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Examining the Associative Learning and Accumbal Dopaminergic Mechanisms of Caffeine Reinforcement

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ABSTRACT

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Caffeine is the most consumed psychoactive substance in the world, and most caffeine consumption in coffee and energy drinks is intended to produce a psychoactive effect. However, caffeine is not a primary reinforcer in preclinical paradigms – non-human species do not reliably take the drug to produce a psychoactive effect. However, caffeine is a 'reinforcement enhancer' in preclinical models; the effects of caffeine increase the motivation to obtain other non-drug reinforcers. The overall goal of this project was to determine if these reinforcement enhancing effects of caffeine could promote caffeine self-administration and to subsequently investigate the behavioral and neurochemical underpinnings of this effect. We hypothesized reliable caffeine self-administration would occur by adventitious pairing of caffeine with saccharin, a primary reinforcer. Second, we hypothesized that caffeine enhances reinforcement by increasing the salience of incentive stimuli, which are stimuli that come to evoke approach behaviors through associative learning (e.g., Pavlovian conditioning). Finally, incentive salience is moderated by dopamine release in the nucleus accumbens (NAc), an area highly involved in reward-learning and substance dependence. Therefore, we hypothesized that if caffeine enhanced control of approach behavior by incentives, then it would increase the ability of incentive stimuli to evoke dopamine in the NAc. These studies show that intravenous delivery of caffeine with oral saccharin increases operant relative to control groups responding for intravenous caffeine or oral

saccharin. The effect was also dose-dependent, confirming that the psychoactive effects of caffeine increased behavior. We also extended this effect to an oral model of caffeine self-administration, which included a simple sweetener (saccharin) or a complex oral vehicle (saccharin with decaffeinated coffee) to mask the bitter taste of caffeine. Presenting caffeine with oral saccharin promoted self-administration, relative to saccharin alone and did not depend on the nature of the complexity of the vehicle. Caffeine also dose-dependently increased approach to an incentive stimulus and this effect was associated with increased extracellular dopamine in the NAc. These findings suggest caffeine enhances incentive motivation and that this effect may result from increases in CS-evoked striatal dopamine.

DEDICATION

This work is dedicated to my dad, William Bradley. He was proud and he let me know it. He didn't see me finish school so I couldn't show him what I would do with my Psychology degree. Regardless, he was beside me the whole time. I wouldn't be where I was today if it wasn't for his love and parenting. You best believe I'll continue to make you proud. I love you dad.

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INTRODUCTION

Human Caffeine Use

Caffeine Consumption

Caffeine is often described as the most consumed psychoactive substance in the world (Glade, 2010). As of 2010, the United States Food and Drug Administration (FDA) estimates that persons 2 years or older consume 131.9 mg/day on average (Somogyi, 2010). In the United States, consumption of caffeinated soft drinks alone has increased from 11 gallons/year to 49 gallons/year per person over the past fifty years (Frary, Johnson, & Wang, 2005; Somogyi, 2010). Although caffeine is most often consumed via caffeinated beverages such as coffee and soft drinks, the market for caffeine-infused products has grown substantially in recent years (Frary et al., 2005; Somogyi, 2010). For example, caffeine can be consumed in a wide variety of products such as energy drinks, alcoholic beverages, waffles, gum, sweets, and smokeless tobacco. Possibly potentiating caffeine consumption, caffeine infused products are becoming a growing public health concern as noted by multiple investigations by the FDA (FDA, 2010, 2015).

Caffeine Use Disorder

Currently recognized by the American Psychiatric Association (APA) as a condition for further study, Caffeine Use Disorder (CUD) and Caffeine Dependence Syndrome (CDS) has become a growing concern in the United States and is recognized by the World Health Organization (WHO, 1992). In The International Statistical Classification of Diseases and Related Health Problems (ICD-10), the WHO defines CDS as behavioral, cognitive, and

physiological symptoms that develop after repeated caffeine use (WHO, 1992). These symptoms include drug craving, an inability to control drug use, persistence despite maladaptive health consequences, increased tolerance, decreased priority of social responsibilities, and withdrawal after periods of cessation (WHO, 1992). The APA recognizes diagnostic symptoms found in the ICD-10 for CUD but requires the three clinical indicators of substance dependence to achieve diagnosis: Persistent use despite unsuccessful quit attempts, continued caffeine use despite negative physical and mental health consequences, and caffeine withdrawal or avoidance of withdrawal via caffeine ingestion (APA, 2013).

