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Delayed Punishment Discounting: Evaluation of Sex Differences and Contribution of Orbitofrontal Cortex

Anna Liley Vongphrachanh

DISCOUNTING OF DELAYED PUNISHMENT: EVALUATION OF SEX DIFFERENCES AND CONTRIBUTION OF ORBITOFRONTAL CORTEX

by

Anna Vongphrachanh

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Preface

This thesis is an original, intellectual product of the author, A. L. Vongphrachanh. The introduction and findings for experiments 1 – 3, Figures 1, 2, 5 – 10, and Tables 1 and 2 have been modified and were previously published in *eNeuro* under the article "Sex Differences and Effects of Predictive Cues on Delayed Punishment Discounting" (Liley et al., 2019). Results from experiment 4 are in preparation to be submitted to the *Journal of Neuroscience*. As such, all citations and references throughout this thesis reflect the publishing requirements for the *Journal of Neuroscience*.

Abstract

The majority of the research studying punishment has focused on an aversive stimulus delivered immediately after an action. However, negative consequences often occur long after a decision has been made. The delayed punishment decision-making task was developed to address this gap in literature. Rats chose between a small reinforcer and a large reinforcer accompanied by a mild foot shock. The shock was preceded by a delay, which increased throughout the session. Rats discounted the negative value of delayed punishment, as indicated by increased choice of the punished reward as the delay preceding the shock lengthened. Female rats discounted delayed punishment less than males. The addition of a cue significantly decreased the undervaluation of delayed consequences for both sexes. There was no correlation between the discounting of delayed punishments and a traditional reward delay discounting task for either sex. Finally, pharmacological inactivation of the orbitofrontal cortex significantly attenuated delayed punishment discounting.

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Introduction

Punishment describes the relationship between an action and a resultant aversive outcome (Simon et al., 2009; Jean-Richard-Dit-Bressel et al., 2018). The majority of the research studying punishment has focused on consequences received immediately after an action; however, punishment often occurs long after a decision has been made. For example, an individual that spends their entire paycheck at a bar may not experience immediate consequences but will face eviction weeks later for being unable to pay rent. Preceding consequences with a delay evokes undervaluation of the impending punishment, diminishing its influence over behavior (Yates and Watts, 1975; Strathman et al., 1994; Murphy et al., 2001; Rodríguez et al., 2018). I label this time-evoked transformation in punishment valuation as "delayed punishment discounting". Understanding this construct is fundamental for addiction neuroscience, as consequences often manifest long after the onset of drug seeking.

Previous work has shown that humans discount costs as a function of delay, and that the magnitude of discounting is distinct from delayed reward discounting (Murphy et al., 2001). Early research reported that animals favor postponed foot shocks relative to prompt foot shocks (Deluty et al., 1983). Rodríguez et al. (2018) began to address delayed punishment discounting during economic decision-making in rats, observing that rats preferred a smaller reward as a function of delay compared with a large, punished reward during ascending and descending delays. In rhesus monkeys, aversive histamine injections weakened cocaine self-administration, an effect attenuated by incremental delays preceding histamine (Woolverton et al., 2012). To further investigate sensitivity to delayed consequences in rats, I developed a two-choice behavioral paradigm called the Delayed Punishment Decision-making Task (DPDT). Rats were trained to choose between a single-pellet reinforcer and a three-pellet reinforcer accompanied by

a mild foot shock (0.35 mA). As the task progressed, a delay was introduced preceding the shock that was systematically increased throughout the session (0, 4, 8, 12, 16 s), followed by a final block in which the shock was no longer present.

Male and female rats respond differently to both punishment and non-contingent aversive stimuli (Gruene et al., 2015; Orsini et al., 2016; Jean-Richard-Dit-Bressel et al., 2018). A subset of females displayed heightened locomotion when anticipating foot shock, whereas males more consistently demonstrate attenuated locomotion (Archer, 1975; Gruene et al., 2015). Chowdhury et al. (2019) observed that female rats are more sensitive to probabilistic punishment during reward seeking than males. During economic decision-making, female rats choose rewards associated with risk of punishment less than males, and omit trials associated with risk more than males (Orsini et al., 2016). Currently, little is known about sex differences in sensitivity to delayed punishment during decision-making. To address this, I compared males and females in the DPDT, and examined effects of estrous cycle on DPDT performance in female rats.

Rewards and punishments are regularly associated with environmental cues, which enable outcome-specific representations that bias decision-making toward positive outcomes and away from aversive outcomes (Barberini et al., 2012). Cues predicting delayed rewards have been shown to affect delay discounting acquisition and sensitivity to pharmacological and neuronal manipulations (Cardinal et al., 2000; Zeeb et al., 2010). Cues can also serve as "conditioned punishers", signaling impending punishment and driving avoidance behavior in the absence of punishing stimuli (Oleson et al., 2012). These cues may increase the salience of impending punishment, thus reducing punishment discounting. I tested this by inserting a punishment-predictive cue that bridged the gap between action and consequences in the DPDT, then measuring sensitivity to delayed consequences in male and female rats.

Assessment of the neuronal and pharmacological correlates of discounting rewards as a function of delay is a fundamental area of interest in decision neuroscience (Ballard and Knutson, 2009; Roesch and Bryden, 2011; Simon et al., 2013; Cardinal et al., 2000). However, it remains unclear whether the value transformation of delayed rewards and delayed punishment share a common mechanism. To address this on a behavioral level, I compared performance in the DPDT with reward preference in a version of the traditional delay discounting task.

The following experiments addressed gaps in the current literature by establishing an effective preclinical model of economic decision-making guided by delayed punishment. They also enabled assessment of the neurobiological mechanisms underlying this critical phenotype, as well as rigorous determination of the causal relationship between delayed punishment discounting and substance use.

The Delayed Punishment Decision-making Task (DPDT)

There has been a wealth of preclinical work dissecting the cognitive and neuronal processes underlying delay discounting of rewards, yet there remains a paucity of knowledge on underestimation of delayed punishment. To begin to address this, I developed the Delayed Punishment Decision-making Task (DPDT) to characterize the tendency of rats to discount impending consequences as a function of delay length during reward-seeking. During this task, rats choose between a small, single pellet reward and a large, three pellet reward accompanied by a mild foot shock. As the task progresses, the shock is preceded by brief ascending delays (0, 4, 8, 12, 16 seconds). Published data demonstrated that rats avoided the punished option when the foot shock was delivered immediately, but systematically shifted preference toward the punished reward with increasing delay, indicative of reduced sensitivity to the shock after a delay (Liley et al., 2019). Establishment of a reliable task that measures discounting of delayed punishment

enables the assessment of behavioral and biological factors that regulate this phenotype, which I will begin to address in this thesis.

Sex Differences in Decision-making

Preclinical data on substance use and relevant cognitive phenotypes is often restricted to male subjects (Lynch, 2018). While this has provided well-controlled insight about drug vulnerability and reinforcement, it precludes information about the neurobiology underlying sex differences in addiction vulnerability. To achieve a more complete assessment of addiction in the population, preclinical data requires information that includes the behavior and pharmacological responses of females.

Male and female rats respond differently to punishment (Jean-Richard-Dit-Bressel et al., 2018; Gruene et al., 2015; Orsini et al., 2016). For instance, female rats tend to choose large, risky rewards significantly less than males, and omit trials associated with risk more than males (Orsini et al., 2016). Furthermore, female rats have significantly longer latencies when choosing a risky option compared to male rats (Orsini et al., 2016). However, there were no sex differences when observing delay discounting of rewards (Orsini et al., 2017). The first experiment in this project compared males and females in the discounting of delayed punishment. In addition, I observed any variations in response suppression and fear expression by analyzing freezing behavior and response omissions for both male and female rats during the DPDT.

Sex hormones contribute to punishment and reward sensitivity in both humans and animals (Mulligan et al., 2018; Wallin-Miller et al., 2017; Orsini et al., 2016). To test if fluctuations in estrogen are involved with discounting of delayed punishment during decision-making, I measured the cycle of all female rats after behavioral testing. The ovulation cycle for

female rats occurs every 4–5 days and consists of 4 phases: pro-estrus (12-14 hours), estrus (25–27 hours). met-estrus (6–8 hours), and di-estrus phase (55–57 hours) (Westwood, 2008). I tested the relationship between ovulation and DPDT by comparing discounting of delayed punishment within female subjects across all 4 phases of the estrous cycle. As estrous cycle has not been shown to mediate other forms of delay or punishment based economic decision-making, I predicted that discounting of delayed punishment would not differ as a function of estrous phase (Orsini et al., 2017).

