduction

Modeling Decision-Making in Rodents

Behavioral economics is a field of research that evaluates how the relative value of incentives drives decisions and behavioral patterns (Bickel et al., 2014; Vlaev et al., 2019). In animal studies, economic decision-making is most commonly assessed using two choice tasks, in which subjects choose between two options of known, differing value. To model the complexity often observed in real-world decision-making, costs are often added to one or both options. These may include a time delay preceding the reward (McClure et al., 2007), an effort requirement (Hart and Izquierdo, 2019), risk of reward omission (Cardinal and Howes, 2005), or risk of physical punishment (Simon et al., 2009). While these models have yielded a significant amount of translatable information about the role of the brain in evaluating costs and benefits, studies of delay discounting have primarily been limited to delayed rewards, failing to identify the mechanisms underlying delayed punishment.

Our current understanding of the neural processes underlying sensitivity to delayed rewards has been achieved using Delay Discounting tasks in both humans (Murphy et al., 2001; Takahashi et al., 2007; Bickel et al., 2014; Hamilton et al., 2015; Samuel F. Acuff, M.S.1, Kathryn E. Soltis, M.S.1, Ashley A. Dennhardt, Ph.D.1, Brian Borsari, Ph.D.2, Matthew P. Martens, Ph.D.3, and James G. Murphy, 2016) and animals. These tasks classically offer the option between a small, immediate reinforcer and a large reinforcer that is preceded by a delayed, which often increases as the task progresses (Mazur, 1988; Murphy et al., 2001). Delay discounting tasks are useful for measuring “impulsive choice”, defined as preference for immediate, small rewards over large, delayed rewards.
(Simon et al., 2007, 2013). This phenotype is commonly observed in SUD, and is a
critical element of the reinforcer pathology hypothesis (Bickel et al., 2014). Further,
impulsive choice has a bidirectional relationship with substance use in rodents: impulsive
choice predicts drug acquisition and acceleration/dysregulation of drug intake, and is also
enhanced by chronic exposure to drugs of abuse (Perry and Carroll, 2008; Setlow et al.,
2009).

Although tasks that measure delay discounting are typically limited to delayed
rewards rather than punishment, there are multiple preclinical approaches to investigating
reward seeking influenced by negative consequences. The first series of tasks utilize risk
of reward omission/lack of reward availability as punishment. In the widely-used
Probability Discounting tasks, subjects choose between a small, certain reward and a
larger, uncertain reward, with preference for this option indicative of risk-taking (Larkin
et al., 2016). The Rat Gambling Task (RGT) is a more complex task analogous to the
Iowa Gambling Task in humans (Brevers et al., 2013). Animals have four choices, two of
which lead to an immediate, larger reward followed by risk of a “time-out” in which
reinforcement is not available, whereas the other two options have a smaller reward with
shorter time-out periods (Rivalan et al., 2013). Due to the lengthy time-outs, selection of
riskier options in this task causes a reduction of total trials. Thus, this task defines
maladaptive risk-taking as choices that minimize cumulative reward output. The “Loss-
Chasing Task” was derived from the RGT and models the desire to recover losses during
gambling. During this task, rats are presented with pellet rewards associated with the risk
of a “time-out”. When faced with a time out, rats are given a decision between “quitting”
and “loss-chasing”. Trials in which rats wait for the time-out period to elapse are
recorded as “quit responses”, while loss-chasing responses involve 50/50 chances of either no time-out at all or the time-out lasting twice as long as previously signaled (Rogers et al., 2013). Thus, as in gambling, chasing losses often leads to a greater net loss of reward. Finally, there is a rat version of the Balloon Analog Risk Task (BART), in which animals can earn increasing amounts of rewards through accumulated key press responses. However, reaching a random number of responses will cause reward forfeiture and trial failure (Ashenhurst et al., 2012). The quick decision-making dynamic necessary for optimal outcomes during each trial in the rat-BART and the formerly mentioned tasks equate to high validity for observing reward associated risk-taking phenotypes commonly observed in SUD and other psychiatric illnesses (Ashenhurst et al., 2012).

While the aforementioned tasks have contributed greatly to our understanding of cost/benefit decision-making, they do not fully reflect real-world scenarios involving the threat of discrete punishment. When utilizing reward omission (Mai and Hauber, 2015; Dalton et al., 2016; Drozd et al., 2016; Jo and Jung, 2016; Larkin et al., 2016) or time-out periods (Rivalan et al., 2013; Ferland et al., 2018, 2019; Langdon et al., 2019) as an aversive stimulus, reward and punishment are delivered as the same modality (receiving versus losing food). While this is an effective model of casino-based gambling behavior, in which money is either won or lost, the identities of rewards and consequences of decision-making are often distinct. For example, running a red light while driving can cause the reward of saving time, but can also evoke the punishment of receiving a ticket or causing an accident. Furthermore, in probability and omission decision-making tasks, there is no discrete punishment; rats begin each trial with no reward, and this situation does not change when rats take a risk, and the reward is omitted. Therefore, tasks that
utilize physical punishment during economic decision-making reflect a more accurate
depiction of risk/reward contingencies observed in most environments.

Several rodent behavioral paradigms have been designed to explore preference for
rewards coupled with physical punishment. Reward/punishment conflict tasks, such as
the Geller-Seifter test, are typically limited to a single reward option punished by a foot
shock, measuring willingness to endure punishment to earn reward (Floresco et al., 2008;
Bali and Jaggi, 2015; Jean-Richard-Dit-Bressel et al., 2018). A recent update of this is the
Punishment Risk Task which pairs a reward with a probability of foot shock that
increases throughout three blocks (Tara G. Chowdhury, Kathryn G. Wallin-Miller, Alice
A. Rear, Junchol Park, Vanessa Diaz, Nicholas W. Simon, 2019). Finally, the Risky
Decision-making Task (RDT) adds an economic decision-making element to punished
reward-seeking, presenting rats with the choice between a safe lever with a small reward
and a “risky” lever with a large reward accompanied by an immediate, foot shock that
increases in probability as the task progresses, with choice of the punished reward
indicative of increased risk-taking (Simon et al., 2009). This form of risk-taking has been
shown to predict several other behaviors associated with vulnerability to SUD, including
impulsive action, sign-tracking, nicotine sensitivity, and cocaine self-administration
(Mitchell et al., 2014; Olshavsky et al., 2014; Gabriel et al., 2018).

Several other punishment-based decision-making tasks use commonly misused drugs,
rather than food pellets, as the primary reinforcer. In one example, alcohol preferring rats
that were instrumentally trained on a chained schedule of alcohol reinforced seeking and
taking, and were later introduced to a probabilistic chance of foot shock (0.25–0.45 mA,
0.5s) to measure resilience or vulnerability to compulsive alcohol seeking(Giuliano et al.,

8
Another is the Electric Barrier–Induced Voluntary Abstinence Model in which rats are taught drug self-administration paired with a cue, then an electric barrier with increasing intensity is placed in front of the drug associated lever to dissuade drug-seeking and promote voluntary abstinence. Drug seeking relapse behavior is then measured one day after exposure to the electric barrier, while different abstinence periods after barrier exposure can be measured as well (Fredriksson et al., 2021).

The Delayed Punishment Discounting Task

Despite the wealth of experiments assessing punishment influence on decision-making, few studies have utilized punishment that occurs later in time, and (to our knowledge) none of these investigated the brain regions that regulate response to delayed vs immediate punishment. One experiment utilized a two lever choice (one safe, one punished) temporal discounting of shock task, with multi-colored cue lights correlated to changes in delay time to foot shock throughout the task (0, 5, 10, 20 and 40s; Rodríguez et al., 2018). Another experiment used histamine injections as a punishment caused by cocaine self-administration in monkeys in an immediate versus delayed context revealed preference for the delayed punishment option in a delay discounting paradigm (Woolverton, William L., Freeman, Kevin B., Myerson, Joel, Green, 2012). These tasks revealed that rats were more likely to choose rewards associated with delayed than immediate punishment, confirming that, as in humans, animals discount delayed punishment.

To investigate how the brain regulates sensitivity to delayed vs. immediate punishment can influence decision-making, I developed the rat Delayed Punishment Decision-making Task (DPDT; Liley et al., 2019). In DPDT, rats are trained to choose
between two levers, one resulting in immediate delivery of a single pellet, and the other resulting in the immediate delivery of three pellets in addition to a mild foot shock. Early in the session, the shock occurs immediately after a choice, then subsequent blocks introduce an incrementally increasing delay that precedes shock (0, 4, 8, 12, and 16s). Rats initially avoid the large, punished reward, but as the delay increases, they shift preference toward the punished reward despite the consequence (Liley et al., 2019) (Figure 1a-b). Thus, rats discount the negative motivational value of delayed versus immediate punishment, and this task provides a reliable tool with which to study this maladaptive phenotype. Critically, the discounting of delayed punishment during reward seeking is uncorrelated with reward delay discounting, suggesting that these forms of temporal discounting may employ distinct neuronal substrates (Liley et al., 2019).

One concern that arises from DPDT is that subjects is that rats are not actually discounting the value of punishment due to delay. Instead, rats may be unaware that delayed shocks are impending, which could account for increased preference for these options. To address this, I measured locomotor activity during the delay preceding the shock, as interruption of exploratory locomotion is a component of conditioned fear (Fanselow, 1980) during the delay period preceding punishment (Liley et al., 2019), suggesting awareness of impending shock (Figure 1c). I observed reduced locomotion during this pre-punishment period in comparison to a comparable delay period with no impending shock (Liley et al., 2019), suggesting that rats were aware of the upcoming punishment, but still discounted its negative motivational value during decision-making. Furthermore, rats were trained in a version of DPDT in which cues were added to bridge the gap between the decision and punishment. In this task, rats continued to choose
rewards associated with delayed punishment, despite the cue signaling that punishment was impending. This suggests that rats increase choice of delayed vs immediate punishment due to delay discounting rather than diminished punishment expectation.

DPDT has also revealed that males discount delayed punishment more than females, as indicated by increased choice of delayed (but not immediate) punishment (Figure 2a). Critically, estrous phase does not influence discounting of delayed punishment (Liley et al., 2019; Figure 2b-c). Thus, DPDT not only enables identification of the brain regions responsible for integration of rewards with delayed punishment during decision-making, but also allows investigation of the neuronal correlates underlying a robust sex difference in behavior.

