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Effects of a psychological stressor on methamphetamine seeking in rats.

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EFFECTS OF A PSYCHOLOGICAL STRESSOR ON METHAMPHETAMINE SEEKING IN RATS.

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ABSTRACT

Although methamphetamine abuse and fatality rates are on the rise in the United States, there are currently no FDA approved drugs to treat methamphetamine use disorder (MUD). To better understand factors contributing to addiction, researchers have designed various rodent models of stress including the use of footshock, social defeat and maternal separation, however, these models involve physical or early life stress exposure and thus are less translatable to human psychological stress. The present study implemented predatory odors as a model of psychological stress and examined whether chronic exposure to these stressors enhanced subsequent vulnerability to a subthreshold dose of methamphetamine. Results of the current study suggest using coyote urine can enhance vulnerability to drug reward/motivation in the CPP drug paradigm, as shown by increased drug-seeking behavior in animals given methamphetamine. Specifically, methamphetamine animals exposed to coyote urine displayed a resistance to extinction in comparison to other stress groups. In conclusion, coyote urine may serve as a sufficient psychological stressor to alter motivation for subthreshold doses of methamphetamine and potentially other drugs of abuse.

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1. Introduction

Amphetamine-type stimulants are one of the most commonly used class of illicit drugs worldwide and methamphetamine is the most frequently used psychostimulant (*World Drug Report*, 2022). In 2021, about 2.5 million Americans reported using methamphetamine in the past 12 months, with an estimated 0.2%-0.5% of 8th, 10th and 12th graders reporting use as well (SAMHS, 2023; Miech et al., 2023). Methamphetamine abuse poses a number of negative health consequences, as well as significant public health challenges and social harms for society (Gonzalez, Mooney, & Rawson, 2010). At low to moderate doses (5-30mg), the clinical response to methamphetamine includes euphoria, arousal, reduced fatigue, as well as positive mood, however, at frequent and high doses the consequences of methamphetamine abuse are detrimental to the individual causing memory loss, aggression, anxiety, malnutrition, dental issues, and potential psychosis (Courtney & Ray, 2014; NIDA, 2019). Methamphetamine abuse also poses a substantial economic burden. In the United States alone, the intangible cost of addiction including drug treatment and other healthcare costs, premature death, crime and other harms resulting from its production, methamphetamine abuse and its consequences totaled approximately $23.4 billion in 2005 (Nicosia et al., 2009). Tragically, the National Institute on Drug Abuse reports that overdose fatality rates involving methamphetamine have quadrupled from 2011 to 2017, and have continued to rise since 2019 (NIDA, 2019; NIDA, 2023). In 2021 alone, approximately 32,537 individuals overdosed on psychostimulants other than cocaine, primarily methamphetamine (Ahmed et al., 2023). Despite the serious need for pharmacological treatments for methamphetamine abuse there are currently no FDA approved drugs designed to treat methamphetamine use disorder (MUD).

In order to try to help curb the methamphetamine epidemic, research examining the factors contributing to the increasing number of users and fatal overdoses is critical. It is well known that environmental factors play a large role in human drug abuse, with adverse life experiences, or excessive and/or prolonged stress playing a critical role in the development and progression of drugs of abuse (Mantsch et al., 2016). Historically, stress is defined as a set of responses to (noxious) demands upon the body, or with respect to the central nervous system, alterations in psychological homeostatic processes (Selye, 1936; Burchfield, 1979). Several models of addiction have proposed that stress increases the risk of drug abuse and relapse, and there is ample evidence from animal studies that stressful events increase addiction vulnerability (Sinha, 2001; Jee-Yeon et al., 2018). Koob and colleagues (2014) hypothesize that the emergence of a negative emotional state that drives negative reinforcement in drug addiction is derived from dysregulation of key neurochemical elements, such as corticotropin-releasing factor (CRF), involved in the brain's stress systems. Excessive drug taking activates CRF in the extended amygdala, as well as the medial prefrontal cortex, which may be responsible for the increased anxiety-like states and decreases in executive function with respect to compulsive-like behavior in drug-seeking and taking. Another popular model of stress and addiction is the stress-coping model of addiction (Wills & Shiffman, 1985). This model suggests that individuals use drugs in order to cope with negative emotional states, or to increase positive affect, albeit a maladaptive form of coping. These models both focus on the need to alleviate the negative emotional states (e.g. anxiety, depression, etc.) that drive drug-seeking and craving. Several animal studies report that chronic stress increases vulnerability to substance abuse disorders, however, there is a lack of research focusing on the impact of stress on methamphetamine use (Brielmaier et al., 2012; Ahmed & Koob, 1997; Buczek et al., 1999; Burke et al., 2011; Holly et

al., 2016; McFarland et al., 2004; Avalos et al., 2022; Lemos et al., 2021). With rates of methamphetamine use rising, it is critical to examine how stress affects methamphetamine use.