Cue Control of Behavior

The occurrence of rewards and punishments is regularly associated with environmental cues, which enable outcome-specific representations that bias decision-making toward positive outcomes and away from aversive outcomes (Barberini et al., 2012). Conditioned reinforcers are cues associated with an outcome that acquire motivational salience, rendering these cues as reinforcing even in the absence of reward (Cardinal et al., 2000). For example, environmental cues become highly salient conditioned reinforcers for drugs of abuse (Caggiula et al., 2001), and can lead to maladaptive behaviors such as drug relapse (Wardle et al., 2018). Cardinal (2006) states that cues appearing during a period of delay before a reward become imbued with reinforcing properties and develop into conditioned reinforcers, which aid in linking actions to outcomes across delays. This has clinical implications for substance use, which is characterized by an inability to appropriately value delayed outcomes (Acuff et al., 2017; Rung and Madden, 2018; Chiou and Wu, 2017; Bulley and Gullo, 2017). For instance, coercing participants to visualize impending positive outcomes has shown to reduce temporal discounting, as well as reduce alcohol demand and nicotine use (Acuff et al., 2017; Rung and Madden, 2018; Chiou and Wu, 2017; Bulley and Gullo, 2017).

Cues can also serve as “conditioned punishers”, signaling impending punishment and driving avoidance behavior in the absence of punishing stimuli (Oleson et al., 2012). As with conditioned reinforcers, it is likely that the presence of conditioned punishers to bridge gaps between actions and consequences will reduce underestimation of delayed consequences. To address this, I integrated punishment-predictive cues into the DPDT. During the cued version of the DPDT, a cue light appeared over the punished lever after a choice of the punished reward and remained illuminated until the foot shock occurred. I predicted that the addition of a conditioned punisher to DPDT would reduce choice of the punished lever by serving as a reminder of delayed punishment.

Contrasting Delayed Punishment with Delayed Reward

Evidence from human studies suggests that the discounting of rewards and consequences involve distinct cognitive processes (Acuff et al., 2017; Daugherty and Brase, 2010). To further establish the independence of these constructs while also testing the construct validity of the rat DPDT, I compared performance in this task with a traditional delay discounting task (modified from Simon et al., 2013). Both tasks used identical sets of delays (0, 4, 8, 12, 16s) and comparable reward parameters (1 vs 3 pellets). As with DPDT, identity of levers was counterbalanced across groups. Percent choice of the delayed reward was used as a measure of impulsive choice, with lower preference for the delayed reward indicative of elevated impulsivity (Orsini et al., 2017). Rats ran on this task until they reach stable performance (30 days). As with humans, I predicted that the discounting curves obtained from these forms of behavioral discounting would be unassociated.

Orbitofrontal Cortex and Delayed Punishment

Animal models allow the precise investigation of the role of individual brain regions involved with behavior and cognition. A region that likely plays a regulatory role in sensitivity to delayed punishment is the orbitofrontal cortex (OFC). The OFC is a region in the prefrontal cortex that contributes to outcome expectation and arbitrates valuation during decision-making (Kolb and Whishaw, 2014; Jean-Richard-Dit-Bressel et al., 2018). Critically, OFC lesions reduce discounting of delayed rewards (Winstanley et al., 2004), and functional neuronal activity in OFC encodes time discounting of rewards (Roesch et al., 2006). Furthermore, OFC is involved in the expectation of both rewards and consequences during decision-making (Orsini et al., 2015; Xue et al., 2013; Roesch et al., 2007), and provides an integrative reward/punishment signal that guides decision-making (Morrison and Salzman, 2009). As the OFC attenuates the value of rewarding outcomes after delays and guides avoidance behavior in the face of punishment, it is likely that OFC also mediates the value of delayed aversive outcomes. To test this, I temporarily inactivated the OFC via micro-infusions of a cocktail consisting of the GABA agonists baclofen and muscimol, which suppress neuronal activity, prior to DPDT testing. I predicted that OFC inactivation would attenuate delayed punishment discounting in this experiment, expressed as reduced choice of the punished reward throughout the session.

Hypotheses

By using the previously discussed measures, I explored the following research questions: (1) Do rats discount the negative value of delayed punishment as a function of delay during decision-making? (2) Are there sex differences associated with delayed punishment? (3) Do environmental cues bridge the gap between actions and consequences to reduce delayed punishment discounting? (4) Is there a relationship between the discounting of delayed

punishments versus delayed rewards? (5) Does the orbitofrontal cortex play a role in the discounting of delayed punishment?

I hypothesized the following: (1) Rats would discount punishment as a function of delay. (2) Males would discount delayed consequences more than females. (3) The addition of a cue would decrease the discounting of delayed punishment. (4) Discounting of delayed punishment would not be correlated with discounting of delayed reward. (5) Inactivation of the OFC would reduce the discounting of delayed punishment, reflected as bias away from the punished reward.

Methods

Subjects

20 male and 20 female Long-Evans rats (n = 40, Envigo) aged approximately 70 days upon arrival were pair housed, with some individually housed in cases of excessive aggression or food domination. All rats were kept on a reversed 12-hour light/dark cycle (lights off at 8 A.M.), with all procedures conducted during the dark cycle to maximize activity. During behavioral testing, rats were maintained at 85% of their free-feeding weight (with allowances for growth) and had free access to water. All animal procedures were approved by the university Institutional Animal Care and Use Committee (IACUC).

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. Directly above these levers were cue lights that would signal an oncoming foot shock when the punished lever was selected during the cued version of the DPDT. 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.

Experiment 1: Establishing the Delayed Punishment Decision-Making Task and Characterizing Sex Differences

Rats (n = 20, 10 male and 10 female) completed behavioral testing in the following order: magazine training, lever press shaping, nose-poke training, 1 vs 3 pellet reward discrimination, delayed punishment, cued delayed punishment, delay discounting, and cued delay discounting (Figure 1).

Shaping

Initial behavioral training procedures were identical to those described previously (Simon et al., 2009). In brief, rats were first taught to associate the food trough with food pellets. They were 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. This was then succeeded by further 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. This nose poke 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 were extinguished, and the lever retracted. Rats trained to achieve a minimum of 30 presses of each lever in a 60-minute time span.

Reward discrimination

Once performance in this task was stable, rats completed 1 vs. 3 pellet reward discrimination. During this task, one lever dispensed a single pellet of food while the other dispensed three pellets of food with no risk of shock. Incorporating this training allowed rats to become familiar with the counterbalanced reward levers before starting the Delayed Punishment Decision-making Task.

Delayed Punishment Decision-making Task (DPDT)

The delayed punishment task measured the influence of punishment on reward magnitude-based decision-making. In brief: rats chose between a small reward and large reward accompanied by a foot shock, which occurred later in time as the task progressed.

Sessions consisted of six blocks with 12 trials each. Each trial began with the house light and food trough light illuminating, after which rats were required to nose poke into the lit trough within a 10 second period to initiate the trial (failure to initiate resulted in the trial being scored as an omission). A nose poke extinguished the trough light, then caused either a single lever or two levers on both sides of the trough to extend. The first two trials of each block were forced choice trials, with only a single lever available to establish the reward/punishment parameters of each lever individually within the current block. After forced choice trials, the following 10 trials were free choice trials in which both levers extended simultaneously, allowing rats to choose a preferred lever/reinforcement schedule.

Choice of one lever resulted in distribution of a single pellet delivered immediately, and the other caused distribution of three pellets immediately delivered over a three second period, accompanied by a mild foot shock (.35 mA). Identity of levers (left vs. right) was fixed across all sessions and counterbalanced between subjects. During the first block, the shock occurred immediately after lever press; subsequent blocks introduced a delay preceding shock that was progressively extended to 4, 8, 12, and 16s across blocks. If the unpunished lever was chosen, the inter-trial interval (ITI) was increased by a period equivalent to the delay preceding shock (4, 8, 12, or 16s) to maintain consistency of trial length regardless of choice. After food delivery, delay, and shock (when large reward was chosen), the house light extinguished and an ITI of 10±2s preceded the next trial. Figure 2 displays the progression of a single DPDT free choice trial. If rats did not engage an extended lever within the allotted 10 seconds, the trial was scored as an omission and followed by the ITI. After completion of all five blocks, rats performed a sixth block in which the large reward was no longer accompanied by a foot shock to confirm a preference for the large reward in the absence of punishment.

Foot shock amplitude began at 0.1 mA and was increased by .05 mA in the following session if rats completed greater than 85% of trails. This incremental increase in shock intensity limited omissions and allowed all rats to acquire task parameters. Upon reaching the final shock intensity of .35 mA, subjects trained for a minimum of 20 consecutive sessions, or until stable choice performance was achieved, defined as no significance in a repeated-measures day by block ANOVA over the final 5 days of behavior. After rats reached stability, decision-making was compared between male and female rats.

DPDT and Female Estrous Cycle

A second cohort of rats (n=20, 10 male and 10 female) was trained on shaping procedures, reward discrimination, and the DPDT (see Experiment 1). To ensure that estrous cycle did not influence decision-making behavior, female rats were smeared with vaginal lavages immediately after each test session for 2-week period once the maximum shock amplitude was reached. This allowed us to track the stability of the estrous cycle over the course of testing. Samples were placed onto microscope slides and observed under a light microscope to determine cell types during pro-estrus, estrus, met-estrus and di-estrus characterization (Lebrón-Milad et al., 2013). Identifying criteria for the four stages of the estrous cycle included: 1) pro-estrus: cells were nucleated and had a granular appearance 2) estrus: cells were cornified (rounded, with jagged edges) 3) met-estrus: cells contained both cornified cells and leukocytes 4) di-estrus: cells were leukocytes with some nucleated cells (Orsini et al., 2016; Goldman et al., 2007). If an estrous phase occurred on more than one occasion, behavioral data for that phase was averaged together during data analysis (Orsini et al., 2016). Decision-making data from all phases of estrous were compared to behavioral performance during anestrus cycle for each female.