Prevalence. A survey conducted in the United States estimated 9% of the population meets diagnostic criteria for CUD (Hughes, Oliveto, Liguori, Carpenter, & Howard, 1998) and a study conducted in Italy estimated prevalence rates closer to 6% (Ciapparelli et al., 2010; Hughes, Oliveto, et al., 1998). A 6-9% prevalence rate would make CUD one of the top three substance abuse disorders in America according to the DSM 5; behind Tobacco Use Disorder (13%) and approximately equivalent to Alcohol Use Disorder (8.5%) (APA, 2013). In addition, rates of CUD are highest among clinical populations. Individuals who have been diagnosed with mood disorders, anxiety disorders, schizophrenia, or eating disorder have a 17% prevalence of CUD (Ciapparelli et al., 2010; Hughes, McHugh, & Holtzman, 1998). Furthermore, comorbidity of CUD is highest amongst individuals who regularly use illicit substances (20%) (Striley, Griffiths, & Cottler, 2011). This may be caused by caffeine's ability to sensitize a person to the effects of other drugs, such as cocaine, as seen in preclinical work (Green & Schenk, 2002; Horger, Wellman, Morien, Davies, & Schenk, 1991). Caffeine Use Disorder, together with the staggering demographics of public caffeine consumption, suggests caffeine may be a public health concern that warrants further study. A thorough understanding of the effects of caffeine on

the brain and behavior is needed to understand the factors influencing the prevalence of caffeine use and why this might be a concern for public health. For example, there is some debate about whether the pharmacological effects of caffeine are reinforcing, whether caffeine influences reward learning, and what effects caffeine has on the brain's 'final common pathway' for substance use disorders: the mesolimbic dopamine system.

Caffeine Reinforcement in Humans

In congruence with survey data of caffeine consumption and maladaptive psychological conditions caused by unhealthy caffeine consumption, caffeine functions as a negative reinforcer in human subjects (Griffiths & Chausmer, 2000; Schuh & Griffiths, 1997). Drugs are considered to be negatively reinforcing when they are self-administered in order to escape or avoid the negative consequences of abstinence (i.e., a withdrawal syndrome; Markou, 1999). The negative reinforcement perspective of caffeine offers the strongest support for the need to label caffeine as a drug of abuse. For example, caffeine functions as a negative reinforcer in participants with histories of moderate-to-high caffeine consumption (Griffiths, Bigelow, & Liebson, 1989). Participants who blindly consume caffeinated coffee report decreases in withdrawal symptoms and simultaneous increase in subjective liking when compared to consumption of decaffeinated coffee (Griffiths, Bigelow, & Liebson, 1986). Additionally, moderate caffeine users who were currently abstinent and experiencing withdrawal symptoms were willing to pay to avoid ingesting a placebo or pay for a caffeine pill (Griffiths et al., 1989).

A negative reinforcement perspective of caffeine use still does not explain acute episodes of consumption, the acquisition of chronic caffeine use leading to unhealthy caffeine consumption, or relapse of caffeine use after the abstinence syndrome have abated (Griffiths &

Woodson, 1988). On the other hand, human models of caffeine use via positive reinforcement produce findings which are incongruent with the prevalence of public caffeine use. Consistent caffeine self-administration only occurs in certain subsets of participants (Griffiths & Woodson, 1988). Conversely, participants prefer caffeine over placebo in the absence of symptoms associated with caffeine withdrawal (Griffiths et al., 1989). More specifically, participants prefer caffeinated coffee or capsules after two weeks of caffeine cessation. Caffeinated coffee and capsules maintain higher rates of self-administration compared to decaffeinated coffee and placebo capsules (Griffiths et al., 1989). Taken together, a strictly negative reinforcement viewpoint of caffeine use does not offer a comprehensive explanation of caffeine use among research with human subjects. Yet, clinical and preclinical models of caffeine self-administration via positive reinforcement are weak and incongruous with caffeine consumption by the general population. A deeper look into preclinical and clinical caffeine research is warranted to understand the incongruous research findings and patterns of public caffeine consumption.