Animal models provide the most efficient and effective way to measure the effects of stress on methamphetamine use. Rodent models investigating the effects of stressors on drug abuse vulnerability traditionally use physical restraint or electric pulses/shocks delivered to the feet or tail to mimic stressors (Erb et al., 1996; Pliakas et al., 2001; Faraday et al., 2005; Nawata et al., 2012; Alam & Chaudhary, 2020). Footshock and immobilization are useful methods for physical stressors, however, the need for more translatable methods has resulted in the use of maternal separation, social defeat, and other witnessing models (Alves et al., 2020; Covington & Miczek, 2001, Warren et al., 2013). The social defeat paradigm involves social dominance between animals that naturally occurs in the wild. While social defeat is a popular model for examining social stress effects on measures of drug abuse, research indicates that drug consumption following social defeat may better reflect episodes of binging as opposed to daily habitual use (Covington & Miczek, 2001). Moreover, most research using the social defeat paradigm examine other drugs of abuse such as cocaine which may be more likely to be administered in binge-sessions. While social defeat introduces a new way of implementing stress, there is often still a physical component due to the assertion of dominance between animals. A more promising social defeat model, known as the witnessing model, implements the same type of stressor on the animal without the physical stress. In the witnessing model, the target mouse is separated and observes two other mice who are clearly in distress nearby. According to Iniguez and colleagues (2018), witnessing the defeat of a male conspecific is salient enough to activate the HPA axis resulting in behaviors similar to those observed in post-traumatic stress disorder such as social avoidance, anhedonia, and despair. Witnessing models

are typically used with respect to mood-related disorders, and unfortunately, there is a lack of research using witnessing models, specifically in the field of addiction. Additionally, several studies have implemented a pharmacological stressor such as the α_2-adrenoceptor antagonist, yohimbine, to activate the HPA axis, however this method is less translatable to the human experience and more useful for therapeutic research (Brown et al., 2012; Funk et al., 2006). The aforementioned methods have helped our understanding of the biological bases of stress and how it may play a role in other disorders; however, the addiction field lacks a psychological stress paradigm that inflicts little to no external stressors. This is a huge detriment to the field of stress and addiction as much of the stress humans experience is not physical and can have a cumulative and lasting effect on the individual's mental health, including the development of drug addiction.

The goal of the present study was to use novel predator odor stressors to model psychological stress and examine whether chronic exposure to these stressors enhance subsequent vulnerability to methamphetamine reward in rats. Studies report that exposing a rat or mouse to predator urine (e.g., cat) elevates levels of corticosterone, indicating increased stress reactivity (Munoz-Abellan et al., 2009). Moreover, studies using the current predator odors to model psychological stressors indicate that they effectively elicit threat avoidance behaviors such as freezing and/or threat detection behaviors such as risk assessment (Maestas-Olguin et al., 2021; Maestas-Olguin et al., 2022). Weera and colleagues (2020) revealed similar predator odors such as bobcat urine not only elicit avoidance in rodents, but increased alcohol self-administration, alcohol-resistance aversion, hyperalgesia, and anxiety-like behavior in comparison to unstressed controls. The present study examined the effects of chronic predator odor exposure on expression of methamphetamine reward using the CPP paradigm. Evidence shows that HPA activity is related to the characteristics of the stressor (e.g., nature, severity,

duration), therefore the present study used both a synthetic (PEA) and natural derived (coyote urine) odor to characterize potential differential effects of the predator odor stress on expression of methamphetamine reward (Belda et al., 2015; Daviu et al., 2014; Maestas-Olguin et al., 2021). Lastly, to test whether predator odor stress effects on drug-seeking behaviors persist, the present study examined rates of extinction following the expression test trial.