Experiment 2: Effects of Punishment-Predictive Cues on DPDT

Once a 20-day period of stability was achieved, rats trained for an additional 10 days on a cued version of DPDT. This task was identical to the initial DPDT, except succeeding the selection of the punished lever during the task, either the left or right cue light activated (counterbalanced across groups). This cue remained illuminated until the foot shock was delivered, thus serving as a conditioned punishment signal that bridged the gap between the lever press and delayed foot shock (0, 4, 8, 12, 16s).

Experiment 3: Comparing Discounting of Punishment with Discounting of Reward

Measuring the discounting of delayed rewards, or impulsive choice, was similar to procedures described earlier (Simon et al., 2010). In brief, sessions consisted of five blocks with 12 trials each. Each block commenced with two forced choice trials (one for each lever), followed by 10 free choice trials. Rats chose between a small, immediate reward (1 pellet) or a large reward (3 pellets) delivered after a delay that increased (0, 4, 8, 12, 16 s) with each block. As with DPDT, identity of levers was counterbalanced across groups. Percent choice of the delayed reward was used as a measure of impulsive choice, with lower preference for the delayed reward indicative of elevated impulsivity (Orsini et al., 2017). Rats ran on this task until they reach stable performance (30 days).

Experiment 4: Role of Orbitofrontal Cortex in the Discounting of Delayed Punishment

DPDT prior to OFC inactivation

All behavioral training was identical to experiment one; however, shock intensity was individualized to each subject to induce linear discounting curves from all subjects. This eliminated floor or ceiling effects prior to OFC inactivation, which would preclude the observation of bidirectional effects.

Foot shock amplitude began at 0.1 mA and was increased by .05 mA in the following session if rats completed greater than 85% of trials without displaying a discounting curve (i.e. choice of large reward was equivalent across all blocks). However, shock intensity was maintained if rats showed a distinction in reward choice between blocks one (immediate punishment) and five (delayed punishment), such that block five was associated with greater choice of the punished reinforcer. Further, shock amplitude was reduced if choice of the large reward ceased or if omissions were greater than 15% of total task trials.

Pharmacological inactivation of OFC

A new cohort of rats (n = 20, 10 male and 10 female) was trained on shaping procedures and DPDT. Rats that acquired DPDT (n = 7 male) received stereotaxic surgery to implant bilateral cannulae into OFC. Following a week of recovery and re-establishment of the task, this region was temporarily inactivated to observe any changes in discounting of delayed punishments during decision-making (Figure 3).

Surgery and micro-infusions

Rats had ad libitum access to food for 3-7 days before surgery. 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 solution was provided throughout surgery via a nose cone. Scalps were shaved and cleaned with a chlorohexidine/isopropyl alcohol swab, then an anterior to posterior incision was made over the skull. Guide cannulae were bilaterally implanted in the lateral region of OFC at 3.0 mm anterior to bregma, 3.2 mm lateral, and 4.0 mm ventral to the surface of the brain (Roesch et al., 2006). Cannulae were held in place by a dental cement headcap anchored by four bone screws. Once surgery was completed, rats were subcutaneously given .5 mL of sterile saline, while food was moistened in a solution of Acetaminophen and H2O and placed in a dish 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.

After 1 week of recovery, rats resumed training on the DPDT. Once three-day stability of performance on the task was re-established, rats received either bilateral micro-infusions of GABA agonists baclofen and muscimol dissolved in sterile saline (250 ng of each drug per each µL of saline) or bilateral saline vehicle micro-infusions in the OFC via an osmotic mini-pump

and 2-µl Hamilton syringe (Bianchi et al., 2018). Bilateral microinjections were performed at a volume of 0.5 µl per side over 1 minute for each rat (Bianchi et al., 2018). Rats then tested in DPDT after a 15-minute absorption period. Using a smaller volume of this cocktail helped restrict the dose to the target region (Churchwell et al., 2009). A total of two inactivation and two saline micro-infusions were conducted on separate days, and order of drug was counterbalanced for each rat. Multiple infusions were performed to reduce the influence of any residual side effects of stress from infusion, and to test if there were any additive effects of repeated inactivation on behavior. All infusions were followed by a non-drug baseline day to control for any performance after effects of the infusion, resulting in an 8-day protocol (Figure 4).

Experimental design and statistical analyses

All behavioral data were compiled using custom-made MATLAB scripts, and all statistical analyses were conducted using IBM SPSS Statistics 24. Any violations of Mauchly's Test of Sphericity were taken into account and statistical reporting was adjusted by reporting Greenhouse-Geisser values, with degrees of freedom adjusted accordingly.

Stable decision-making in either the DPDT or delay discounting task was tested using a day x block ANOVA across the final five days of testing and was defined as: 1.) lack of main effect of day, 2.) lack of significant day by block interaction. The average percent choice of punished reward across these five days of stability as well as the slope of percent choice of the punished reward from blocks 1 through 5 were calculated as complementary measures of delayed punishment discounting. Sex differences were assessed using a sex x block mixed ANOVA. I also evaluated sex differences in task acquisition using a three-way ANOVA comparing sex, delay block and training phase (using the means of days 1-5, 6-10, 11-15, and 16-20 as each training phase). Furthermore, data collected from females from each day of the

estrous cycle (proestrus, estrus, metestrus and diestrus) were compared using an estrous cycle x block ANOVA.

Locomotion was used as an indirect measure of expectation of delayed punishment during the DPDT and was compared during the delay preceding foot shock with locomotion during the matched delay after small reward delivery on punishment-free trials. Locomotion for punished and safe levers was measured as total percentage of time spent moving, was averaged across all delay lengths (individual blocks could not be analyzed because some rats never chose the punished reward with certain delays), and then compared using a paired samples *t*-test. Data from three female rats were removed from locomotion analysis because of avoidance of the punished lever. The effects of conditioned cues on behavior were measured using a task (cued versus uncued) x block repeated-measures ANOVA. To compare the discounting of delayed punishment with delayed reward, a bivariate correlation was run to compare (1) slope of large reward preference across all blocks for both DPDT and delay discounting tasks, and (2) area under the curve (AUC) across all blocks for each task. The rate of discounting between reward and punishment was compared using the absolute value of the slopes between tasks using a two-way mixed sex x task ANOVA. Absolute value was used to control for the direction of the curve, as DPDT produces an upward curve whereas delay discounting produces a downward curve.

Paired sample *t*-tests comparing the means of the two OFC inactivation days and two saline days were conducted to test for any differences between treatment days. In the absence of effects, OFC inactivation and both saline vehicle micro-infusion days were averaged together, then analyzed via a two-way treatment x block repeated measures ANOVA. Another set of paired sample *t*-tests comparing OFC inactivation with saline were also performed on latency to

decide for either the punished or safe lever, total locomotion, and omissions during inactivation and saline sessions.

Results

Experiment 1: Delayed Punishment Decision-Making Task (DPDT)

The mean number of days to complete pre-training tasks (FR1 schedule for both levers, nose poke, and 1 vs 3) was 10 for females and 6.1 for males. Days to stable performance after reaching .35 mA of shock was 20.3 for females and 20 for males. Female rats required more sessions than males to achieve stable responding since the beginning of magazine training (t (20) = 5.243, $p < .001$; female mean: 32.3 sessions, male mean: 28 sessions).

After rats achieved stability, a repeated measures ANOVA of the five-day average revealed a significant effect of punishment delay (F (2.269, 36.306) = 17.766, $p < .001$), such that rats selected the punished reward more frequently during blocks in which punishment was delayed, with this preference increasing during longer delays (Figure 5a-b). Thus, delayed punishment did not influence reward preference as substantially as immediate punishment, indicating that rats discount the negative value of delayed punishment.

Rats demonstrated a significant decrease in locomotion during the delay after selection of the punished lever compared with the safe lever (t (16) = - 3.85, p = .001, Figure 5c). This suggested that subjects were aware that delayed punishment was impending after reward delivery. There was no difference in latency to decide between the punished or safe reward trials (t (16) = - .45, p = .66).

Sex Differences in Delayed Punishment Discounting

A two-way mixed ANOVA was conducted to assess the impact of sex across the six different punishment latency blocks. While there was no main effect of sex, (F (1, 18) = 2.066, p = .168), there was a significant sex x block interaction, (F (2.327, 41.879) = 3.090, p = .049; Figure 6a). Post hoc t-tests revealed that males and females did not differ in choice of the punished reward during the first four blocks of DPDT, but males chose this reward more than females during the 16 second delayed punishment and no punishment blocks (Table 1). Thus, males and females demonstrated comparable devaluation of the punished reward when the shocks were delivered immediately or with shorter delays, but males discounted punishment more with the longest delay. Moreover, females were more likely to avoid the large reward than males even after removal of punishment during the final block. Additionally, there was a near significant effect of sex on slope between blocks 1 (0 s delay) and 5 (16 s delay; t (18) = - 2.02, p = .06), with females showing a mean slope of 7.26, and males demonstrating a steeper mean slope of 13.03. Finally, there were no differences between sexes in task acquisition (training phase x sex interaction: F (1.980, 29.693) = .462, p = .633; training phase x block x sex interaction: F (5.212, 78.177) = 1.539, p = .185; Figure 7).