Neurobiological Substrates of Delay Discounting

While discounting of delayed punishment has not been well-studied, there is a substantial amount of literature on the neural substrates of reward delay discounting. Sensitivity to delayed rewards seems to depend upon corticolimbic circuitry (i.e., prefrontal cortex, hippocampus, and amygdala), which integrate cognition with emotion to generate flexible behaviors that can adapt to environmental circumstances (Cardinal et al., 2004; Rusbridge, 2020). In humans, functional magnetic resonance imaging (fMRI) has shown more frequent activation of limbic structures (the hypothalamus, thalamus, amygdala, and hippocampus) during immediate versus delayed rewards than during tasks with two delayed rewards (Wittmann et al., 2010). Moreover, both the lateral prefrontal cortex and posterior parietal cortex activate regardless of delays; and one task observed that the lateral orbitofrontal cortex (LOFC) and ventral striatum are engaged when there are immediate rewards compared to no rewards (Tanaka et al., 2004; Wittmann et al.,
2010). Positron emission tomography (PET) and fMRI have revealed correlations of low levels of midbrain and striatal D2-type dopamine receptor (D2 and D3) availability with both self-reported impulsivity and impulsive choices during reward-based decision-making tasks, while available striatal D2-type receptors have been positively correlated with cognitive flexibility and inhibited motor response (London, 2020). Gambling research in human with lesions in the ventromedial prefrontal cortex (vmPFC) has also found that these individuals are insensitive to future (positive and negative) consequences (Bechara and Dolan, 2002), while lesions in the LOFC have been reported to increase impulsive behavior during these tasks (Bechara et al., 1998, 1999).

While human lesion and imaging work has shed light upon the brain circuitry recruited during delay discounting, animal research has been necessary to further elaborate the neurobiological mechanisms underlying this form of cognition. For this dissertation, I will use a multidimensional approach to investigate the role of LOFC, a brain region implicated in sensitivity to delayed rewards (Mobini et al., 2002a; Roesch et al., 2007; Zeeb et al., 2010), in sensitivity to immediate versus delayed punishment.

*Orbitofrontal Cortex (OFC)*

OFC is a prefrontal cortical brain region that receives input from all major sensory systems (auditory, visual, olfactory, gustatory, and somato-sensory), in addition to influences from limbic regions (Carmichael and Price, 1995) and circuits responsible for social and emotional behaviors (Mcdonald, 1991). Thus, the OFC is ideally situated to integrate sensory-motor information with motivation (Öngür and Price, 2000). Functional changes in OFC neuronal firing indicate concurrences between distinct environmental stimuli and the motivational salience of impending rewards, which allows for the
adaptation of learning and behavior (Schoenbaum et al., 2000; Wallis, 2007; Van Wingerden et al., 2010). This region also enables humans to process decisions and act based on outcome expectations (Schoenbaum and Roesch, 2005a) while also driving emotional and visceral responses to the environment (Bechara et al., 1996, 1997; Öngür and Price, 2000; Milne and Grafman, 2001; Black et al., 2002). Finally, many aspects of OFC function and connectivity are conserved across rats, primates, and humans (Krettek, JE and Price, 1977; Guldin and Markowitsch, 1984; Goldman-Rakic and Porrino, 1985; Ray and Price, 1993; Öngür and Price, 2000; Petrides and Pandya, 2002; Murray et al., 2007; Moorman and Aston-Jones, 2014), suggesting that studying the rat OFC has strong translational value.

Humans with OFC lesions have demonstrated poor decision-making and impulsive choice behavior (Rogers et al., 1999). Results have been less consistent in rodent studies, with OFC lesions either increasing or decreasing preference for immediate gratification (Mobini et al., 2002a; Winstanley, 2004; Rudebeck et al., 2006a; Sosa et al., 2021). Moreover, the effects of OFC inactivation varied based on individual differences in impulsive choice and the presence of cues during the pre-reward delay (Zeeb et al., 2010). Single unit recording in rat and primate OFC encode information about delay length preceding rewards, and discriminate between rewards delivered immediately and after a delay (Roesch and Olson, 2005; Schoenbaum and Roesch, 2005b). Collectively, these studies all suggest that OFC regulates sensitivity to delayed rewards.

Previous research also demonstrates that OFC plays a crucial role in reward-punishment integration during delayed punishment discounting. Neurotoxic lesions of rat OFC during RDT decreased choice of rewards associated with risk of punishment, and
was unrelated to reward magnitude discrimination or effortful requirements measuring appetitive motivation (Orsini et al., 2015b). Pharmacological inactivation of OFC using GABA agonists baclofen and muscimol resulted in increased punished lever responding during an instrumental aversive learning task in rodents (Jean-Richard-Dit-Bressel and McNally, 2016). Additionally, individual neurons in primate OFC simultaneously encode both appetitive and aversive information about predictive cues, and these signals predict subsequent behavior (Morrison and Salzman, 2009). As OFC has been shown to contribute to both delay discounting of rewards and reward/punishment integration, it is a likely candidate to regulate the discounting of delayed punishment during economic decision-making.

Since a wide variety of these types of stimuli can be received simultaneously, they are processed by two reciprocally related, although anatomically and functionally distinct, medial and lateral OFC (mOFC and LOFC) subregions that contribute to varying aspects of goal-directed behavior (Fettes et al., 2017). Rodent delay discounting of reward studies have stated that lesions to mOFC and LOFC have distinct effects on impulsive choice (Mar et al., 2011; Burton et al., 2014). Human studies have found that LOFC is activated by and helps process memories for experiences with nonrewarding and/or punishment-oriented stimuli, while the mOFC functions similarly for rewarding stimuli (O’Doherty et al., 2001; Suzuki et al., 2017; Xie et al., 2021). Finally, LOFC activity appears to encode information about both reward value and reward/delay integration (Roesch et al., 2006). Based on its role in both reward and punishment, I will specifically target the role of LOFC in delayed punishment-based decision-making.
The role of LOFC in sensitivity to delayed punishment will be interrogated by both measuring and manipulating LOFC neuronal activity. I first tested if LOFC is necessary for delayed punishment discounting by bilaterally inactivating this region prior to behavioral testing (Experiment 1). Then, I measured if LOFC activity encoded delayed punishment using single unit electrophysiology (Experiment 2). Finally, I determined how suppression of LOFC activity during specific phases of the decision-making process affected sensitivity to delayed punishment using optogenetics (Experiment 3).

**Purpose and Specific Aims**

**Experiment 1: Assessing the role of LOFC in decision-making throughout DPDT**

This experiment tested the role of LOFC in delayed punishment discounting via pharmacological inactivation prior to DPDT. Pharmacological inactivation is accomplished by infusing an inhibitory drug directly into a brain region via a surgically implanted cannula. If this affects a behavior of interest, one can infer that activity in the inactivated region is involved with that behavior. A common method of inactivation is to stimulate inhibitory receptors such as δ-aminobutyric acid (GABA) to hyperpolarize neurons in that region, preventing the initiation and propagation of action potentials (Olsen, 2018; Garzola, 2019). This transient form of cellular inhibition is advantageous over permanently lesioning brain regions (Orsini et al., 2015b) because it enables within-subjects comparison of the same region both intact and inactivated, and also avoids potential damage to fibers of passage from other brain regions. Here, I micro-infused a drug cocktail consisting of GABA agonists baclofen (active at GABA_B receptors) and
muscimol (active at GABA\textsubscript{A} receptors) dissolved in sterile saline into LOFC prior to DPDT performance.

Based on the role of LOFC in punishment sensitivity as well as delay discounting of rewards (Mobini et al., 2002b; Roesch et al., 2006; Zeeb et al., 2010), I hypothesized that LOFC activity would be necessary for the discounting of delayed punishment. Accordingly, I predicted that LOFC inactivation would decrease delayed punishment discounting, reflected as elevated choice of the punished option as punishment delay was increased.

**Experiment 2: LOFC encoding of decision-making during DPDT**

Neurons are cells in the brain that communicate in part through electrical impulses called action potentials. Measuring the rate and pattern of these signals enables understanding of how populations of neurons respond to different events, such as movement, goal-directed action, or learning. Characterizing the relationship between neural activity and behavior is necessary to understand how the brain detects and responds to challenges in the environment, which in turn can facilitate development of neural manipulations to improve maladaptive behavior in psychiatric disorders.

One method of investigating how action potentials represent, or “encode” information within specific brain regions is single unit electrophysiology. This involves implanting microwire electrode arrays into the brain to detect extracellular fluctuations in electrical activity emitted by neurons or groups of neurons (Cousens and Muir, 2006). Action potentials can be isolated from this activity by identifying similar patterns of measurable waveforms (Roesch et al., 2006; Stüttgen et al., 2011; Simon et al., 2015a; Nimitvilai et al., 2017) that differ from electrical noise in the cortex and recording environment.
(Figure 3a-b). Each putative neuron is labeled as a “unit”, as it is difficult to determine if these signals are being produced by a single neuron, or a cluster of neurons. After electrical signals are detected by the electrode, the signal is passed through a head stage to a digital head stage processor, which converts the electrical activity to digitized signals (Simon and Moghaddam, 2015; Seo et al., 2019). These signals are visualized and recorded using Omniplex data acquisition software (Plexon), which is interfaced with our standard behavior software (MedAssociates). This enables the synchronization of unit activity with decision-making and other behaviors at extremely high temporal resolution.

Previous research utilizing single unit electrophysiology in LOFC reported that neuronal activity reflects impending reward value (Schoenbaum and Roesch, 2005b; Van Duuren et al., 2008), provides information about delay length preceding reward (Roesch et al., 2006), and appears to reflect convergence between expected reward and punishment (Morrison and Salzman, 2009). Therefore, I hypothesized that activity in LOFC during DPDT would encode the motivational value of reward and punishment prior to a decision and would reflect changes in this value caused by delay preceding punishment. Notably, although I previously observed sex differences in this task (Liley et al., 2019), this dissertation was restricted to male rats due to difficulty with task performance post-surgery in female subjects (Liley & Simon, Unpublished observation).

**Experiment 3: Time-specific inhibition of LOFC during different facets of decision making in DPDT**

Decision-making is a complicated process consisting of multiple components, including the deliberation prior to a decision, the decision itself, and the post-decision
outcome (Orsini et al., 2019). However, the majority of the research on economic decision-making has utilized neural or pharmacological manipulations that uniformly affect the entire decision-making sequence (Cardinal et al., 2004; Orsini et al., 2015a; Winstanley and Floresco, 2016). This experiment used optogenetics to determine how inhibition of LOFC activity during different events regulated decision-making with delayed punishment. Optogenetics is a technique that enables manipulation of neural activity with fine temporal precision, whereas the pharmacological inactivation used in Experiment 1 inhibits brain activity throughout an entire session. Understanding how the brain regulates behavior during individual events in decision-making is necessary for precise, event specific neurobiological treatments that affect behavior.