2. Methods and materials

2.1. Animals

Subjects were adult male Long-Evans hooded rats between postnatal days (PNDs) 75-112, bred and reared in the Department of Psychology Animal Research Facility at the University of New Mexico. Following weaning, rats were pair-housed in standard home-cages (21.6 x 45.7 x 17.8 cm) in a temperature-controlled colony room (21-24 C) with a 12-hour light/dark cycle (lights on at 1000 h) and food and water available ad libitum in their home cages. Prior to testing, animals were handled 4-5 times per week for 2 weeks to minimize potential handling stress during testing. All husbandry and procedures adhere to the Guide for the Care and Use of Laboratory Animals (The *Guide*, 2011), and all experimental procedures were reviewed and approved by the University of New Mexico Institutional Animal Care and Use Committee.

2.2. Odorants

Rats were randomly assigned to a control group, a natural odor group, a synthetic odor group, or a combined synthetic and natural odor group (8-12 per group). The control odor group was exposed to distilled water (5ml), the natural odor group was exposed to coyote urine (5ml; Main Outdoor Solutions), the synthetic odor group was exposed to 2-phenylethylamine (PEA; 500ul; Sigma Aldrich), and the combined odor group was exposed to both coyote (5ml) urine

and PEA (500ul) on alternating days. The amount of each odorant was based on previous studies reporting these amounts increased anxiety-like defensive behaviors in adult rats (Maestas-Olguin et al., 2021).

2.3 Drugs

Methamphetamine hydrochloride (Sigma Aldrich) was dissolved in 0.9% sterile saline and injected intraperitoneally (i.p. 1 ml/kg). The dose of methamphetamine (0.125 mg/kg) was based on previous research (Zakharova et al., 2009) and preliminary data in our laboratory confirming that 0.125 mg/kg is the threshold dose to obtain methamphetamine-induced CPP. Saline injections were administered using the same route of administration at a volume of 1 ml/kg.

2.3.1 Behavioral testing, Stress

The predator odor apparatus used to confine the animals consisted of an operant conditioning cage (30 x 25 x 30 cm; Coulbourne Instruments). Briefly, a small petri dish containing the odorant was placed and secured under the grid floor for a 10-minute exposure period. The same procedure was conducted at the same time each day across 10 consecutive days. Between each trial the apparatus was cleaned with a 70% ethanol solution and a new petri dish was used for each trial. All sessions were conducted under red light to minimize potential environmental/light stress (see figure A).

2.3.2 Behavioral testing, Drug conditioning

Conditioning was conducted in 4 Plexiglass apparatuses each composed of two equal sized compartments (25 x 25 x 45 cm) and divided by a removable solid partition. On baseline and expression test days, the solid partition was removed and replaced with a partition containing a small opening (8 x 8 cm) to allow for movement between compartments. The 2 compartments

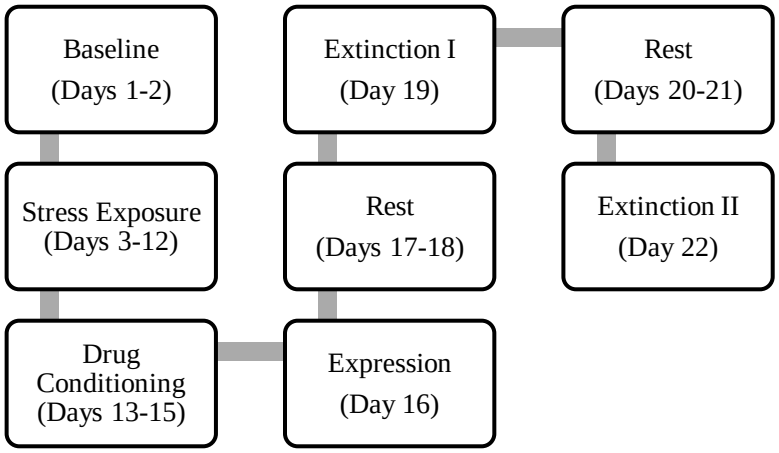
were distinguishable by color, texture and odor. More specifically, one side had white walls with a wire mesh floor and corncob bedding, while the other side had black walls with a metal bar floor and pine bedding. All CPP sessions were monitored and recorded using Lorex infrared surveillance cameras and a Lorex LHV210800 DVR. All testing procedures were conducted in a dimly lit room under red light during the rats' dark cycle. A white noise generator was set at 15 dB to minimize potential noise disturbances. Between every trial each apparatus was cleaned and wiped down with a 70% ethanol solution to eliminate any residual olfactory cues.