A Levene's test for equality of variances revealed no difference in variability between male and female rats in area under the DPDT curve (F = .003, p = .325). However, three females demonstrated complete avoidance of the large reward, even after removal of punishment during the final block, suggesting that females were more likely to use an avoidance-based strategy than males. A two-way sex x delay mixed ANOVA revealed that there were no significant sex differences in locomotion after choice of either reward (F (1, 15) = .124, p = .729; Figure 6b), nor was there a sex x delay interaction (F (1, 15) = .604, p = .449). Thus, males and females

demonstrated comparable reduced locomotion during the delay preceding punishment, suggesting that sex differences were not related to inability to anticipate impending shock. Finally, in female rats, there was no main effect of estrous cycle on punishment discounting (F (1.531, 15.314) = 2.024, p = .172), or cycle x delay interaction (F (2.790, 27.902) = 1.076, p = .372; Figure 8b), indicating that female rats performed similarly across all four stages of estrous.

Experiment 2: Cued DPDT

I next measured the influence of a visual cue bridging the gap between selection of the large reward lever and the delayed shock. Addition of this cue light significantly reduced choice of the punished reward across all subjects (F (1, 17) = 16.012, p = .001; Figure 9a). There was also no cue x block interaction for all rats (F (2.158, 36.691) = 1.242, p = .303), indicating that while the cue light reduced choice of the punished reward, it did not affect the shape of the discounting curve. The cue x sex interaction was not significant (F (1, 17) = 1.099, p = .309), indicating that presence of a cue exerted comparable effects on both male and female rats. Finally, there was no significant interaction between delay block and cue location (F (1.957, 29.354) = 1.602, p = .219), suggesting that cue location did not bias task performance. When divided into groups based on sex, both males and females showed a main effect of task (male: F (1, 9) = 7.545, p = .023; female: F (1, 9) = 8.617, p = .017; Figure 9b-c). In summary, a punishment-predictive cue caused an overall reduction in choice of the punished reward without affecting the shape of the discounting curve across both sexes.

Experiment 3: Comparing DPDT with Delay Discounting

Rats were trained in a small vs large, delayed reward discounting paradigm. Two females and one male were removed from data analyses due to enduring avoidance of the large reward. A mixed sex x delay ANOVA revealed a significant main effect of delay (F (4,60) = 44.251, p <

.001), indicating that rats discounted the large reward as a function of delay (Figure 10a-b). There were no sex differences in reward choice during delay discounting (main effect of sex: F (1,15) = 0.021, p = .888; sex x delay interaction: F (1.760,26.399) = 1.213, p = .309). When analyzed separately, both females and males showed a significant main effect of delay block (females: F (4, 28) = 31.504, p < .001; males: F (1.560, 12.478) =19.935, p < .001).

I then analyzed the relationship between large¸ delayed reward choices in the delay discounting task with large, punished reward choice in DPDT. There was no correlation between area under the curve for the DPDT and delay discounting tasks (r = -.049, n = 17, p = .852; Figure 10c); nor was there relationship between slopes of the discounting curves for each task (r = .160, n = 17, p = .539; Figure 10d). When rats were separated into males and females, there were no correlations for either sex between task slopes (female: r = -.207, n = 8, p = .623; male: r = -.189, n = 9, p = .626) or areas under the curve (female: r = .008, n = 8, p = .985; male: r = -.098, n = 9, p = .802; Table 2). I also compared large reward choice between tasks at each individual delay and observed no significant correlations within all rats or either sex (for full statistics, see Table 2). Thus, delayed punishment discounting appears to be independent of delayed reward discounting.

To determine whether there was a difference in the rate of discounting between rewards and punishments, I compared the slopes of the respective discounting curves using a two-way sex x task mixed ANOVA. There was no difference in slope between discounting of reward and punishment (F (1, 18) = 1.572, p = .226; Figure 10e); however, there was a sex x task interaction (F (1, 18) = 5.126, p = .036; Figure 10f). Within-subjects t-tests revealed that females had a greater discounting curve slope for rewards than punishments (t (9) = 2.941, p = .016), whereas males showed no difference between task outcomes (t (9) = -.630, p = .544). Therefore, female

(but not male) rats demonstrated more rapid discounting of delayed rewards than delayed punishments.

Experiment 4: Pharmacological inactivation of OFC

To test the role of OFC in delayed punishment discounting, I either inactivated OFC with a GABA agonist cocktail or infused sterile saline 15 minutes prior to behavioral testing. Results reported were taken from male rats only (n=7); data collection from female subjects is ongoing. Prior to testing the effects of OFC inactivation, I confirmed that the micro-infusion procedure did not influence decision-making using a treatment (saline micro-infusion vs no treatment) x block ANOVA, which denoted no significant effect of micro-infusion on behavior (F (5, 15) = 1.108, p = .397). No significant differences were found when t-tests were conducted to compare mean choice of the punished lever during OFC inactivation 1 and 2 (t (6) = 1.475, p = .191), as well as for saline infusion 1 and 2 (t (6) = -.447, p = .671). Thus, I used the average of the two saline infusions and the two OFC infusions for analysis.

Next, I tested the effects of OFC inactivation on DPDT. A two-way micro-infusion type X block repeated measures ANOVA did not produce a significant main effect of drug (F (1, 6) = .320, p = .592). However, there was a significant infusion X block interaction (F (5, 30) = 3.365, p = .016), such that OFC inactivation reduced preference for the punished reward as punishment delay increased. Thus, OFC inactivation reduced delayed punishment discounting without affecting avoidance of a reward associated with immediate punishment (Figure 11).

Paired samples t-tests were conducted to evaluate any significant differences between OFC inactivation and saline average latency to choose the safe or punished lever, locomotion after a choice, and trial omissions. There were significant differences between inactivation and saline on latency to choose the punished lever (t (6) = 2.912, p = .027), as well as the safe lever (t

(6) = 3.429, p = .014). In both cases, OFC inactivation increased the latency to make a decision (Figure 12). There were also no significant differences between inactivation and saline omissions (t (6) = 1.783, p = .125) or other measures.

Discussion

There is a wealth of research dissecting the cognitive and neuronal processes underlying delay discounting of rewards yet remains a paucity of information on discounting of delayed punishment. This cognitive construct is critical for understanding the prevalence of drug seeking despite severe consequences that manifest later in time. To address this, I developed the Delayed Punishment Decision-making Task (DPDT). I observed that rats discount the negative value of delayed consequences relative to immediate consequences. Female rats discounted delayed punishment less than males, and this was not influenced by phase of the estrous cycle. Moreover, addition of a punishment predictive cue light decreased choice of delayed punishment for both sexes. There was no predictive relationship between the discounting of delayed rewards and punishments, although females selectively discounted reward at a faster rate than punishment. Finally, OFC inactivation reduced discounting of delayed punishment in male subjects.

Experiment 1: Delayed Punishment Decision-Making Task

The DPDT revealed that, on average, rats avoided a large reward associated with an immediate shock in favor of a small, safe reward. However, rats shifted preference toward the punished reward when the shock was delayed. This demonstrates that, like in humans (Murphy et al., 2001), rats discount delayed consequences relative to immediate consequences, and that this value transformation increases as a function of delay. The current task also revealed substantial

individual differences in propensity to discount delayed punishment, which may be a promising avenue for future study.

One concern was that the delay caused the shift in preference via failure to associate actions with the delayed outcomes, rather than discounting of delayed consequences. To address this, I measured locomotion following choice of the large reward, then compared this with a time-matched delay after choice of the small reward. In previous literature, rats have demonstrated freezing behavior during expectation of an aversive stimulus, quantified as near complete lack of mobility and crouching posture (Fanselow, 1980). Although we did not directly assess freezing, cessation of normal locomotion is an integral portion of the freezing response; therefore, locomotion has utility as a proxy of freezing. Rats demonstrated a consistent reduction in locomotor activity during the pre-punishment delay, suggesting that rats were indeed aware of impending punishment, yet still discounted the negative value during reward choice.

While delayed punishment discounting is relatively understudied, other studies have observed this factor in different animal models of decision-making. A recent study observed two-choice delayed punishment discounting during a decision-making task in rodents but varied in numerous ways from the task at hand (Rodriguez et al., 2018). The current study used different reward size and delay lengths, different ITI length, and included a punishment-free block as an additional control measure. Additionally, Rodriguez et al. (2018) utilized multi-colored cue lights to signify a change in delay time to foot shock, whereas our experiment was replicated in both uncued and cued conditions. Another experiment involving rhesus monkeys used histamine as a punishment to reduce the potency of a cocaine reward and observed that delaying the histamine infusions increased choice of the punished cocaine reinforcer (Woolverton et al.,

2012). These variations of delayed punishment demonstrate that this phenomenon occurs across multiple species and rat strains.