Optogenetics genetically alters neurons in a specific brain region, then manipulates neuronal activity when a laser or LED light shines directly into the brain to activate opsins, which are membrane-bound, light sensitive proteins (Karl Deisseroth, 2015). An opsin used to suppress activity is halorhodopsin, a chloride pump that can be activated by yellow or green light to hyperpolarize a cell causing synaptic inhibition by moving chloride ions inward (Guru et al., 2015). I introduced halorhodopsin to cells by bilaterally infusing an adeno-associated virus (AAV) containing halorhodopsin and a fluorescent tag into LOFC neurons. After this infusion, optical fibers able to shine light into the brain were implanted into LOFC. After a 30 day incubation period, viral transduction allowed the neurons infected with this AAV to express halorhodopsin (Naso et al., 2017). Typically, light stimulation does not affect neurons; however, neurons that express halorhodopsin can be inhibited by LED stimulation at the proper wavelength during specific time-points in DPDT. This allowed for evaluation of exactly when LOFC was
critically involved with delayed punishment discounting, which is useful in understanding its role in this form of cognition.

Optical inhibition occurred at three different time points in the DPDT: 1.) pre-decision (when levers extended at the beginning of a trial), 2.) pre-punishment delay, and 3.) intertrial interval (ITI; as a control condition). I hypothesized that LOFC activity both before a decision and during pre-punishment delay regulates delayed punishment discounting. I predicted that pre-decision inhibition of the LOFC would attenuate preference for punished rewards, whereas inhibition during the pre-punishment delay would increase choice of delayed punishment by disrupting punishment expectation. Similar to experiment 2, it would have been likely that differential effects would be observed in female rats during this experiment; however, due to time constraints and difficulty training females post-surgery this experiment was restricted to male rats.

**Method**

**Experiment 1: Assessing the role of LOFC activity in decision-making throughout DPDT**

**Brief Summary**

I investigated how LOFC broadly contributes to delayed punishment discounting during decision-making by temporarily inactivating LOFC immediately prior to DPDT performance.

**Subjects**

I used 32 Long Evans rats aged 70 days upon arrival (16 females and 16 males). Rats were food restricted to 85% free feeding weight one week prior to behavioral training,
with free access to water throughout the experiment. All rats were individually housed and maintained on a 12-hour reverse light/dark cycle. All methods were approved by the University of Memphis Institutional Animal Care and Use Committee.

**Surgery**

Prior to behavioral assessments, rats were implanted with a cannula in each hemisphere to enable bilateral LOFC inactivation. Rats were anesthetized in an isoflurane gas induction chamber, then placed into a stereotaxic apparatus (Kopf) while resting on a heating pad adjusted to 40 degrees C. Isoflurane was provided throughout surgery via a nose cone. An anterior to posterior incision was made over the skull, and guide cannulae were bilaterally implanted in the lateral region of OFC (3.0 mm AP, 3.2 ML, and 4.0 DV from skull surface (Roesch et al., 2006)). Cannulae were held in place by a dental cement headcap anchored by three bone screws. Once surgery was completed, rats were subcutaneously given 1mL of sterile saline, and a solution of Acetaminophen and H2O was placed in a dish along with a dish of moistened food during recovery. Rats were closely monitored for signs of infection or distress during the next week, with cage bedding changed daily for the first 3 days. All behavioral training and testing took place after one week of recovery.

**Apparatus**

Testing was conducted in standard rat behavioral test chambers (Med Associates) housed within sound attenuating cubicles. Each chamber was equipped with a recessed food pellet delivery trough fitted with a photo beam to detect head entries, and a 1.12 watt lamp to illuminate the food trough. Food pellets were delivered into the food trough, 2 cm above the floor centered in the side wall. Two retractable levers were located on the
left and right side of the food trough, 11 cm above the floor. A 1.12-watt house light was mounted on the opposing side wall of the chamber. Beneath the house light was a circular nose poke port equipped with a light and photo beam to detect entry. The floor of the test chamber was composed of steel rods connected to a shock generator that delivers scrambled foot shocks. Locomotor activity was assessed throughout each session with infrared activity monitors located on either side of the chamber just above the floor. Test chambers were interfaced with a computer running MedPC software, which controlled all external cues and behavioral events.

Shaping Procedures

Prior to acquisition of DPDT, rats underwent a series of shaping procedures. Rats were first taught to associate the food trough with food pellets during magazine training. They then trained to press a single lever (left or right, counterbalanced across groups) to receive one pellet of food. After performing 50 reinforced lever presses within 30 minutes, rats then trained to press the opposite lever under the same criterion. Next were shaping trials in which both left and right levers were retracted, and rats were required to nose poke into the food trough during a period of illumination from both the house and food trough lights. Nose poking evoked the extension of a single lever (either left or right in pseudorandom order). A subsequent lever press was reinforced with a single pellet. After the lever was pressed, the house and trough lights extinguished, and the lever retracted. After achieving a minimum of 30 presses of each lever in a 60-minute time span, rats progressed to magnitude discrimination training. The 30-minute reward magnitude sessions utilized 2 levers with counterbalanced presses producing either 1 or 3
pellets. Once rats achieved >75% preference for the large reward, they began DPDT training.

Reward discrimination

Once adequate performance on all shaping procedures was achieved, rats completed sessions of 1 vs. 3 pellet reward discrimination. Trials began with illumination of the house light and food trough, after which rats were required to nose poke into the lit trough within a 10s period to initiate the trial. A nose poke turned off the trough light and caused both levers to be extended simultaneously. A press on one lever dispensed a single pellet, while the other dispensed three pellets, with both levers retracting after a choice. There were 5 blocks, with 8 forced choice and 10 free choice trials in each block for a total of 90 trials. Identity of levers (left vs right) were counterbalanced between subjects. This training continued across multiple sessions until rats demonstrated preference of >85% for the large reward. Incorporating this training allowed rats to become familiar with the rewards evoked by each lever before starting the Delayed Punishment Decision-making Task. Additionally, rats underwent pharmacological inactivation of LOFC after this phase of training to test the effects of region-specific inhibition on reward discrimination behavior.

Delayed Punishment Decision-making Task (DPDT)

During DPDT, rats chose between a small reward and larger reward associated with punishment preceded by varying delays. DPDT methodology was comparable to magnitude discrimination above, with choice between small and large food pellet reinforcers. However, in this task the large option was accompanied by a mild, 1 second...
foot shock. This shock initially occurred immediately after a choice, then systematically takes place later in time throughout the task (Figure 4).

Trials began with illumination of the house light and food trough, after which a nose poke into trough caused one or both levers to be extended simultaneously. A press on one lever dispensed a single pellet, while the other dispensed three pellets with a 1 second mild foot shock. After all outcomes were delivered, the house light extinguished, and the trial proceeded to an ITI of 10±2s. The session was divided into 6 blocks, with 2 forced choice and 10 free choice trials in each block for a total of 72 trials. The first 2 trials of each block were “forced choice” trials in which only a single lever was available, establishing the reward/punishment parameters within that block. The following 10 trials were “free-choice” trials in which both levers were extended, allowing rats to choose a preferred lever. During the first block, shock occurred immediately after lever press. In each subsequent block, a delay in introduced preceding shock that extended to 4, 8, 12, and 16 seconds (Figure 4). Then, in the final block, the shock was eliminated. Notably, on trials in which the unpunished lever was chosen, the ITI was increased by a period equivalent to the delay preceding shock in that block (4, 8, 12, 16s) to maintain consistency of trial length regardless of choice. Finally, to confirm that the order of delays did not affect performance, a subset of animals (n = 16) trained in a reversed DPDT, in which all parameters were similar to the original DPDT, but the trial began with no shock, then shock delays descend from 16 to 0s across blocks (16, 12, 8, 4, 0s).

During task acquisition, shock amplitude began at 0.05 mA, then increased 0.05 mA in the following session if rats completed >85% of trials. This incremental increase in shock intensity limited omissions and allowed all rats to acquire task parameters. To
minimize individual differences in performance and avoid excessive omissions, shock intensity was titrated for each individual rat until their decision-making was between floor (0% choice of punishment) and ceiling (100% choice of punishment). Upon reaching the final shock intensity, subjects trained for a minimum of 2-3 days until they achieved stability, which consisted of no more than a 10% overall shift in daily choice behavior.

*LOFC Inactivation*

After rats reached stable performance in DPDT, they underwent at least one habituation session to acclimate them to the handling that occurred during the infusion procedure. On the next day, they received bilateral drug micro-infusions to inactivate the LOFC. A drug cocktail of GABA agonists baclofen (Reis and Duarte, 2006) and musicmol (Chandra et al., 2010) dissolved in sterile saline (concentration: 250ng/μl, .5 μl infusion volume over 1 minute (Piantadosi et al., 2017; Orsini et al., 2018) was administered into each hemisphere via an automated infusion pump and 2 50μl Hamilton syringes. Behavioral testing commenced after a 15-minute absorption period. After a day of baseline testing with no treatment, subjects were infused with bilateral saline micro-infusion (5μl infused at .5μl/min). Drug/saline order was counterbalanced across subjects.

LOFC inactivation was first performed after acquisition of magnitude discrimination. This served as a control measure to confirm that inactivation did not cause gross impairments to overall task engagement or reward expectancy. Then, LOFC was again inactivated after completion of either standard DPDT or DPDT with reversed delays.
Histology

Rats were euthanized with Euthasol, and perfusions were conducted with saline and 10% formalin solution. Brains were extracted, stored in 10% formalin solution, sliced at 60-150 μm using a Cryostat, and mounted onto slides. Cannulae placements and infusion localization was confirmed via light microscopy (Sara E. Morrison, Alexandre Saez, Brian Lau, 2012).

Experimental Design and Statistical Analysis

Custom-made MATLAB scripts were used to compile behavioral data, and all statistical analyses were conducted using IBM SPSS Statistics 24. If Mauchly’s test of sphericity was violated, Greenhouse-Geisser values and degrees of freedom were used accordingly. If a rat failed to make any choices during a block of the task, the slope of that subject’s curve was used to extrapolate that missing data point. If two or more blocks of behavioral data were missing, that rat was removed from analysis due to excessive omissions.