The acquisition and expression phases of CPP occurred over 6 consecutive days immediately following odor exposure as described previously with minor modifications (Madden et al., 2020; 2021; see figure A). Briefly, CP testing consisted of 2 days of baseline, 4 drug conditioning days, and a CPP preference expression test trial. Rats were randomly assigned to either side of the apparatus (black or white) for their initial placement and the starting compartment was counterbalanced across the 2 days of baseline. During the 2 baseline days, rats were placed in either side of the apparatus and allowed to roam freely for 15 minutes to explore both compartments and the amount of time spent on each side was recorded and averaged across both days. The lower average recorded from both days of baseline was defined as the initially non-preferred side, which is operationally defined as the compartment in which the rat spent less than 50% of their total time. Entry into the compartment was determined by the presence of both the rats' head and shoulders and was recorded until the head and shoulders enter the other compartment. Conditioning trials occurred immediately following the 2 baseline days. Rats from each odor group were randomly assigned to either a methamphetamine group or a saline group. Each conditioning day consisted of 2 30-minute sessions where the rats were confined in each compartment, receiving methamphetamine in their initially non-preferred side and saline in their

initially preferred side. Daily conditioning sessions were separated by 4 hours and the order of drug administration was counterbalanced across sessions. The day after conditioning, the closed partition was removed so the rats are free to roam the apparatus for a single 15-minute expression test session during which the amount of time spent in each side was recorded. After the initial expression test, 2 additional 15-minute extinction trials were conducted with two days between each trial. Drugs were not administered during the expression or extinction trials. Expression trials were recorded for 15 minutes.

Figure A: Experimental Timeline

Figure A depicts the experimental timeline from start (day 1; baseline) to finish (day 22; last extinction trial).



2.4 Data analysis

Methamphetamine-induced CPP was operationally defined as a significant increase in the duration of time spent in the initially non-preferred side post-conditioning compared to the pre-conditioning baseline. To test if the test chambers were biased, an independent sample *t*-test comparing the baseline preferences (black vs. white means) of all animals regardless of group was conducted. Additionally, a univariate analysis was conducted to examine whether groups

differed in their baseline preferences prior to conditioning. To determine if the methamphetamine dose was subthreshold, an independent samples t-test comparing percent change of DI water/methamphetamine groups and DI water/saline groups was used as well as separate paired sample t-tests comparing baseline to expression for the DI water/saline and DI water/methamphetamine groups. Percent change is operationally defined as the amount of increase or decrease the final value has from the initial value out of 100, and was calculated using the formula [(A2-A1)/A1 * 100], where (A1) represents the time spent in the nonpreferred side preconditioning and (A2) represents the time spent in the nonpreferred side postconditioning. Next, a repeated measure ANOVA was used to examine differences across expression/extinction trials with test session as the repeated measure, and drug and odors groups as the between subjects variable. Methamphetamine group differences in percent change were measured using separate one-way ANOVAs for each test expression/extinction day. When appropriate, Fisher's Least Significant Difference (LSD) tests were used to further analyze group differences. All analysis was performed using SPSS version 29. Data are presented as mean ± standard error. Alpha was set at $p < .05$ for all comparisons. Adjustments to degrees of freedom were made when unequal variances between groups existed (for example, Greenhouse-Geisser correction).

3. Results

The independent sample t-test examining whether the chambers were biased revealed no significant difference, t (58) = 1.279, p = .206 in baseline preference for the black side (M = 407.680, SE = 6.673) versus the white side (M = 396.257, SE = 5.855) regardless of groups (see table 1). As shown in figure 1, separate univariate analysis of variance indicated there were no differences in baseline preference preconditioning in stress [F (3, 53) = 1.565, p = .209; figure

1A] or drug groups [F (1, 53) = .055, p = .816; figure 1B] groups. Next, when collapsing across DI water exposed groups, the independent samples t-test examining percent change from baseline in saline (M = 18.183, SE = 6.389) and methamphetamine (M = 27.490, SE = 4.807) conditioned groups indicated that methamphetamine failed to produce reward [t (18) = -1.187, p = .251; see figure 2]. Collectively, these data confirm that the dose of methamphetamine was subthreshold.