Sex Differences in Delayed Punishment Discounting

To my knowledge, this experiment was the first to report sex differences in delayed punishment discounting. It was observed that male rats discounted delayed consequences more than females, as indicated by an increased shift in preference toward punishment-associated rewards when punishment was delayed. This did not appear to be a function of reduced overall sensitivity to punishment, as males and females demonstrated comparable avoidance of the punished reward when punishment was immediate, as well as displayed comparable freezing behavior during punishment anticipation.

These data expand upon previous research that reported sex differences in response to punishment during reward seeking. Orsini et al. (2016) found that female Long Evans rats significantly preferred small, safe rewards over large, risky rewards compared to males during the Risky Decision-making Task, and that this was unrelated to body weight or reward motivation (RDT). Altered sensitivity to punishment in females is not limited to nociceptive stimuli, as female rats are more sensitive to reward loss than males (Van den Bos et al., 2012; Chowdhury et al., 2019).

The menstrual/estrous cycle mediates reward-seeking, cue sensitivity, and evoked dopamine release in females (Johnson et al., 2019; Becker and Hu, 2008; Calipari et al., 2017). Furthermore, there is evidence that women vary in discounting of delayed rewards during different phases of estrous (Hosseini-Kamkar and Morton, 2014). Thus, it was important to test if estrous contributed to delayed punishment discounting. No difference was evident in DPDT during any of the four phases of estrous, consistent with estrous cycle playing no role in other

punishment-related decision-making tasks (Orsini et al., 2016). Therefore, it is unlikely that hormonal fluctuations contribute to sex differences in delayed punishment discounting.

The majority of seminal studies in behavioral neuroscience have been restricted to male subjects (Beery and Zucker, 2011). Unfortunately, this relegates many overarching theories of behavior to a singular male perspective, disregarding both the differences in brain structure/function and the discrepancies in vulnerability to disease between males and females (Becker and Hu, 2008; Shansky, 2019; Grissom and Reyes, 2019). The current study provides novel evidence that male behavior is not fully generalizable to females during economic decision-making, further underscoring the importance of evaluating behavior in both sexes to optimize treatment of maladaptive decision-making in psychopathology.

Experiment 2: Cue Influence on Delayed Punishment Discounting

In humans, providing reminders of delayed rewarding outcomes reduces both temporal discounting and vulnerability to substance use (Murphy and Dennhardt, 2016). Rodent models of reward-based delay discounting have demonstrated that introduction of a cue that bridges the gap between an action and the outcome affects task acquisition and sensitivity to drugs and brain region inactivation (Cardinal et al., 2000; Zeeb et al., 2010). Accordingly, I tested if exposure to cues reminding subjects of impending negative outcomes affects delayed punishment discounting. It was determined that the addition of a punishment-predictive cue reduced punishment discounting in both sexes, reflected as a shift in preference away from punished rewards preceded by delays. This finding reinforces the idea that environmental cues heavily influence temporal decision-making. Furthermore, this suggests that providing reminders of impending consequences may have utility for attenuating high levels of punishment discounting,

which may be maladaptive in disorders characterized by pathological reward seeking in the face of consequences.

Experiment 3: Delay Discounting and Delayed Punishment Discounting

Preference for immediate gratification, often referred to as delay discounting or impulsive choice, is prevalent in many mental disorders, including substance use disorder (Winstanley et al., 2004; Orsini et al., 2017; Brevers et al., 2012; Garavan and Hester, 2007). While there is substantial literature in humans and animals investigating delayed rewards during decision-making, there is very little research investigating delayed consequences in animal models. Evidence from human studies suggest that the discounting of rewards and consequences are distinct cognitive processes that occur at different rates (Murphy et al., 2001). To further evaluate the independence of these constructs, I compared delayed punishment performance in this task with delayed reward performance in a traditional delay discounting task (modified from Simon et al., 2013). Both tasks used identical sets of delays (0, 4, 8, 12, 16s) and comparable reward parameters (1 vs 3 pellets). As in humans, no association between discounting of rewards or punishments was observed. Interestingly, humans discount delayed rewards at a faster rate than delayed costs (Murphy et al., 2001). While this was not observed across all subjects, females showed a steeper discounting curve for rewards than punishments, being more likely to shift away from delayed rewards than to shift toward punishment at comparable delays. This sex-selective species difference may be a related to a critical difference in task outcomes: in the current study, the delayed consequence is a physical foot-shock, which is distinct from the delayed reward of pellets. In humans, both the reward and consequences manipulate the same outcome: either gaining or spending money, respectively. This provides further evidence of sex

differences in delayed outcome processing, which suggests differences in recruited neuronal circuitry or functional activity during decision-making.

Multiple brain regions are implicated in mediating time-discounting of rewards, including the orbitofrontal cortex, nucleus accumbens, and basolateral amygdala (Roesch et al., 2006; Cardinal, 2006; Bickel et al., 2014). The current research suggests that delay discounting does not occur at a comparable rate between rewards and punishment; thus, it is likely that the neuronal mechanisms underlying punishment discounting diverge from those involved with transformation of reward value. Further research is necessary to confirm if there are distinct circuits involved across both reward and punishment delay discounting, or if comparable circuits encode delayed outcomes independent of valence.

Experiment 4: Pharmacological Inactivation of OFC

I observed that OFC inactivation via GABA agonist cocktail reduced delayed punishment discounting. Although this study is the first to discover a potential neural substrate of delayed punishment discounting, multiple studies have observed that OFC contributes to temporal discounting of reward. Mobini et al. (2002) discovered that OFC lesions induce preference for immediate vs. delayed reinforcers. Winstanley (2004) observed the opposite effect, with OFC lesions increasing preference for delayed rewards. Zeeb et al. (2010) incorporated environmental cues into the delay discounting task and found that OFC lesions in highly impulsive rats increased selection of delayed rewards when no reward-predictive cue was provided, but increased avoidance of delayed rewards in less impulsive rats in the presence of predictive cues. Despite the differential effects between these experiments, they each observed that OFC plays a fundamental role in reward delay discounting. Furthermore, research involving humans has demonstrated that OFC damage leads to increased impulsive gambling task performance and

maladaptive decision-making with delayed rewards (Damasio, 1994; Bechara et al., 2000; Rogers et al., 1999). The current study extends our knowledge of OFC function, suggesting OFC involvement in temporal discounting of punishment as well as reward.

Roesch et al. (2006) further analyzes the role of OFC in delay discounting using single unit electrophysiology. This study found that reward-responsive OFC neurons were less sensitive to delayed rewards, suggesting this as a potential neurophysiological mechanism that drives preference for immediate over delayed gratification. This attenuated OFC encoding of delayed outcomes may also occur during delayed punishment in DPDT, reducing the salience of delayed consequences and shifting decision-making preference toward these options. Future research involving single unit electrophysiology during DPDT will determine how functional activity in OFC responds to delayed punishment during decision-making, and how this compares with delayed reward encoding. It is important to note that punishment discounting in DPDT was uncorrelated with delayed reward discounting; therefore, despite the likelihood that OFC encodes time discounting of both punishments and rewards, it may encode them in distinct fashion. This could be a result of different patterns of encoding delayed reward and punishment in OFC, or differences in projections to and from OFC that correspond with rewarding and punishing stimuli (Groman et al., 2019).

One possibility is that OFC inactivation did not affect reward discounting, but instead altered overall sensitivity to punished rewards. However, this is unlikely because OFC inactivation did not influence choice during blocks with immediate or short delays, but shifted choice away from punishment with longer delays. Furthermore, Jean-Richard-Dit-Bressel and McNally (2016) found that OFC lesions increased punished responding for reward – therefore, if our results were solely related to changes in reward/punishment integration, OFC inactivation

would be more likely to increase punished option rather than the decrease observed here. Collectively, this suggests that OFC inactivation only influences cost-benefit decision-making when punishment is delayed, suggesting that value transformation of delayed punishment is likely dependent upon OFC.

Another potential explanation for the reduced preference for delayed punishment is that OFC inactivation impaired the ability to detect changing contingencies within a session. This inability to process task changes may have caused rats to maintain a consistent reward preference even as delays preceding punishment increased. However, previous cost-benefit decision-making research suggests that this is unlikely. Medial pre-frontal cortex (mPFC) rather than OFC lesions appear to drive the ability to update outcomes throughout a session, whereas OFC is implicated in general reward discounting processes (Orsini et al., 2015; Orsini et al., 2018; St. Onge and Floresco, 2010). To address this concern, a follow up experiment is in progress testing subjects on a reverse version of DPDT in which the delay sequence is 0s (no shock), 16s, 12s, 8s, 4s, 0s. I predict that OFC inactivation will shift preference away from the delayed punishment regardless of delay sequence, which would suggest that OFC inactivation affects punishment discounting rather than response flexibility.