Following task acquisition, stable decision-making for DPDT and REVDPDT were measured using a day x block repeated measures ANOVA, quantified as lack of effect of day and a significant effect of block. Effects of micro-infusions on behavior were analyzed via sex x infusion (drug vs saline) x block ANOVA. Latency to lever press during testing was evaluated using a mixed sex x safe vs punished lever ANOVA.
**Experiment 2: LOFC encoding of decision-making during DPDT**

**Brief Summary**

To determine how LOFC processed information about decision-making with delayed vs immediate punishment, I measured functional neuronal activity in LOFC during DPDT using single-unit electrophysiology.

**Subjects**

A cohort of male Long Evans rats aged 70 days upon arrival (n = 6) was food restricted to 85% free feeding weight with free access to water. All rats were individually housed and maintained on a 12-hour reverse light/dark cycle. All methods were approved by the University of Memphis Institutional Animal Care and Use Committee.

**Behavioral Testing: Modified DPDT Procedure**

Operant chambers were comparable to in Experiment 1, except they were fitted with taller food troughs to avoid contact with the electrode/headcap during reward consumption. Also, there was a hole in the top of the chamber to enable tethering of rats to the Omniplex system via headstage cable.

DPDT was modified slightly in Experiment 2 for single unit electrophysiology. Each block began with forced choice trials (2 safe, 2 punished; counterbalanced across blocks), followed by 30 free choice trials/block. This increased trial count was instated to minimize variability in neuronal data and improve signal to noise ratio resulting from effluxes of neural activity. In addition, rats were required to repeat any incomplete trials until they reached 30/block.

The 3 blocks were comparable except for changes in punishment delay, consisting of: 1) immediate shock, 2) 8s delayed shock, and 3) 16s delayed shock. Half of the subjects
were given ascending delays (beginning at 0s), and the others were given descending delays (beginning at 16s; Figure 5). As in Experiment 1, if the unpunished lever was chosen, the ITI increased by a period equivalent to the delay preceding shock (16 or 8s) to maintain consistency of trial length regardless of choice. After all outcomes were delivered, the house light extinguished, and the trial proceeded to an ITI of 10±2s.

During task acquisition, shock amplitude began at 0.05 mA and increased 0.05 mA in the following session if rats completed >85% of trials. Shock amplitude was raised until 0.35 mA was reached, and subjects trained until they achieved stable behavior for a minimum of three days; thus, the task was well-learned prior to electrophysiology.

Recording occurred throughout the sessions, but first-pass analyses focused on neuronal activity evoked during the “pre-decision”, “delay”, and “ITI” time segments.

Surgery

Electrodes were implanted after rats acquired the task. General surgery procedures were comparable to those stated for Experiment 1. However, for electrode implantation, 16 channel drivable microelectrode arrays (Innovative Neurophysiology) were implanted for single unit recording. Electrodes were unilaterally implanted into OFC (+3.0 mm AP, +3.1 ML, and -4.5 DV from skull surface (Roesch et al., 2006)), with left vs right hemisphere counterbalanced across subjects and held in place by a dental cement headcap anchored by four bone screws. Recovery procedures were also comparable to Experiment 1. After one-week, food restriction was re-established followed by three days of habituation to the headstage cable, then rats re-trained in DPDT until achieving stability (Field et al., 2019) for 3 consecutive days.
Single Unit Electrophysiology

After a week of recovery, rats re-trained on DPDT until a stable post-operative baseline was established. In subsequent sessions, electrical signals recorded from electrodes implanted into LOFC were buffered by a headstage amplifier, then amplified through an analog band pass filtered via preamplifier. Lightweight and unobtrusive electrode arrays, and a rotating commutator (Plexon, Dallas, TX) connected to the headstage cable ensured free movement. Critically, previous studies have found that comparable electrodes, headstage cable, and commutators do not alter action latency or task engagement in adult or smaller adolescent rats (Totah et al., 2013; Simon et al., 2015b; Yunbok Kim, Nicholas W. Simon, Jesse Wood, 2016). Prior to each session, electrodes were lowered .1mm (until they reach the ventral end of the brain region (Paxinos and Watson, 1997)) to identify new units. During testing, the MedPC behavioral system controller sent TTL pulses to the neural data acquisition system to synchronize behavioral events with neural data. After recording, high pass filtered neuronal data, or “spikes”, were analog filtered between 300 Hz and 8 kHz, digitized at 40 kHz, and sorted using Plexon Offline Sorter. Finally, neuronal data was separated into 50ms time bins, smoothed with a 5-point filter to reduce noise, and Z-score normalized to a five-sec window of the ITI.

Histology

Rats were euthanized with Euthasol, and perfusions were conducted with saline and 10% formalin solution. Brains were extracted, stored in 10% formalin solution, sectioned into 60-150 μm coronal slices using a Cryostat, and mounted onto slides. Electrode
placements were verified under a light microscope as previously described (Simon et al., 2015b; Wood et al., 2017).

**Experiment 3: Time-specific inhibition of LOFC during different facets of decision making in DPDT**

*Brief Summary*

I investigated how LOFC contributes to decision-making during specific time periods within the decision-making process by temporarily suppressing LOFC activity during DPDT using optogenetic inhibition.

*Subjects*

This experiment utilized two cohorts of male Long Evans rats (n = 8/group) to include groups for halorhodopsin and an inactive control viral vector. Housing conditions were similar to experiments 1 and 2.

*Surgery*

Surgery procedures were comparable to Experiment 1. Guide cannulae were bilaterally anchored in place and an injection needle was lowered into each cannula to deposit either AAV5-CAMKIIα-eHpNR3.0-mCherry or AAV5-CAMKIIα-mCherry1 (Orsini et al., 2017a) into LOFC (+3.0 mm AP, +3.1 ML, -4.5 dorsal and -4.7 ventral DV from skull surface (Roesch et al., 2006)) into the LOFC (0.3 μl at the ventral DV coordinate and 0.3 μl at the dorsal DV coordinate, at a rate of 0.5 μl/min). Following each infusion, the needle remained in place for five minutes to ensure adequate diffusion of the virus. Next, optic fibers were inserted into LOFC to allow LED modules to deliver lime green light and express halorhodopsin. All optical stimulation occurred after 3-4
weeks to enable sufficient time for expression of halorhodopsin (Gardner et al., 2018; Adler et al., 2020a).

Behavioral Testing

After recovering from surgery, all rats trained in a modified DPDT (Figure 6). In this version, there were 3 blocks with either ascending (0, 8, 16s) or descending delays (16, 8, 0s) with 4 forced choice and 30 free choice trials in each block.

Optogenetic Inhibition during DPDT

After rats reached stability performance in DPDT for a minimum of 3 consecutive days, testing occurred across 4 sessions. Rats were tethered to a commutator, which connected directly to the implanted fibers and allowed free movement. This commutator was connected to an LED generator. In the first session, 560 nm light pulses were emitted into LOFC through the implanted fiber for 1000 msec at the “pre-decision” point before a choice was made on all free choice trials (Figure 6). In session 2, 560 nm light pulses were emitted throughout the pre-shock “delay” on all trials, and in session 3 light pulses were emitted throughout the same delay period for safe trials to serve as a control after food collection (Figure 6). During session 4, pulses were emitted for 1000 msec during a random time of the “ITI”. As in Experiment 1, between each session, there was a non-treatment baseline session. This schedule was performed both for subjects given halorhodopsin and subjects given mCherry alone as a control for non-channel activation light-evoked changes in behavior. A computer running PlexBright (Plexon) and Med-PC software was interfaced with each LED to enable synchronization of behavior and optical inhibition.
**Histology**

Future confirmation of halorhodopsin in LOFC will be achieved through procedures adapted from previous work (Gardner et al., 2018; Adler et al., 2020b). Rats will be euthanized with Euthasol and perfused with 0.9% saline and 10% neutral buffered formalin. Brains will be kept in formalin then transferred to a 30% sucrose solution of 10% formalin. Expression of halorhodopsin or non-channel control will be confirmed using a fluorescent microscope.

**Data Analyses**

*Experiment 1:* Stability in DPDT was measured using a day x block ANOVA, quantified as lack of effect of day across three days. Effects of micro-infusions on behavior was analyzed via sex x infusion (drug vs saline) x block ANOVA, and individual comparisons were conducted to further evaluate any significant effects. Nonparametric tests were utilized if any assumptions of ANOVA were violated.

*Experiment 2:* Activated and inhibited units were identified as units with a Z score greater than 1 or less than -1 for a minimum of three consecutive time bins during the event of interest. Firing rate was Z-score normalized to a five second period of the ITI and smoothed with a 7-point filter. Recording occurred throughout the entire session and across all events, but analyses focused on: the **decision point** (the .5 second window surrounding a choice) and **delayed punishment expectation** (the 16 second pre-punishment delay following food delivery in delayed vs immediately punished trials).

Analyses was performed on multiple levels. First, mean firing rate from all recorded units was averaged together each event of interest, and this population firing rate was compared between delay blocks and choices of either reward type (“does population
firing rate differ between choice of the safe vs. punished reward”, “does activity before a choice differ based on delay length preceding the punishment”, etc.). In addition, because population activity often obscures certain effects found in subpopulations of neurons, I probed for units that demonstrated phasic activation or suppression (see (Simon and Moghaddam, 2015) for criteria), and tabulated the total units that were activated or suppressed during each event of interest. Criteria for activation or suppression was three consecutive bins with a Z score >1 (for activated) or < -1 (suppressed). These totals were then compared across different task events (for example: choice of small, safe reward vs choice of large, punished reward) using chi-square tests.

*Experiment 3:* Effects of suppression of LOFC activity on decision-making were analyzed for each of the four sessions using a mixed ANOVA (virus type x stimulation x delay block). Upon detection of main effects or interactions, individual comparisons were used to compare effects between delay blocks. Notably, because the final sample size was insufficient, I did not report stats on this experiment.