Table 1: Pre-conditioning baseline preference

Baselines were computed as the average of the two baseline sessions. **BAR** indicates the black side of the apparatus, while **GRID** indicates the white side. The nonpreferred side was distributed equally amongst the black and white sides indicating that the apparatus where not biased.

Non-Preferred side	N	Percent of rats	Mean ± SEM
Bar/Black	25	41.7%	407.680 ± 6.674
Grid/White	35	58.3%	396.257 ± 5.855

Figure 1: Pre-conditioning baseline preferences for drug and odor groups

Figure 1A shows time spent in the nonpreferred side between animals conditioned with saline and methamphetamine. Figure 1B shows time spent in the nonpreferred side between animals assigned to the four stress groups. The graphs show no initial biases in either drug group or stress group.

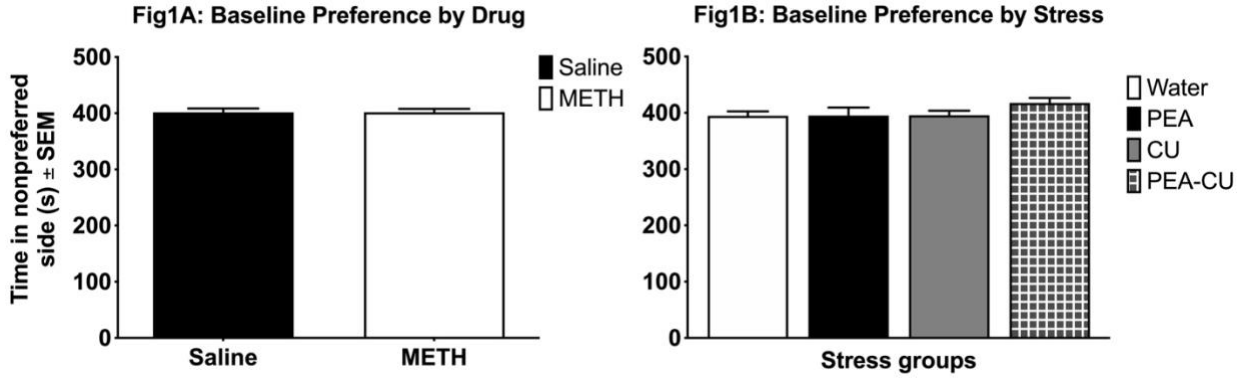
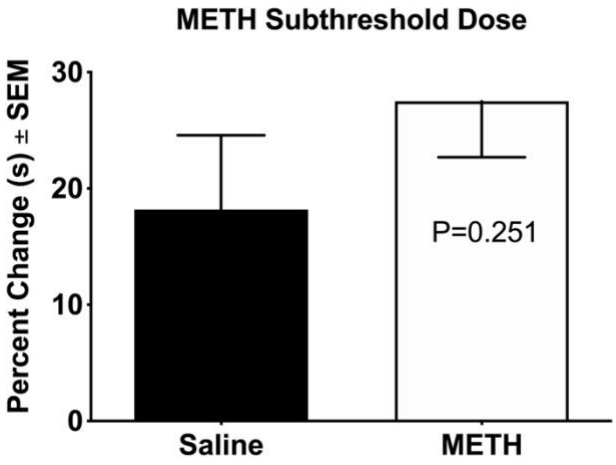


Figure 2: Methamphetamine alone does not produce CPP

Figure 2 shows the percent change from baseline between DI water/saline and DI water/methamphetamine groups postconditioning. Results indicate that the dose of methamphetamine did not produce a significant increase in the time spent in the initially nonpreferred side indicating that the dose of methamphetamine was subthreshold.