Following OFC inactivation, I observed increased latency to decide, which was similar for choice of punished and safe levers. Orsini et al. (2015) also found that OFC lesions increased decision latency during a risky decision-making task; however, this was limited to trials with choice of the large, punished reward. This subtle difference may be a function of task design; both experiments involved a comparable reward magnitude discrimination and the presence of punishment, but the current study integrated delays that precede punishment. Addition of a delay likely drives increased recruitment of OFC during outcome evaluation prior to decision-making,

as OFC is heavily implicated in processing of delayed outcomes (Bechara et al., 2000; Roesch et al., 2007). This could explain the gross increase in decision-time across all trials (rather than solely punished trials).

The question remains: why has the mammalian brain evolved to discount the value of delayed punishment? This might be perceived as disadvantageous, as both immediate and delayed punishment exert comparable physical costs to the subject. Delayed punishment discounting likely developed to facilitate focus on current needs for survival (food, water, shelter, sex, sleep) despite the potential long-term occurrence of aversive outcomes. Critically, in real-world situations, delayed outcomes may not be guaranteed; thus, it is often beneficial to weigh immediate, tangible punishment or reward over delayed, hypothetical outcomes during decision-making. However, as society has evolved, delayed consequences associated with substance use or other risky legal or financial decisions have grown more inevitable, making discounting of delayed punishment a less advantageous strategy. Thus, it is imperative to understand the fundamental neurobiological processes underlying discounting of delayed punishment.

Limitations

A current limitation of this study is that the sample used in the OFC inactivation analyses consists of all male subjects. This is partially due to extenuating factors involving female subjects taking longer to re-acquire stable task performance after recovering from surgery. However, preliminary data including both sexes revealed that females differ at baseline performance but show the same inflexible behavior pattern (flatter curve) during OFC inactivation. Thus, despite the baseline sex differences in delayed punishment discounting, OFC likely governs DPDT comparably in males and females.

Additionally, histology associated with this experiment is unavailable due to on-going research involving a reverse delay version of the DPDT and further comparison of OFC inactivation. Therefore, the accuracy of surgical implantation of bilateral cannulae is not known and cannot currently be cross-referenced with behavioral data. Nonetheless, completed data analyses from this experiment will be made public once in hand.

Conclusion

Overall, development of the delayed punishment decision-making task: (1) has demonstrated that rats discount the negative value of delayed consequences as a function of delay, (2) revealed novel sex differences in decision-making, (3) is modulated by punishment predictive cues, (4) is independent of reward delay discounting, and (5) is affected by inactivation of the OFC during task performance, which significantly influences behavioral shifting patterns based on outcome prediction and may contribute to reduced delayed punishment discounting.

Delayed punishment discounting is a critical aspect of substance use disorders and other forms of pathology, during which future consequences are often undervalued in favor of immediate rewards. This task will enable assessment of the neurobiological mechanisms underlying this critical phenotype, including further exploration into the role of OFC in punishment discounting. Finally, a rat model of delayed punishment discounting allows rigorous determination of the causal relationship between this cognitive phenotype and substance use.

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Figures

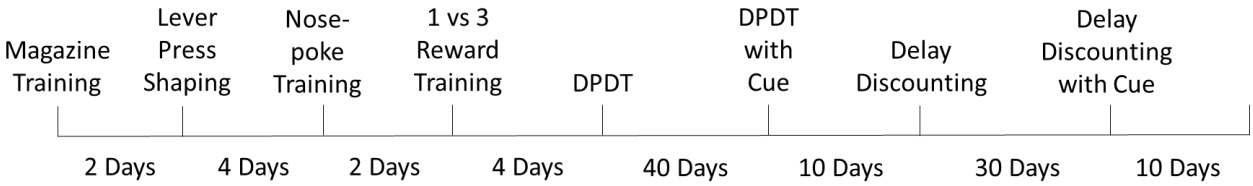

Figure 1. Timeline of experiments 1, 2, and 3.

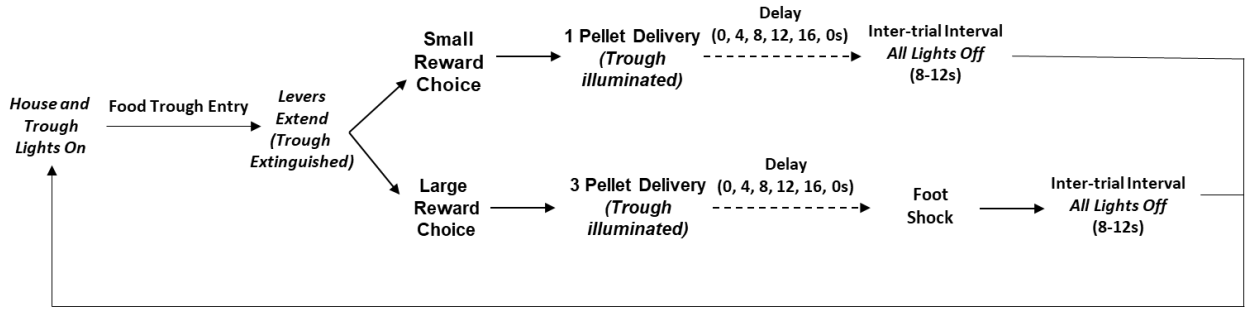

Figure 2. Delayed Punishment Decision-making Task. Rats chose between two levers, one lever delivering a one pellet reward and the other delivering a three pellet reward accompanied by a delayed foot shock (0, 4, 8, 12, 16 seconds). There was no shock associated with reward in the final block.

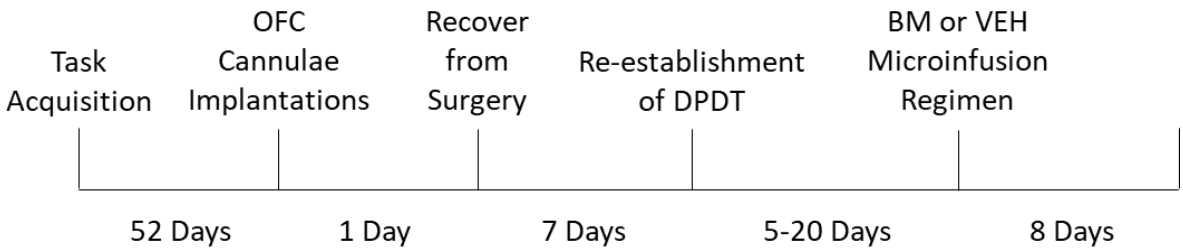

Figure 3**.** Experiment 2 timeline.

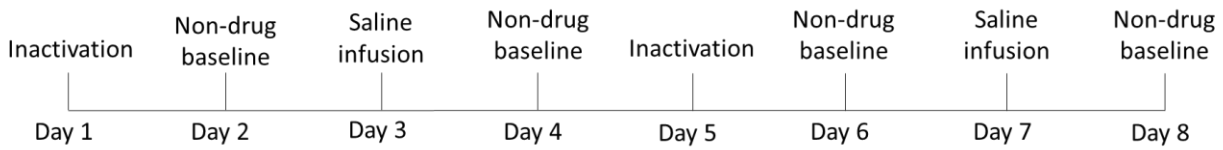

Figure 4. OFC inactivation and saline micro-infusion timeline. Counterbalanced across subjects.

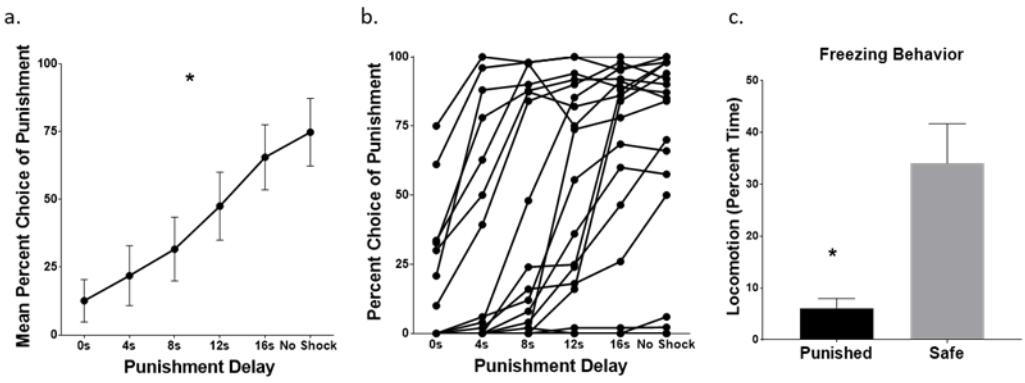

Figure 5. Delayed Punishment Decision-making Task (DPDT). a) On average, rats shifted behavior toward the punished reward as the delay increased, indicative of underestimation of delayed punishment. Each marker represents mean choice of the large reward ±SEM. b) Individual differences in DPDT performance, with each line representing a single subject. c) Percent time spent performing locomotion during the delay period after punished and safe levers were pressed and reward was delivered. Locomotion significantly decreased after choice of the punished relative to safe reward, suggesting that rats anticipated impending shock.