**Results**

1. **Experiment 1: DPDT/REVDPDT Acquisition and Stability**

   The mean number of days to complete shaping procedures prior to DPDT (magazine training, FR1 for both levers, nose poke, and magnitude discrimination training) was 8.63 for females (n = 7) and 7.00 for males, with significant differences between sexes (t (24) = 1.24, p = .011). Females required significantly more training sessions to reach stability on DPDT (female mean = 42.88 days, male mean = 15.67 days; t (24) = 3.63, p < .001). After successful training, there were significant effects of block
for all subjects in DPDT, such that subjects shifted choice preference from the safe reward to the punished reward as punishment delay increased (LOFC group: $F_{(2.159, 21.587)} = 29.304, p < .001$). There were no significant differences between sexes in DPDT performance (LOFC: $F_{(5, 50)} = .627, p = .680$).

A separate group of rats trained in REVDPT, in which punishment delays were presented in descending instead of ascending order (Figure 1). There was no difference in length of shaping for REVDPT between males and females (female mean = 9.27 days, male mean = 7.61 days; $t (27) = 1.62, p = .157$). Unlike in DPDT, there were no sex difference in sessions required to achieve stability for REVDPT (female mean = 30.36 days, male mean = 30.67 days; $t (27) = .03, p = .750$). After task acquisition, there were significant effects of block (LOFC group: $F_{(2.716, 27.164)} = 18.059, p < .001$), such that rats shifted choice away from the punished option as punishment became less delayed/more proximal to the action. There were no sex differences in REVDPT (LOFC group: $F_{(5, 50)} = .657, p = .657$).

Figure 1. a) Delayed punishment decision-making task (DPDT and REVDPT). Rats chose between two levers, one delivering a one-pellet reward and the other delivering a
three-pellet reward accompanied by a delayed foot shock (delay sequence: 0, 4, 8, 12, 16 s, No Shock/Delay for DPDT; No Shock/Delay, 16, 12, 8, 4, 0 s for REVDPDT). b) A six-day micro-infusion schedule showing inactivation and saline order (days 3 and 5) counterbalanced across subjects.

A two-way analysis of variance was performed to observe the impact of sex and task (DPDT vs REVDPDT) on differences in titration of foot shock mA (0.05 - 0.55). There was no significant difference between males and females, although there was a trend toward males having higher terminal shock levels (F(1, 53) = 2.757, p = .10; male DPDT: .28 mA; male REVDPDT: .30 mA; female DPDT: .24 mA; female REVDPDT: .27 mA).

1.1. Effects of LOFC Inactivation on DPDT

We assessed the effects of acute pharmacological LOFC inactivation on sensitivity to delayed punishment prior to DPDT using 9 male and 3 female rats, with bilateral cannulae placement in LOFC confirmed before any analyses (Figure 2a). There was a main effect of block (F(2.793, 27.931) = 26.736, p < .001; Figure 3a), such that rats chose the punished reward more frequently when punishment was delayed. There was no effect of sex (F(1,10) = .018, p = .897), inactivation x sex interaction (F(1, 10) = 1.024, p = .335), or inactivation x block x sex interaction (F(5, 50) = .663, p = .653), so males and females were merged for all analyses (Figure 3b-c).
Figure 2. Histologic confirmation of cannulae placements in a) lateral orbitofrontal cortex.

OFC inactivation reduced overall choice of the punished reward ($F(1, 10) = 5.888, \ p = .036$). Critically, there was also an inactivation x block interaction ($F(5, 50) = 3.261, \ p = .013$; Figure 3a), such that LOFC inactivation only reduced large reward choice when punishment occurred after long delays, but not when punishment occurred immediately or after a short delay. Further investigation using two-tailed paired samples $t$-tests (see Table 1a) revealed no effects of inactivation in the first three blocks, a near significant difference between drug vs saline for the 12 second delayed shock ($p = .074$), and a significant difference for the 16 second delayed shock ($p = .005$). Finally, there was no effect of LOFC inactivation during the final, unpunished block ($p = .16$), suggesting that LOFC inactivation did not cause gross motivational deficits or inability to discriminate reward magnitude.
Figure 3. a) Lateral orbitofrontal cortex (LOFC) inactivation reduced choice of rewards with delayed punishment without affecting choice of immediate or short-delay punishment. b-c) Females and males showed comparable reduction in choice of delayed but not immediate punishment after LOFC inactivation. All panels display data as mean 6 standard error of the mean (SEM).
### LOFC

#### a.

<table>
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<tr>
<td>4s</td>
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#### b.

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**Table 1:** t-test results comparing average selection of the punished lever following inactivation versus saline during lateral orbitofrontal cortex (LOFC) DPDT a) and t-test results comparing average selection of the punished lever following inactivation versus b) saline during LOFC REVD PDT.
Next, we assessed the effects of LOFC inactivation on omitted trials during DPDT. There was a main effect of inactivation \( F(1, 10) = 10.494, p = .009; \) (Figure 4a) such that omissions were greater following inactivation compared to saline infusions. There was also an effect of block \( F(1.706, 17.061) = 5.734, p = .015 \), with subjects omitting more trials early in the session wherein punishment had shorter delay times. There was no inactivation x block interaction \( F(1.590, 15.901) = 2.220, p = .148 \). There was a trend toward a main effect of sex in which females displayed more omissions throughout the task than males \( F(1, 10) = 4.395, p = .062 \); (Figure 4b-c). There was also an inactivation x sex interaction \( F(1, 10) = 9.655, p = .011 \), with females showing increased omitted trials after LOFC inactivation compared to males.
Figure 4. a) Lateral orbitofrontal cortex (LOFC) inactivation increased free choice omissions compared with saline, and omissions were most prevalent when punishment occurred after shorter delays. b-c) Females displayed more free choice omissions throughout the task than males. d) LOFC infusions did not affect latency to choose either lever. e-f) Females took longer to choose the punished lever after LOFC inactivation than males. All panels display data as mean ± SEM.

We next investigated the effects of LOFC inactivation on latency to choose a lever. There was no significant difference in latency to choose safe vs punished levers ($F_{(1, 11)} = .073, p = .792$; Figure 4d), nor was there an effect of LOFC inactivation ($F_{(1, 11)} = .003, p = .960$). However, there was a trend toward an inactivation x lever type interaction ($F_{(1, 11)} = 4.435, p = .059$), such that inactivation lengthened the time required for subjects to choose the punished but not safe lever. There was also an effect of sex ($F_{(1, 11)} = 8.871, p = .013$; Figure 4e-f), with females taking longer than males to make a choice, but no sex x lever type interaction ($F_{(1, 11)} = 2.319, p = .156$).

1.2. Effects of LOFC Inactivation on REVDPDT

To test that the effects observed with DPDT were not solely due to inflexible behavior (leading to a “flattened” discounting curve), we trained rats in a reversed version of DPDT (REVDPDT) with descending delays preceding punishment (blocks: no shock, 16s, 12s, 8s, 4s, 0s). Analysis of brain sections determined that 5 female and 9 male rats ($n = 14$) had accurate cannula placement in LOFC (Figure 2a, see above). Importantly, as with DPDT, there was a significant main effect of block ($F_{(5, 60)} = 21.468$,
such that rats shifted choice away from the punished reward as delay decreased. There was no effect of sex ($F_{(1, 12)} = 3.119, p = .103$), inactivation x sex interaction ($F_{(1, 12)} = .002, p = .961$), or inactivation x block x sex interaction ($F_{(5, 60)} = .381, p = .860$), so males and females were again merged for subsequent analyses (Figure 5b-c).

**Figure 5.** a) Inactivation of Lateral orbitofrontal cortex (LOFC) during REVDPDT shifted choice away from the punished reward as delays decreased. b-c) Females and males displayed similar reduction in choice of the punished lever compared with safe when delays decreased during LOFC inactivation. All panels display data as mean 6 (SEM).

There were no effects of inactivation on choice of the punished option ($F_{(1, 12)} = 1.920, p = .191$), nor an inactivation x block interaction ($F_{(5, 60)} = 1.128, p = .355$).

However, based on the LOFC inactivation exerting the most substantial effects during the 16 second delayed punishment in the standard DPDT (Figure 5a), we probed for an effect of inactivation on this block during REVDPDT. We observed that LOFC inactivation did indeed reduce choice of the punished reward in this block ($t (13) = -$
2.816, p = .015), with no differences observed in other blocks (Table 1b, see above). This revealed that, as in the original task, LOFC inactivation reduced choice of the punished reward when punishment occurred after a long (16s) delay, but not when punishment coincided with choice after shorter (0-12s) delays.

We next investigated the effects of LOFC inactivation on omissions during REVDPDT. This revealed a near significant effect of inactivation ($F_{(1, 12)} = 4.690, p = .051$), such that LOFC inactivation increased omissions compared to saline. There was also a significant effect of block ($F_{(2.363, 28.355)} = 4.277, p = .019$) with omissions increasing after the first, unpunished block (Figure 6a). There was no inactivation x block interaction ($F_{(2.760, 33.122)} = 2.160, p = .116$). There was an effect of sex ($F_{(1, 12)} = 11.974, p = .005$), with females omitting more trials than males. There was also an inactivation x sex interaction ($F_{(1, 12)} = 4.887, p = .047$), with females, but not males, demonstrating an increase in omissions after LOFC inactivation (Figure 6b-c).
Figure 6. a) Inactivation of Lateral orbitofrontal cortex (LOFC) during REVDPDT increased free choice omissions during inactivation compared with saline. b-c) Females omitted more free choice trials than males throughout the task. d) There were no differences in latency to choose either the punished or safe levers for both inactivation and saline infusions during REVDPDT. e-f) Females required more time to select the punished lever than males. All panels display data as mean ± SEM.

Finally, we examined the effects of LOFC inactivation on response latency. There was no effect of inactivation ($F_{(1, 11)} = 3.403, p = .092$) or inactivation x lever type interaction ($F_{(1, 11)} = .300, p = .595$; Figure 6d). There was a difference in time taken to choose a lever ($F_{(1, 11)} = 11.619, p = .006$). There was also a main effect of sex ($F_{(1, 11)} = 11.420, p = .006$; Figure 6e-f) such that females were slower to respond than males.
However, there were no sex x lever type ($F_{(1,11)} = 1.911$, $p = .194$) or sex x drug interactions ($F_{(1,11)} = 2.745$, $p = .126$).

2. Experiment 2: LOFC encoding of decision-making during DPDT

In this experiment, 9 male rats were first trained in DPDT before undergoing stereotaxic surgery to implant drivable microwire electrode arrays in LOFC of either the left or right hemisphere. To minimize effects of task design, satiation, or shock habituation on neural activity and behavior, five rats were run in DPDT with ascending punishment delays (0s, 16, no shock) and four with descending delays (no shock, 16s, 0s). Of the five trained in ascending DPDT, three performed a two-block design (0s and 16s without the third block), and the other two performed all three blocks. All rats were merged for all analyses comparing blocks one and two, and the rats trained in the two-block design were removed from any analysis including block three. Six rats were implanted with electrodes in LOFC in the left hemisphere, and three in the right.