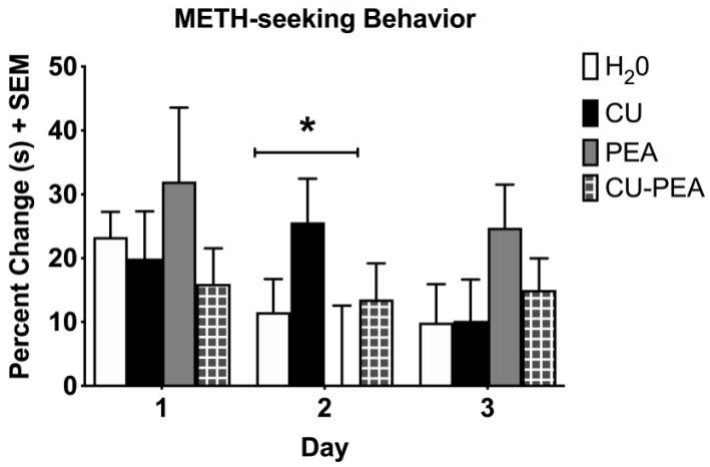


The effects of odor exposure on expression of methamphetamine-induced CPP are depicted in figure 3. The repeated measures ANOVA revealed no significant Day x Stress x Drug interaction [F (2, 53) = 2.320, p = .108], or Day x Stress interaction [F (3, 53) = .879, p = .458], however, there was a significant main effect of Day [F (2.052, 108.759) = 2614.993, p = <.001] and a significant Day x Drug interaction [F (1, 53) = 11.131, p = .002]. Based on the significant trend detected in the three -way ANOVA and our a priori prediction that the predator odors would enhance methamphetamine reward, separate one-way ANOVAs examining percent change on each test day was conducted in methamphetamine treated rats. The ANOVAs indicated a significant difference between stress groups on expression/extinction day 2, F (3, 30) = 4.548, p = .010, while no other test days showed significant differences between groups [Day 1: F (3, 30) = .358, p = .784; Day 3: F (3, 30) = .616, p = .610]. LSD post hoc comparisons revealed the percent change on expression day 2 was significantly higher in the coyote urine

group compared to the DI water (MD = +20.651, SE = 9.392, p = .036) and PEA (MD = +40.244, SE = 11.101, p = .001) groups.

Figure 3: Effects of predator odor exposure on methamphetamine CPP

Figure 3 depicts percent change from baseline for the four stress groups across the three test days in methamphetamine conditioned rats. The ANOVA revealed a significant day by drug interaction with LSD posthoc tests indicating that CU groups were significantly higher than H_20 and PEA groups on day two. Asterisk (*) represents a significant difference between CU, H_20 and PEA groups (p<0.05 in each case).



4. Discussion

Results from the present study indicate that the use of natural predatory odors, specifically coyote urine, can serve as an effective psychological stressor to increase motivation for methamphetamine in the CPP model. Both DI water exposed drug groups (DI water/saline and DI water/methamphetamine) failed to exhibit CPP following methamphetamine conditioning, indicating that the dose of methamphetamine was subthreshold (Figure 2). Therefore, a significant increase in the time spent in the initially nonpreferred side post conditioning can be attributed to the effects of the predator odors enhancing the rewarding effects of methamphetamine. The repeated measures ANOVA revealed that predator odor

exposure failed to significantly increase expression of methamphetamine reward post conditioning on day 1, suggesting that the stressors did not enhance the rewarding effects of a subthreshold dose of methamphetamine (Figure 3). However, coyote urine exposed rats showed elevated methamphetamine-seeking behavior on the second expression test trial. Specifically, rats in the coyote urine stress group that received methamphetamine showed a resistance to extinction, as shown by the increase in drug-seeking behavior on day 2 compared to the PEA and water groups. These results suggest that the stressful effects produced by exposure to coyote urine prior to drug conditioning enhance the incentive motivational effects of a subthreshold dose of methamphetamine. These differences between stress groups across three days of extinction (the rate of extinction) provide evidence that coyote urine, but not PEA, can serve as a psychological stressor to enhance drug-seeking behavior for a subthreshold dose of methamphetamine.

Although the current study provides evidence that coyote urine can be utilized as a psychological stressor, there are several limitations that need to be addressed. First, the PEA stress group is statistically underpowered (n=6) in comparison to other stress groups (n=11/DI water; n=10/CU; n=7/CU-PEA), suggesting that future studies should fully characterize the effects of PEA on methamphetamine CPP. Additionally, in order to further validate the use of predator odors as a psychological stressors to enhance drug reward, future studies should measure corticosterone or CRF following odor exposure. Results from these experiments would also determine if chronic exposure to these odors results in habitation. Indeed, the current design implemented 10 days of repeated, inescapable stress exposure, while previous research indicates that a single exposure produces both unconditioned and conditioned fear in male and female adult and adolescent rats (Maestas-Olguin et al., 2021; Pentkowski et al., 2022). It is important to

understand at what point, if any, rats begin to habituate to the effects of chronic predator stress exposure in the absence of threat and how this may impact subsequent methamphetamine reward. Thus, future experiments can address this knowledge gap by altering the duration and/or number of stress exposures. Finally, while the present study provides evidence for the utility of coyote urine as a model of psychological stress to alter motivation for methamphetamine, only adult male rats were used, and thus future studies are needed to examine potential sex and/or age differences.