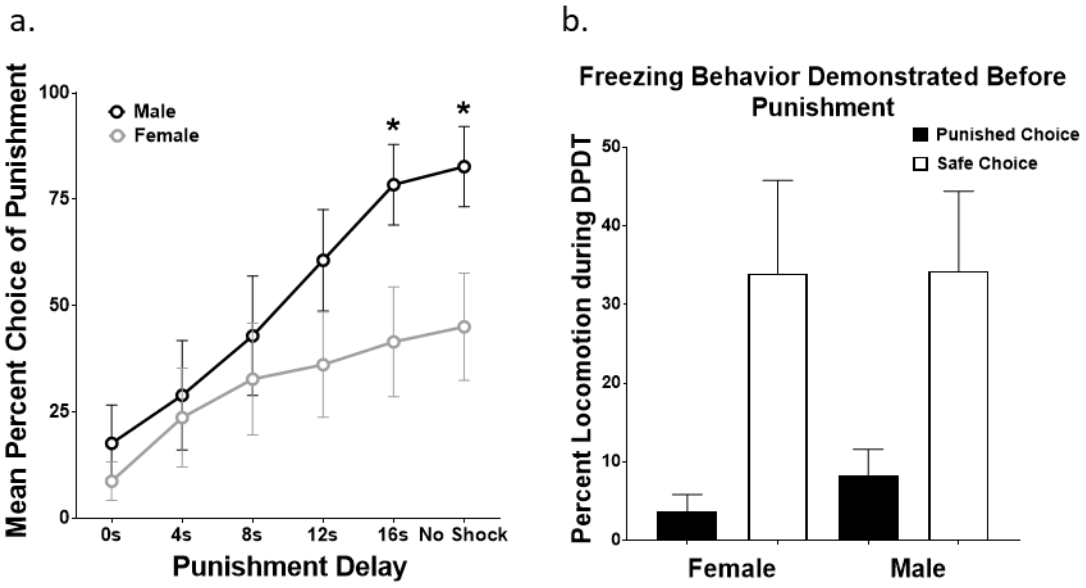

Figure 6. Sex differences in delayed punishment discounting. a) Mean percentage of male vs. female selection of the punished lever through blocks 1-6 of DPDT. Males discounted punishment significantly more than females as delay increased. b) Both females and males showed reduced locomotion during the delay preceding punishment compared to unpunished trials.

Table 1. *t*-test results comparing sex differences in percent choice of the punished reward.

Delay	Sex	Mean	SEM	*t*	df	*p*
0s	Male	17.611	8.98	-0.882	18	0.389
	Female	8.722	4.56			
4s	Male	28.933	12.87	-0.303	18	0.766
	Female	23.677	11.644			
8s	Male	42.933	14.081	-0.53	18	0.603
	Female	32.727	13.155			
12s	Male	60.711	11.934	-1.428	18	0.17
	Female	36.122	12.414			
16s	Male	78.514	9.491	-2.311	18	0.033*
	Female	41.501	12.905			
No shock	Male	82.755	9.446	-2.389	18	0.028*
	Female	45.042	12.65			

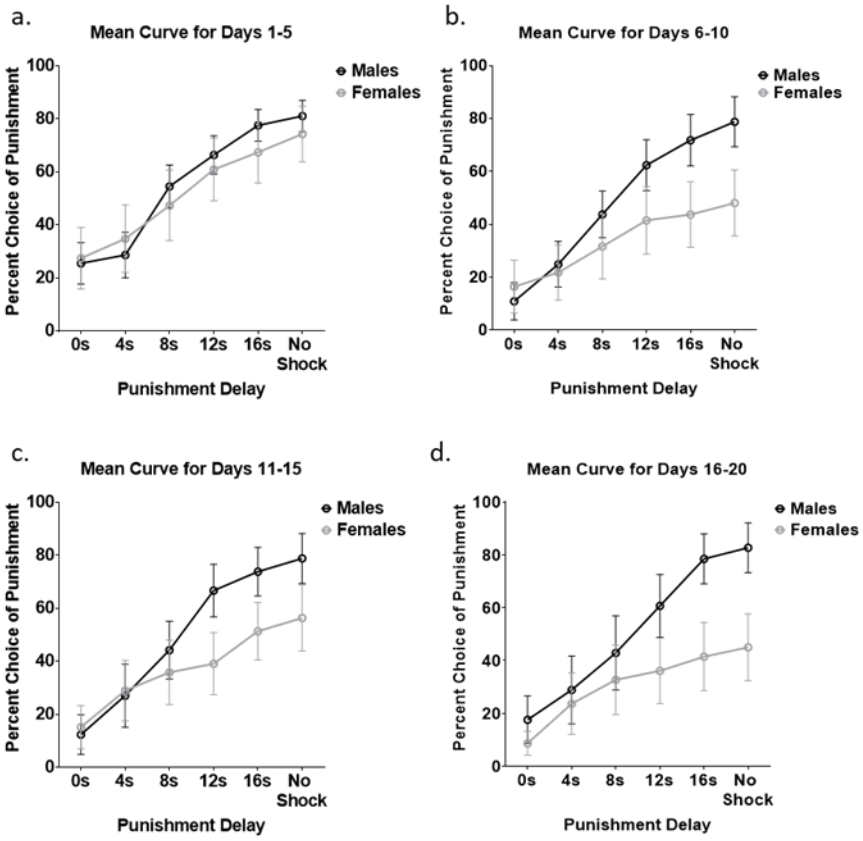

Figure 7. There was no difference between males and females in DPDT acquisition. a) Behavioral training from days 1-5, b) days 6-10, c) days 11-15, d) days 16-20.

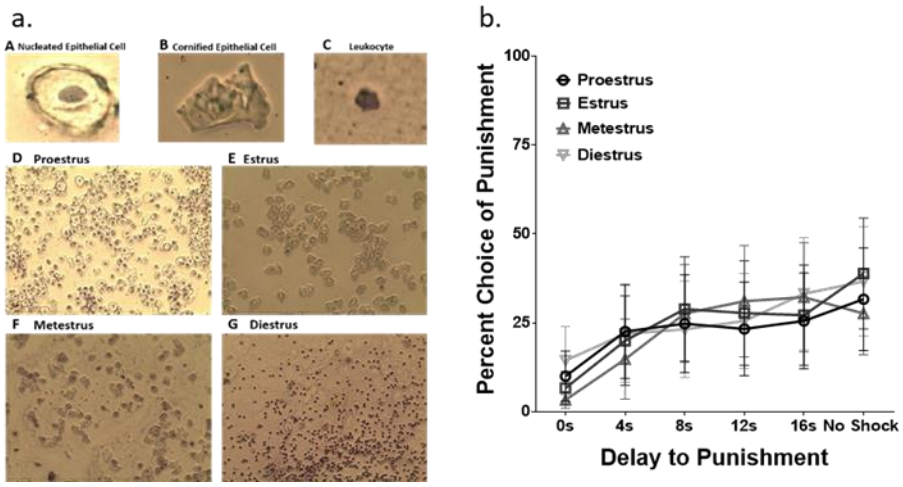

Figure 8. a) Appearance of nucleated epithelial cells, cornified epithelial cells, or leukocytes was used to determine stage of estrous cycle. b) Female rats did not differ in percent choice of the punished lever during the DPDT across proestrus, estrus, metestrus, and diestrus stages of the estrous cycle.

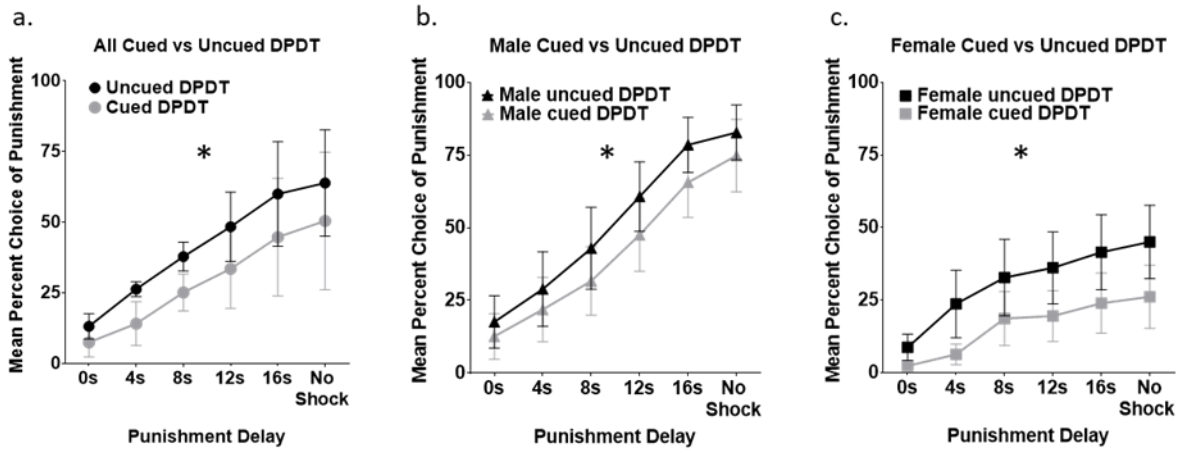

Figure 9. Cued Delayed Punishment Decision-making Task. a) Mean percentage of male vs. female selection of the punished lever through blocks 1-6 of DPDT and cued DPDT. b) Addition of a cue light attenuated choice of the punished lever for male rats c) and female rats across all delays.