Collectively, I recorded activity from 633 units across 85 total sessions. Of these units, 497 were obtained from rats trained in the three-block task that includes the no shock control (see 2.2.1.1). Event-evoked phasic signals from each block of the task were evaluated in numerous neuronal subpopulations to identify either a decrease (Figure 7a) or increase in firing rate (Figure 7b) during or following reward and time-related events during the task.
2.1. Delayed Punishment Decision-making Behavior

Prior to analysis of neurophysiological data, I assessed decision-making for all subjects across all recording sessions. A mixed ANOVA revealed a significant main effect of block ($F_{(2, 6)} = 60.268, p < .001$; **Figure 8a**), demonstrating that rats showed the least preference for the large reward with immediate punishment, increased preference for the delayed punishment, and the largest preference for large reward with no shock.
Further, there was no main effect of task design \( (F_{(1, 3)} = .221, p = .670; \text{Figure 8b-d}) \) showing that rats underestimate delayed punishments regardless of whether the immediate or delayed shocks occurred first in the session. There was also no effect of hemisphere of electrode implantation \( (F_{(1, 3)} = .098, p = .774; \text{histological analysis is ongoing}) \), suggesting that the location of implant did not influence choice behavior. Therefore, as with previous experiments using the traditional DPDT design (Liley et al., 2019, 2022), rats were more likely to avoid rewards associated with immediate than delayed punishment, likely reflective of delay discounting.

**Figure 8.** a) Mean of combined DPDT and REVDPTD effect of block. Rats avoid the large reward with immediate punishment, increase preference for delayed punishment, and display the largest preference for large reward with no shock. b) Each line
represents the mean decision-making of the individual rats that performed all three
delays (rats that performed the two-block version of DPDT are not included). c-d) There
was no main effect of task design between DPDT and REVDPDT, showing that rats
prefer delayed over immediate punishment regardless of whether the immediate or
delayed shocks occurred first in the session.

2.2. Neuronal Activity in LOFC during DPDT

2.2.1.1. LOFC encoding of rewards associated with immediate vs no punishment

To determine if LOFC encoded the expectation of immediate punishment during a
choice, I compared activity evoked by large reward with immediate punishment choice vs
large rewards with no punishment choice. On average, rats showed greater choice of the
unpunished than punished large reward (Figure 8a). There was no difference in mean
population activity between these choices ($F_{(1, 10)} = 1.744, p = .329$; Block x time
interaction: $F_{(1.732, 859.020)} = 1.732, p = .369$; Figure 9c). However, there was a difference
in overall unit engagement during choice, such that more units were phasically modulated
in either direction during unpunished choice ($n = 132$) than punished choice ($n = 96$; $X^2$
(2, $N = 228$) = 7.38, $p < .01$; Figure 9a-b). Comparing individual subpopulations of units
revealed a significant increase in suppressed units during unpunished (n = 98) compared
to punished choice (n = 74; $X^2 (2, N = 172) = 4.05, p < .01$) and no difference in total
activated units between punished (n = 22) and safe choice (n = 34; $X^2 (2, N = 56) = 2.72,$
$p = 0.09$; Figure 9d). Overall, this shows that LOFC activity differentiates between
choice of safe and punished rewards of similar magnitude, suggesting that LOFC encodes
the absence of punishment with increased neuronal inhibition.
Figure 9. a-b) Heat plots comparing activity of individual units before choice of the large reward with immediate punishment (a) and no punishment (b). c.) There was no difference in mean population activity during choice of immediate punishment vs no punishment choice. d) Individual subpopulations of units showed a significant increase in suppressed units during unpunished compared to punished choice, and no difference in total activated units between punished and safe choice.

2.2.1.2. LOFC encoding of immediate vs delayed punishment

Next, I compared LOFC activity evoked by choice of large rewards with immediate punishment to large rewards with delayed punishment. On average, rats showed greater preference for the option with delayed vs. immediate punishment (Figure 8a). There was
no difference in mean population activity during these choices ($F_{(1,631)} = 1.691, p = .194$; Block x time interaction: $F_{(1.025, 646.85)} = .697, p = .408$; Figure 10c). There were also no differences between choices in total suppressed units (block 1: n = 99; block 3: n = 95; 2, $N = 194) = 0.09, p = .76$; Figure 10a-b). However, chi-square revealed an increase in total activated units during choice of delayed (n = 50) compared to immediate (n = 31; $X^2(2, N = 81) = 4.76, p = .03$; Figure 10d). This suggests that LOFC discriminates between delayed and immediate punishment prior to their occurrence, reflected as increased unit activation during choice of delayed punishment (also see Figure 7a-b).

Figure 10. a-b) Heat plots comparing activity of individual units before choice of the large reward with immediate punishment (a) and delayed punishment (b). c) Mean population activity evoked by choice of large rewards with immediate punishment and
large rewards with delayed punishment revealed no difference between options. d) There was an increase in total activated units during choice of delayed compared to immediate.

2.2.1.3. LOFC encoding of immediately punished large rewards vs safe, small rewards

I next compared choice of small, safe vs large, punished rewards. On average, rats demonstrated a preference for the small safe option (Figure 8a). A mixed ANOVA revealed a significant effect of choice ($F_{(1, 496)} = 8.885, p = .003$; Choice x time interaction: $F_{(1.774, 879.738)} = 4.556, p = .014$; Figure 11c), such that firing rate was lower during choice of the small, safe option. There was higher overall unit engagement during safe trials ($n = 176$) compared to punished ($n = 130$; $X^2 (1, N = 306) = 9.12, p < .01$), and more inhibited units during choice of the small, safe rewards ($n = 142$) than large, punished rewards ($n = 99$; $X^2 (1, N = 241) = 9.48, p < .01$; Figure 11d). There was no difference in percent of activated units between choices (block 1 punished: $n = 31$; block 1 safe: $n = 34$; $X^2 (1, N = 65) = 0.15, p = .70$; Figure 11a-b). Therefore, LOFC is more inhibited during small, safe choice than large, punished choice.
Figure 11. a-b) Heat plots comparing activity of individual units before choice of the large reward with immediate punishment (a.) and small reward with no punishment (b). c) There was a significant effect of choice such that firing rate was lower during choice of the safe option. d) There was a higher overall unit engagement during safe than punished trials, with more inhibited units during choice of the small, safe reward than large, punished reward and no difference in total activated units.

2.2.1.4. LOFC encoding of delayed punishment with large rewards vs safe, small rewards

I also compared choice of large rewards with delayed punishment to choice of small rewards during block 2. On average, there was no substantial difference in preference between these options (Figure 8a). There was no difference between these events in mean population activity ($F_{(1, 630)} = .454, p = .501$; Block x Bin ($F_{(1,271, 800.797)} = .439, p =$
.555; **Figure 12c), nor a difference in total units engaged during these choices (block 2 punished: n = 105; block 2 safe: n = 167; \( X^2 (1, N = 272) = 0.06, p = 0.15; \) **Figure 12a-b). There was also no difference in total suppressed (block 2 punished: n = 95; block 2 safe: n = 117; \( X^2 (1, N = 212) = 2.74, p = 0.09 \)) or total activated units (block 2 punished: n = 50; block 2 safe: n = 50; \( X^2 (1, N = 100) = 0.0, p = 1.0; \) **Figure 12d). Therefore, LOFC activity responded comparably to small, safe rewards and large rewards with delayed punishment.

**Figure 12.** a-b) Heat plots comparing activity of individual units before choice of the large reward with delayed punishment (a.) and small reward with no punishment (b). c) Choice of large rewards with delayed punishment vs small, safe rewards during block 2 showed no difference between these events in mean population activity. d) There was also
no difference in total suppressed or total activated units between delayed punished and safe trials.

2.2.2. LOFC encoding of delayed punishment expectation

To determine if LOFC activity tracks upcoming punishment, I measured LOFC activity during the 16 second pre-punishment delay period after food delivery, then compared this to a matched time prior after choice of the immediate punishment with no impending outcome. To determine changes in activity over time, the delay was divided into 1.) early delay (five-second bin after reward delivery), and late delay (2.5 second bin prior to the occurrence of delayed shock). During the early delay, there was a significant difference between trial types \((F(1, 629) = 31.651, p < .001)\), and a trend toward significance for trial type x time interaction \((F(5.907, 3715.523) = 2.088, p = .053); \text{ Figure 13a}\). Further, investigation revealed slightly more activated (block 1: \(n = 138\), block 2: \(n = 133\); \(X^2 (2, N = 271) = 0.12, p = 0.73\) than suppressed (block 1: \(n = 70\), block 2: \(n = 77\); \(X^2 (2, N = 147) = 0.38, p = 0.54\) activity during large reward, immediate punishment and large reward, delayed punishment vs safe.

Furthermore, comparison of the last 2.5 seconds during outcome anticipation between immediate punishment in block 1 vs delayed punishment in block 2 showed increased activity during anticipation of punishment late in the delay \((F(1, 628) = 4.124, p = .043); \text{ Figure 13b-c}\). There were also more activated units during delayed punishment outcome anticipation when shock was impending vs, when shock had already occurred (previous shock: \(n = 36\); delayed shock: \(n = 81\); \(X^2 (2, N = 117) = 19.07, p < .01\); \text{ Figure 13d}\) and more total selective units when shock was impending (previous shock 1: \(n = 96\), delayed
shock: n = 173; \chi^2 (2, N = 269) = 20.99, p < .01; Figure 13d). In summary, there was increased OFC activity after either choice, but this activation was greater during expectation of the delayed punishment. This suggests that OFC activation bridges the gap between a choice and the resulting delayed punishment.

**Figure 13.** a) There was increased OFC firing rate during the anticipation of delayed punishment compared to trials with immediate punishment. b-c) Heat plots comparing activity of individual units after immediate punishment (b) and during punishment delay (c) revealed a clear increase in activation during shock expectation, which was most pronounced in the period immediately preceding shock. d) There were more activated units and total combined activated punished and safe units during delayed punishment outcome anticipation in block 2 than immediate punishment in block 1.
3. Experiment 3: Time-specific inhibition of LOFC during different facets of decision-making in DPDT.