Preclinical Caffeine Research

Caffeine Self-Administration in Non-Human Subjects

Preclinical self-administration paradigms are considered the gold-standard in drug abuse research (Le Foll & Goldberg, 2009). These procedures determine if a drug is a primary reinforcer by observing whether an animal will work to self-administer a drug. If an animal continuously works to receive the drug, it is reasonable to assume a psychoactive property of the drug is perceived as pleasant. The pleasant, or rewarding, effect increases the probability of future instances of the operant behavior contingent upon future infusions of the drug. Although caffeine functions as a primary reinforcer in human participants and over 90% of the human

population consumes the drug regularly (Evans, Critchfield, & Griffiths, 1994; Garrett & Griffiths, 1998), studies with non-human animals provide little to no evidence of abuse liability (see Griffiths & Woodson, 1988 for review). Intravenous caffeine self-administration has been described as erratic (Deneau, Yanagita, & Seevers, 1969; Griffiths, Brady, & Bradford, 1979), inconsistent (Atkinson & Enslen, 1976; Schuster, Fischman, & Johanson, 1981), and non-existent (Collins, Weeks, Cooper, Good, & Russell, 1984; Hoffmeister & Wuttke, 1973). Additionally, route of administration does not appear to influence caffeine consumption. Oral caffeine self-administration, a more ecologically valid model of caffeine consumption, results in weak and incongruent findings for a preclinical model of caffeine abuse liability (Heppner, Kemble, & Cox, 1986; Vitiello & Woods, 1975). Preference for oral caffeine occurs at such low concentrations that above-threshold doses are unobtainable unless a forced consumption paradigm is administered prior to a free choice task (Heppner et al., 1986; Vitiello & Woods, 1975). Furthermore, caffeine self-administration does not appear to be species-specific. Rodent and primate models are unsuccessful in establishing or maintaining intravenous caffeine self-administration (see Griffiths & Woodson, 1988 for review). Interpretation of these results can be complicated due to animals' exposure history to other drugs of abuse (Griffiths et al., 1979; Hoffmeister & Wuttke, 1973; Schuster et al., 1981).

Associative Learning and Caffeine

The stark contrast between caffeine's low abuse liability in preclinical research and high rates of drug use in the general population suggests a primary reinforcing effect of caffeine is not enough to explain the prevalence of public caffeine consumption. Research outside of the self-administration literature suggests caffeine’s effect on associative learning processes may contribute to high rates of caffeine use. Conditioned Place Preference (CPP) is a model used by

preclinical researchers to determine drug abuse liability. In this associative learning paradigm, an environment is repeatedly paired with a psychoactive dose of a drug. If the drug produces some preferred internal state, often labeled euphoria, the environment will inherit some of the drug's conditioned properties. As a result, animals prefer the environment associated with the drug in comparison to a similar context associated with placebo/saline. Common drugs of abuse elicit CPP and verify the model as a preclinical indicator of abuse liability (Tzschentke 2007 for review). Caffeine induced CPP supports the idea that the stimulant has potential for abuse. Caffeine induces CPP in rodents in a biphasic manner (Brockwell, Eikelboom, & Beninger, 1991; Hsu, Chen, Wang, & Chiu, 2009; Patkina & Zvartau, 1998). In other words, low to moderate doses of caffeine induce CPP while higher doses reduce the time spent in the environment paired with caffeine. Also, Hsu and colleagues (2009) discovered caffeine and the adenosine A_{2A} antagonist SCH 28261 induced CPP, while a selective adenosine A_1 antagonist had no effect on place preference (Hsu et al., 2009). Taken together, these findings implicate caffeine's ability to alter reward perception which may occur via adenosine A_{2A} receptor antagonism and the resulting dopamine D_2 receptor activation.