Due to the broad variation between expression scores within stress groups, further categorization of rodents' behavior may provide additional insight into the use of predator odors as a psychological stressor to impact drug reward. More specifically, categorizing animals into avoiders vs. non-avoiders or defensive vs. non-defensive animals in response to odor exposure may help to elucidate the effects of predator odors on drug seeking. For example, Weera et al., (2020) categorized rats into avoiders vs. non-avoiders using predator odor conditioning. Specifically, rats that showed > a 10 second decrease in time spent in the odor-paired context pre- and post-conditioning were classified as 'Avoiders,' and the others were classified as 'Non-Avoiders'. Their findings indicate distinct differences in both the behavioral (increased alcohol self-administration) and biological correlates (increased c-Fos+ cells and CRF) of the avoidance phenotype in comparison to non-avoiders. Using this approach, it is possible that higher levels of methamphetamine-seeking behavior would have been detected in coyote urine and PEA exposed rats that showed higher levels of defensive behavior compared to rats with lower levels of defensive behavior. Future research is needed to explore this possibility.

As previously mentioned, research from our lab indicates that a single exposure to coyote urine and PEA results in elevated stress-induced unconditioned and conditioned defensive

behavior (Pentkowski et al., 2022; Olguin et al., 2021), therefore, it is important to examine if the observed effects can be elicited over fewer days of stress exposure (< 10 days) and if the number of stress exposure trials (10 days) is related to reward and rates of extinction. Moreover, it is important to understand if and how manipulating the duration of stress exposure (10 minutes) and the volume of odors used affects subsequent methamphetamine reward and extinction rates. The present study reveals the ethological validity of coyote urine as a psychological stressor in the context of subthreshold methamphetamine reward, but further directions should focus on manipulating doses of methamphetamine as well as other drugs of abuse to fully examine its potential utility in the context of drug abuse.

One important possibility to consider in the present study is that the effects of the chronic stress exposure could result in memory deficits (i.e., delayed extinction) rather than enhanced motivation. However, previous research from our lab indicates that coyote urine produces conditioned fear (i.e., enhanced memory formation not memory interference; Pentkowski et al., 2022; Olguin et al., 2021) and thus the present effects are likely not due to memory deficits. Although the mechanisms by which stress enhances motivation are not fully understood, evidence suggests that stress potentiates dopamine (DA) release within the mesolimbic pathway, resulting in increased sensitivity to drugs and their associated stimuli (Graf et al., 2013).

Other animal models of stress (immobilization, footshock, social defeat, maternal separation, etc.) can serve as useful tools in examining how stress subsequently increases drug reward and reinforcement (Brielmaier et al., 2012; Ahmed & Koob, 1997; Buczek et al., 1999; Burke et al., 2011; Holly et al., 2016; McFarland et al., 2004; Avalos et al., 2022; Lemos et al., 2021), however, most animal models of stress are not easily translatable to psychological stress (i.e., physical stressors) or are better equipped to model certain types of stress (i.e., early life

stress and maternal separation). This highlights the need for a novel and more valid psychological stressor such as the use of predator odors. Given the limitations of preexisting stressors, coyote urine can be used to probe the neural mechanisms underlying stress effects on motivation for methamphetamine.

5. Conclusions

Due to increasing rates of MUD and the need to understand how stress may modulate addiction, the current study implemented predatory odors as a psychological stressor to examine subsequent methamphetamine reward/motivation. In the current study, coyote urine, but not PEA alone or their combined use, successfully enhanced methamphetamine-seeking postconditioning on day 2. The present findings suggest that the use of coyote urine can be used as a psychological stressor in animals to alter motivation for a subthreshold dose of methamphetamine. Future research should examine various aspects of coyote urine as a stressor by manipulating the duration, severity and frequency of exposure, as well as examining its impact on acquisition, retrieval, extinction using a variety of drugs of abuse.

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