Due to technical issues and equipment malfunction, I was only able to finish performing optical inhibition in a single rat. Preliminary results are presented below as a single-subject case study to demonstrate feasibility for future studies. Halorhodopsin tagged with mCherry will be observed localized to LOFC using a fluorescent microscope.

For this experiment, functional neuronal activity in LOFC was inhibited during four distinct events during decision-making via LED light stimulation during DPDT, then performance was compared with the subsequent DPDT session with no optical manipulation. These four events of interest were: deliberation, a one sec window immediately preceding punished or safe lever selection (Figure 14a); throughout the inter-trial interval (ITI) following the end of each trial before the next trial begins (Figure 14b); after punished choice (lasting 7.5 seconds in the delayed punishment block; Figure 14c), and after safe choice (lasting 4 seconds; Figure 14d).

OFC inhibition during deliberation choice decreased selection of the punished lever when punishment was delayed. ITI inhibition caused a near complete avoidance of the punished lever, an effect that persisted even during the following baseline session with no stimulation. OFC inhibition after punished choice did not affect choice during any block of the task. Finally, OFC inhibition after safe choice caused a strong bias toward continued small reward choice for all blocks of the task. Although these results are insufficient for statistical analysis, they do suggest that optical inhibition of OFC has event-specific effects on decision-making with delayed punishment.
Figure 14. Preliminary optogenetics data taken from a single rat. a) OFC inhibition during deliberation prior to choice decreased selection of the punished lever when punishment was delayed; b) OFC inhibition during the entire ITI caused a near complete avoidance of the punished lever, an effect that persisted even on the baseline session with no stimulation; c) OFC inhibition after punished lever selection (7.5s) did not affect choice, and d. OFC inhibition after small, safe choice (4s) caused a strong bias toward continued small reward choice.
Discussion

While discounting of delayed rewards has been well-studied, little is known about the neural substrates underlying sensitivity to delayed punishment. Here I replicated previous findings that rats undervalue punishment preceded by a delay, reflected as increased choice of rewards with delayed compared to immediate punishment. This increased choice of delayed punishment was comparable between ascending and descending punishment delay schedules. In experiment one, LOFC inactivation reduced choice of delayed rewards with both ascending and descending delays, although the effect was confined to the longest (16s) punishment delay in the descending condition. Electrophysiological recording in experiment two showed that LOFC activity signals several different events during decision-making with delayed vs immediate punishment. LOFC activity 1.) encoded the presence/absence of punishment during a decision, 2.) distinguished between rewards associated with delayed vs immediate punishment, and 3.) demonstrated persistent neuronal activity during expectation of delayed punishment. Critically, LOFC was selected inhibited prior to choice of safe rewards regardless of reward size. Lastly, optogenetic inhibition of LOFC in experiment three was only completed in a single rat; however, this preliminary datum indicated that inhibition before selection of a punished or safe lever reduced choice of delayed punishment. Collectively, these data converge to provide a compelling argument that LOFC contributes to the discounting of delayed punishment.
Experiment 1: LOFC Regulates Sensitivity to Delayed Punishment

Pharmacological LOFC inactivation reduced choice of rewards with longer delayed (but not immediate) punishments, suggesting that LOFC contributes to underestimation of delayed punishment during reward seeking. This is comparable to OFC driving discounting of delayed rewards (Mobini et al., 2002a; Rudebeck et al., 2006b), although effects of OFC manipulation vary based on task design and individual differences in impulsivity (Winstanley, 2004; Zeeb et al., 2010). Notably, a population of neurons in OFC signals reduction in value of delayed rewards (Roesch et al., 2006); it is possible that OFC activity signals discounting of impending punishment in similar fashion. However, based on the lack of correlation between delay discounting of reward and punishment (Liley et al., 2019), it is feasible that OFC encodes delayed outcomes differently based on motivational valence.

One explanation for reduced choice of delayed punishment after LOFC inactivation is impaired ability to adapt to changes in delay. This inability to update task contingencies would likely manifest as a “flattened” discounting curve. However, this is unlikely based on effects of LOFC inactivation during REVDPDT, in which punishment delays decreased throughout the session. As with standard DPDT, LOFC inactivation reduced choice of delayed punishment but not immediate or briefly delayed punishment, resulting in a “steeper” curve. This verifies that LOFC inactivation does not impair behavioral flexibility in this context. Notably, LOFC inactivation in REVDPDT evoked more selective effects than during the standard task, only reducing choice of punishment during the longest (16s) delay. Performing individual comparisons typically requires the presence of an interaction; however, because the 16s delay produced the greatest effect in
standard DPDT, there was strong rationale to selectively probe this data point in REVDPDT. Nonetheless, the effects of LOFC inactivation on REVDPDT are not as substantial as on DPDT. Future replications using longer delays may increase the sensitivity of this task to LOFC inactivation and other experimental manipulations.

It is also possible that reduced choice of delayed punishment following LOFC inactivation was not caused by reduced delayed punishment discounting, but by increased overall sensitivity to punishment. However, this is unlikely because LOFC inactivation did not influence choice when punishment was immediate or after a short delay (0-8s). Another possible explanation for reduced large reward choice is that LOFC inactivation impaired magnitude discrimination, as LOFC has been shown to signal reward value (Van Duuren et al., 2008; Simon et al., 2015b; Ballesta and Padoa-Schioppa, 2019). This is unlikely because both DPDT and REVDPDT include a punishment-free 1 vs 3 pellet block, during which LOFC inactivation did not influence reward choice.

It is possible that rats performing DPDT are not discounting delayed punishment but are instead unaware of impending punishment due to reduced temporal contiguity between action and outcome, leading to increased choice of options with delayed punishment. OFC has a well-established role in outcome representation (Ursu and Carter, 2005; Mainen and Kepecs, 2009; Panayi et al., 2021); therefore, if choice of delayed punishment was driven by reduced punishment expectancy, inactivation of LOFC would further disrupt expectancy and increase choice of delayed punishment. However, LOFC inactivation here had the opposite effect, reducing choice of rewards with delayed punishment. Therefore, the most plausible explanation for the reduced choice of delayed punishment is reduced punishment delay discounting.
While decision-making in DPDT involves delay discounting of punishment, this is superimposed over a reward-based decision-making task. Optimal choice in this task requires two cognitive processes: merging punishment with expected delay to produce a negative motivational value, then integration of this aversive information with the value of appetitive outcomes (1 vs. 3 pellets). It is difficult to disentangle which of these factors is affected by LOFC inactivation. OFC is theorized to encode a dynamic cognitive map of task space that integrates all available action-outcome contingencies to guide decision-making (Wilson et al., 2014; Schuck et al., 2016; Cai, 2021). It is possible that the ability to incorporate delays preceding punishment into this “map” is dependent on LOFC. More granular research of the behavioral components of this task along with assessment of functional neuronal activity is necessary to fully delineate how LOFC drives decision-making in this context.

A previous study determined that males select rewards accompanied by delayed punishment more than females when shock intensity was comparable for all subjects (Liley et al., 2019). Baseline sex differences in decision-making were not observed here, as shock levels were titrated to avoid ceiling or floor effects for each subject (Orsini and Simon, 2020). Surprisingly, evaluation of final shock intensities per group for LOFC did not reveal a sex difference, although there was a trend toward males requiring a higher intensity shock than females. It is important to note that there were 17 more males than females overall, which likely contributed to this lack of main effect of sex. This imbalance was caused by several females failing to complete acquisition of the task and proceed to the testing phase, remaining at 0% choice of the large reward even at extremely low shock intensity. Had these subjects been included, it is likely that we
would have replicated the sex difference observed in Liley et al. (2019). Regardless, the results of the inactivation experiments suggest that LOFC regulates choice in DPDT similarly in both males and females. Due to difficulty with task acquisition, the sample size of females was smaller than males; however, the lack of sex differences in sensitivity to inactivation permitted merging sexes for each experiment.

Female rats omitted more trials than males, consistent with previous data showing that estradiol drives avoidance during punishment-based decision-making (Orsini et al., 2021). Inactivation also increased latency for females to make a choice compared to males. Finally, females required almost three times as many sessions as males to acquire DPDT. This is likely attributable to the first exposure to immediate shock driving avoidance of all options (including safe choice) in females. This subsequently increased the time required for females to be exposed to all task parameters, attenuating the overall rate of task acquisition. Alternatively, when the task began with delayed punishment (REVDPDT), females acquired the task as quickly as males. Therefore, beginning training with the option of immediate punishment may cause females to generalize punishment to both levers and completely disengage from the task early in training.

**Experiment 2: LOFC Encodes Decision-making during DPDT**

In experiment 2, I recorded neuronal activity from over 600 neurons in rat LOFC during a modified version of DPDT. As expected, behavioral performance with this specialized task reflected previous findings of DPDT in that rats discounted delayed punishments (Liley et al., 2019), and reversing the order of delays did not perturb the rate of discounting (Liley et al., 2022). Measuring single unit activity revealed that LOFC
neurons were sensitive to multiple events during DPDT, with a percentage of neurons demonstrating event-evoked phasic inhibition or activation. Critically, I observed that patterns of activation and inhibition during choice and during the pre-punishment delay encoded information about the task, such as the presence of punishment, presence of delay preceding punishment, and safety of the impending outcome.

**LOFC encodes presence of immediate punishment during choice**

First, I compared activity during choice of large rewards with immediate punishment vs the same reward size with no punishment choice. This enabled me to determine if LOFC encodes punishment expectancy during decision-making. Analyses revealed an overall difference in total unit engagement when punishment is immediate compared to non-existent, with a higher percentage of units being inhibited during safe reward choice. OFC is known for its role in signaling anticipated reward expectancy (Schoenbaum and Roesch, 2005a). The current data expands upon other studies by showing that OFC also signals information about upcoming punishment. Interestingly, neuronal subpopulations of units showed increased suppression of units during unpunished rather than punished choice, and no difference in total activation units between punished and safe choices. Therefore, increased OFC inhibition during a choice appears to reflect safety.

**LOFC differentiates between immediate and delayed punishment during choice**

Next, I compared activity during choice of large rewards accompanied with immediate or delayed punishment. LOFC signaled the presence of punishment delay by
increasing the number of units activated during a choice. It is possible that this activation could reflect subjective reward value since rats chose rewards with delayed punishment more frequently than rewards with immediate punishment.