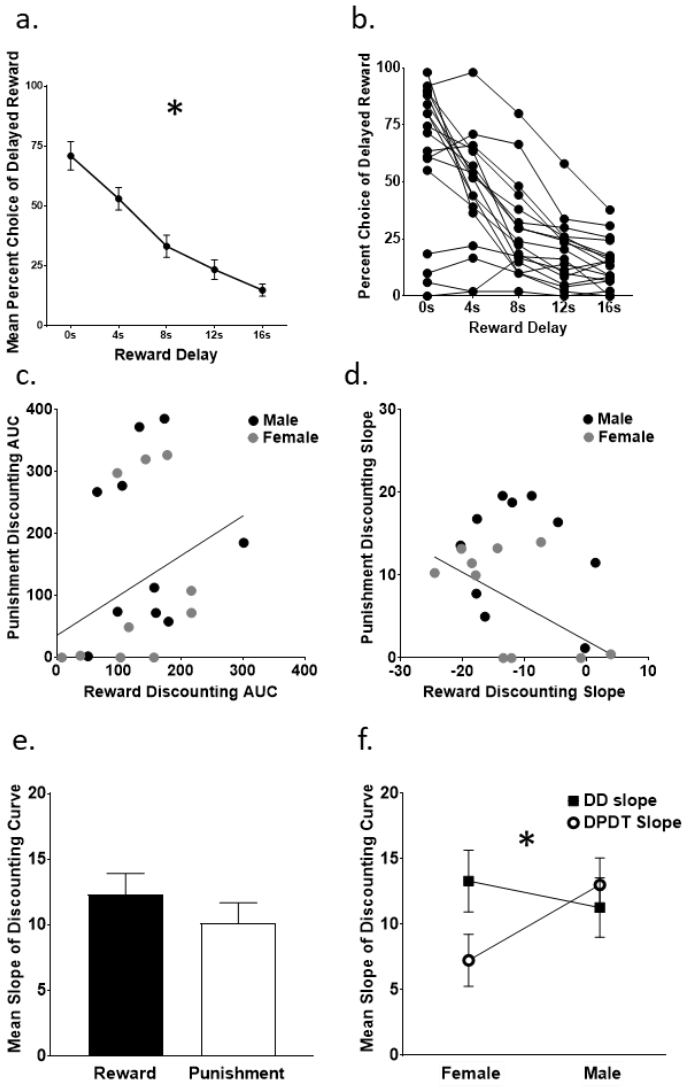

Figure 10. a) On average, subjects shifted behavior toward the immediate reward as the delay increased. b) Individual differences in delay discounting, with each curve representing an individual rat. c) Area under the curve (AUC) for delay discounting was not significantly correlated with AUC during DPDT. d) Slope of % choice of punished reward during DPDT and percent choice of delayed reward during delay discounting were also not correlated. e) Overall, there was no differences in discounting curve slope between reward and punishment discounting. f) Females but not males demonstrated a higher slope for delayed reward than delayed punishment choice, indicating more rapid discounting of delayed rewards vs. punishments.

Table 2. Bivariate correlations were conducted to compare slope, AUC, and mean percentage choice of the punished reward for each delay block between DPDT and the delay discounting task. There were no significant correlations between tasks in any measure for all rats, nor for individual sexes.

Measure	*r* All Rats	*p* All Rats	*r* Female	*p* Female	*r* Male	*p* Male
Slope	-0.173	0.508	0.207	0.623	-0.237	0.539
AUC	-0.049	0.852	0.008	0.985	-0.098	0.802
Mean: 0s	0.386	0.126	0.658	0.076	0.409	0.274
Mean: 4s	-0.134	0.608	0.009	0.984	-0.199	0.608
Mean: 8s	-0.269	0.296	-0.201	0.632	-0.347	0.361
Mean: 12s	0	0.999	-0.021	0.961	-0.082	0.833
Mean: 16s	0.068	0.796	-0.098	0.818	0.052	0.895

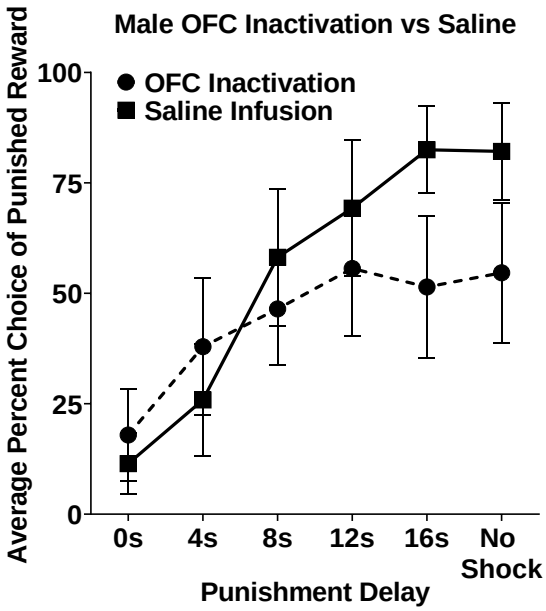

Figure 11. Mean percent choice of the punished lever during OFC inactivations compared to saline vehicle.

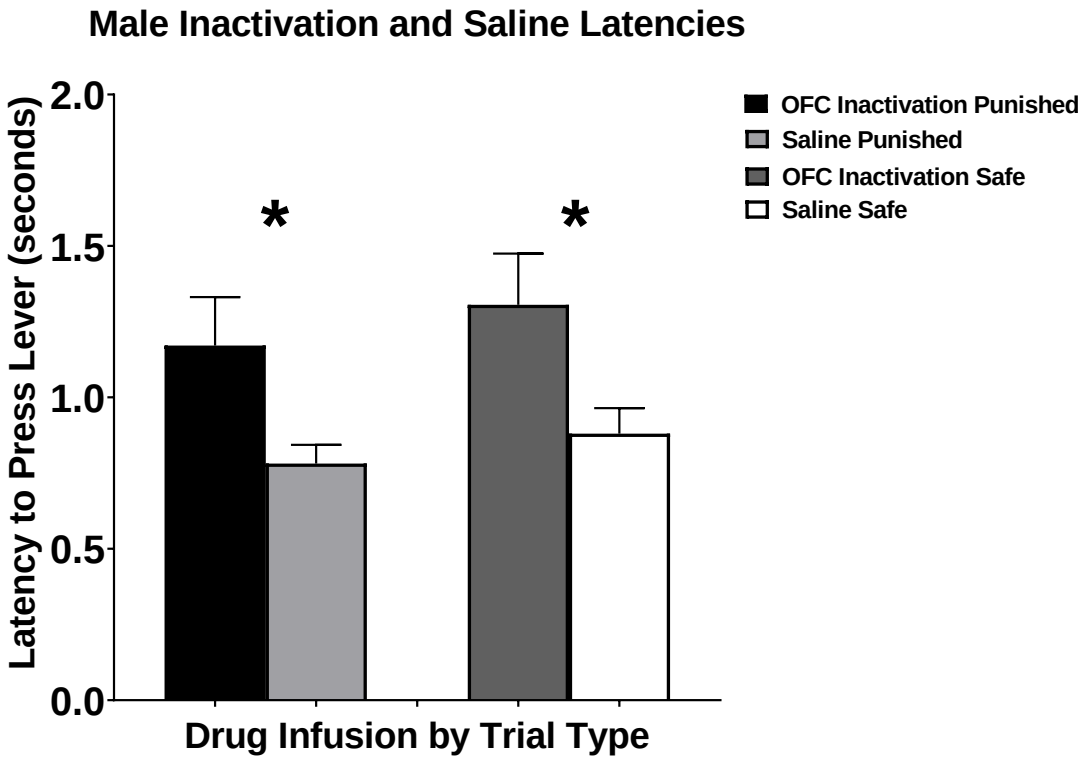

Figure 12. OFC inactivations increased latency to choose both the punished and safe levers compared to saline micro-infusions.

Appendix

IACUC PROTOCOL ACTION FORM

To:	Nicholas Simon
From:	Institutional Animal Care and Use Committee
Subject:	Animal Research Protocol
Date:	September 20, 2019

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 0844

0844 1. Identifying the Neurophysiological basis of risky decision-making (2019-2022). 2. Comorbidity between impulsivity and voluntary oral oxycodone intake across multiple inbred rat strains (2019-2020)

☒ Your protocol is approved for the following period:

From: October 1, 2019 To: September 31, 2022

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: To:

☐ Your protocol is approved with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated for the following period:

From: To:

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated

Amy L. de Jongh Curry, PhD, Chair of the IACUC

Karyl K. Buddington, University Veterinarian and Director of the Animal Care Facilities