Alternately, it is possible that this increase in choice evoked LOFC activation reflects discounting of the negative value of the upcoming delayed punishment. Previous research from Roesch et al. (2006) assessing single-unit activity in LOFC during reward delay discounting reported that short delays rather than long delays prior to reward delivery produced stronger neuronal engagement. This activity was also independent of reward size, suggesting that this change in OFC engagement reflected temporal discounting rather than value. Interestingly, the reduced engagement observed before delayed reward choice was opposite of the increased engagement, I observed during delayed punishment choice. Therefore, choices yielding delayed reward or delayed punishment seem to have opposing effects of OFC activity.

Additionally, Burton et al. (2014) reported that lateral OFC, as opposed to medial OFC, can display selective unit engagement while assessing rewards of different values. This could mean that rats subjectively hold immediate, large rewards succeeded by delayed punishments at a higher reward value than those succeeded by immediate punishment, despite reward and punishment magnitudes being equivalent for both choices. It is possible that large rewards with delayed punishment may be more savored and enjoyed during consumption, leading to increased encoding of reward value in subsequent trials. Alternately, the positive hedonic experience during consumption of the large reward with immediate punishment is dampened by immediate pain of foot shock. It would be interesting for future research to measure the influence of neuronal opioid
activity during DPDT to see if self-administration of mu-opioid activating drugs that elicit antinociception influence choice between immediate punishment or delayed punishment.

**LOFC encoding of immediately punished large rewards vs safe, small rewards**

Comparison of large, punished vs small, safe rewards showed greater LOFC unit inhibition during choice of small, safe rewards. It is feasible that LOFC inhibition reflects the safety of the small reward, indicating that there is not an immediate threat associated with this decision. Another possibility is that this increase in inhibition is not reflective of safety, but is instead providing information about reward magnitude, with increased inhibition reflecting smaller rewards (1 vs 3 pellets). Indeed, OFC has been shown to signal information about upcoming reward size during a choice (Van Wingerden et al., 2010; Roesch and Bryden, 2011; Simon et al., 2015a). However, this is unlikely, as increased inhibition was also observed during choice of safe large rewards. Since safe large AND safe small rewards both cause increased neuronal inhibition compared to punished rewards, this altered activity likely reflects safety rather than size.

These findings are comparable to a recent study observing single-unit LOFC recordings during the Risky Decision-making Task (RDT) that evaluated neuronal engagement during both safe and risky reward choice. This experiment revealed increased inhibition in LOFC when risk of punishment was absent compared to risky trials (Gabriel, 2022). Based on my data in conjunction with Gabriel (2022), increased recruitment and inhibition of neurons in LOFC signifies safety of the current choice.
It is possible that this increased inhibition in LOFC is suppressing brain regions involved with threat response or avoidance, leading to the invigoration of reward seeking. LOFC interacts closely with regions such as the basolateral amygdala (BLA; Groenewegen et al., 1990; Mcdonald et al., 1996), hypothalamus (Hurley et al., 1991; Price et al., 1991; Hardy, 1994; Gabbott et al., 2005) and periaqueductal grey (PAG; (Wyss and Sripanidkulchai, 1984; Hardy, 1986; Ongur et al., 1998; Babalian et al., 2019)). For instance, OFC and BLA are densely interconnected (Price, 2007), and both contribute to decision-making informed by reward and punishment (Jean-Richard-Dit-Bressel and McNally, 2016; Orsini et al., 2017b). Furthermore, connections between LOFC and BLA are critically involved with encoding/retrieving the incentive value of cues and actions to help guide future decision-making (Groman et al., 2019; Malvaez et al., 2019; Sias et al., 2021; Liley et al., 2022).

Additionally, the ventromedial hypothalamus along with its projection to the dorsal periaqueductal grey are responsible for fear response behaviors such as flight, freezing, and panic (Masferrer et al., 2020). Importantly, a previous study observing reversible neuronal inhibition in these two regions reported that the ventromedial hypothalamus is responsible for the encoding of an internal state that elicits motivation for defense responses and dPAG initiates motor patterns to react to such stimuli (Masferrer et al., 2020). Therefore, it is possible that LOFC transmits information about threats to this region during decision-making, and that LOFC suppression serves as a signal in dangerous, dynamic environments that threat is not impending, leading to cancellation of dPAG-regulated threat response.
LOFC encoding of delayed punishment with large rewards vs safe, small rewards

When comparing choice of small, safe rewards with choice of large, delayed rewards (the choice presented in block 2 of the task), I observed no difference in activity during choice. This contrasts with the increased inhibition typically observed during safe choice. It is possible that large rewards with delayed punishment are not considered as hazardous as rewards with immediate punishment, removing the necessity of a safety signal during safe choice. Critically, rats generally showed no preference between small, safe rewards and large rewards with delayed punishment. Thus, it is possible that when two options are considered equal in value, the LOFC does not provide a safety signal for the safer option. Rather, LOFC inhibition to indicate safety may only occur when that safe option is subjectively more valuable than the punished option.

LOFC encoding of delayed punishment expectation

Finally, I measured activity during the delay preceding punishment and determined that activity was elevated during the entire delay. This increase in activation was most evident during the final seconds of the delay when punishment was most imminent. Therefore, LOFC activity appears to bridge the gap between reward and delayed punishment.

This could mean that LOFC activity serves as a “working memory” signal, maintaining a representation of an upcoming outcome until it occurs. Indeed, this sustained increase in neural activity is similar to working memory signals observed in prefrontal cortex (Curtis and D’Esposito, 2003). However, this activation could also represent a “fear” signal that encodes upcoming threats, in this case foot shock. For
example, Liley et al. (2019) showed that locomotion during the delay is significantly reduced, indicative of expectation of an aversive event during delayed punishment choice in DPDT, which may be driven by sustained LOFC pre-punishment activity.

**Limitations**

One caveat of this experiment is the lack of female subjects due to difficulty training them in the electrophysiology compatible DPDT. Female rats have a tendency to be punishment avoidant, so encouraging these subjects to engage with the immediate trial block of DPDT can take longer than males. This avoidance can also cause increased omissions, as well as make decision-making curves to flatten out instead of producing an ascending or descending behavioral curve during DPDT. Importantly, female data is an integral part of experimentation and how females encode decision-making during DPDT will be explored in future endeavors. However, based on the current findings, it should be noted that female rats most likely would perform similarly to those observed during pharmacological inactivation in experiment one by having a lower rate of discounting throughout delay blocks than males. To correct this, I suggest introducing less free choice trials (10 instead of 20/block), using lower shock levels (~.2 mA), and possibly using a sucrose solution instead of pellet delivery as a reward.

Another limitation was that there was only one delayed punishment block (16s) during the DPDT electrophysiology task, whereas the original task incorporated 4, 8, 12, and 16s delays. Recapturing behavioral responses with multiple delays during electrophysiological recording would lead to better determination of whether LOFC encodes information about delay length, or just a general delay signal independent of
length. However, it should be noted that because of the high number of trials in the DPDT electrophysiology task (72 total trials), adding more blocks would be difficult to observe due to food satiation.

It is also worthwhile to note that this experiment only assessed activity in a single brain region. LOFC communicates with several other regions during behavior; therefore, recording from multiple regions would provide a more complete, circuit-based account of the neurophysiology underlying delayed punishment. Future experiments could anticipate interesting findings during the investigation of OFC-BLA and/or OFC-Hypothalamus/PAG circuitry.

The majority of the neurons in LOFC are excitatory pyramidal neurons, but a small subset of neurons is characterized as fast-spiking interneurons. The current study did not differentiate between these cell types. To determine if these functionally distinct neurons encoded events differently during DPDT, future analyses will divide all units into putative pyramidal neurons vs fast spiking interneurons based on waveform properties and baseline firing rate (Connors and Gutnick, 1990; Homayoun and Moghaddam, 2007; Simon et al., 2015b)

**Experiment 3: Time-specific inhibition of LOFC during different facets of decision-making in DPDT**

In experiment three, I used optogenetics to selectively inhibit LOFC activity with LED stimulation during different time points in decision-making. Preliminary data from a single rat suggests that time-specific inhibition during pre-decision, inter-trial interval, post safe lever selection, and post punished lever selection reduce selection of the large,
punished reward compared to saline (Figure 14a-d). Despite these findings and their similarity to the reduction of punished lever selection seen in experiment one (Liley et al., 2022), additional data must be gathered to statistically test these results.

Data collection for this experiment is ongoing due to technical difficulties. For instance, LED fibers implanted into LOFC commonly broke off following surgery, leaving the subjects unable to finish behavioral testing. There were also hardware issues in which patch cables connected to the implanted LED stubs frequently broke, leaving me unable to complete DPDT sessions. To optimize this technique, future experiments should focus on better securing LED fibers with Loctite before creating the headcap over the skull during surgery, and possibly securing the ends of the optogenetic patch cables with either copper wire or soldering silver onto the flimsy covering to prevent bending and breakage.

Conclusion

In summation, suppression of LOFC may drive a bias toward avoidance of punished events. Evidence of this theory was observed in experiment one in which choice of delayed punishment was reduced significantly more than immediate punishment via pharmacological inactivation. Experiment two showed that LOFC neuronal inhibition was more elicited during safe reward rather than immediately punished reward trials. Finally, preliminary data from experiment three suggests that optogenetic stimulated inhibition before selection of punished or safe rewards was attenuated during choice of delayed punishment.
Additionally, LOFC activation may have the opposite effect, initiating willingness to endure punishment. To evaluate this, future experiments could use techniques such as optogenetics via channelrhodopsin to activate neuronal engagement during DPDT during the same pre-decision, ITI, post-safe lever selection, and post-punished lever selection periods. One could also use Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to excite neuronal engagement by modifying muscarinic G-protein coupled receptors (GPCRs) in the brain. This technique has become very popular in recent years and has been an effective tool for dissecting the neural circuitry of behavior (Whissell et al., 2016). While optogenetics allow greater specificity than DREADDs, DREADDs are more established in large mammal models such as primates (Galvan et al., 2019), suggesting that this tool is closer to therapeutic use in humans.

Insensitivity to delayed punishment is a critical aspect of psychiatric illnesses, during which future consequences are often undervalued in favor of immediate rewards. To our knowledge, these experiments are the first assessments of the neurobiological mechanisms underlying this critical phenotype. These data indicate that LOFC appears to drive the underestimation of delayed punishment, and treatments selectively altering LOFC activity may serve as a promising therapeutic target to improve sensitivity to delayed punishment during decision-making.
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