Parallels of Caffeine and Nicotine

Although preclinical models show effective caffeine-conditioned reward via CPP, this preference for the drug-paired environment is small relative to more typical drugs of abuse such as cocaine (Bedingfield, King, & Holloway, 1998). This pattern is reminiscent of another psychomotor stimulant that is widely consumed by humans yet has relatively small reinforcing and rewarding effects in preclinical models - nicotine. For example, caffeine- and nicotine-induced CPP has been best observed in the biased design (Bedingfield et al., 1998; Brielmaier, McDonald, & Smith, 2008; Le Foll & Goldberg, 2005; Patkina & Zvartau, 1998). ‘Biased’ CPP

designs involve pre-tests in which rats are placed in an apparatus with free access to two distinct environments, and the unconditioned preference between environments (i.e., based on time spent in each side) can be measured. During conditioning, the subjects are repeatedly confined to each environment and drug injections are paired with the initially non-preferred side; placebo injections are paired with the preferred side. A shift in preference toward the non-preferred side provides a more sensitive measure of conditioned reward, if interpretive pitfalls are accounted for (e.g., a placebo-only group to account for spontaneous changes in side preference over time). Weak to moderately rewarding drugs would be expected to confer weak conditioned rewarding properties on the drug-paired environment and weaker observed preferences. Conditioned place preference exclusive to biased designs indicate both drugs have weak subjective rewarding effects in rodents, which lends further support for the weak subjective rewarding effects of the drugs observed in humans (Griffiths & Woodson, 1988; Tzschentke, 2007). However, nicotine and caffeine elicit higher rates of CPP when the drug-associated environment is paired with a second reward. For example, nicotine CPP is more robust when the environment contains social stimulus (Thiel, Sanabria, & Neisewander, 2009), cocaine (Buffalari et al., 2014), or sucrose (Buffalari et al., 2014). Similarly, stronger rates of caffeine-induced CPP occur when the environment is paired with cocaine injections (Bedingfield et al., 1998). Taken together, caffeine and nicotine appear to induce weak levels of CPP that are enhanced by co-presentation with rewarding stimuli.

In addition to CPP, nicotine and caffeine have similar effects in other preclinical models of drug- and non-drug reinforcement. Like caffeine, nicotine is a weak primary reinforcer but enhances responding for other rewards (Le Foll & Goldberg, 2009; Sheppard, Gross, Pavelka, Hall, & Palmatier, 2012). For example, nicotine injections support low levels of operant behavior

when they are presented alone, but robustly increase responding for a reinforcing visual stimulus (Donny et al., 2003). Early nicotine self-administration research revealed the importance of cue lights associated with nicotine delivery in maintaining significant and consistent levels of self-administration (Goldberg, Spealman, & Goldberg, 1981). This phenomenon was replicated in later studies (Caggiula et al., 2001), confirming that intravenous delivery of nicotine alone was insufficient to explain nicotine use and dependence. The relationship between nicotine, rewarding stimuli, and self-administration was clarified by Donny and colleagues (2003). First, nicotine enhanced responding for a rewarding stimulus regardless of the contingency for nicotine delivery. Second, responding for a rewarding stimulus was attenuated and reestablished when nicotine was removed and subsequently reinstated. Third, rates of nicotine self-administration were low when delivered alone and significantly higher when paired with a rewarding stimulus. Put together, these results confirm that nicotine functions as a weak primary reinforcer, potentiates the efficacy of other reinforcers, and promotes high rates of self-administration through co-presentation with other reinforcers.

Behavioral Mechanisms of the Reinforcement Enhancing Effects of Nicotine

Donny and colleagues (Donny et al., 2003) interpretation of increased responding for rewards as reinforcement enhancement was challenged as ‘rate dependent’ increases based on the behavioral activation commonly observed in psychostimulants (Frenk & Dar, 2004). Since this criticism, nicotine's ability to increase operant responding for a reinforcer has been replicated and observed with diverse primary reinforcers as well as conditioned reinforcers (see Caggiula et al., 2009 for review). Nonetheless, to better understand the circumstances in which nicotine enhanced responding for reinforcing stimuli, Palmatier, O'Brien, and Hall (2012) systematically altered reinforcer intensity (sucrose concentration), conditioning history, and schedule of

reinforcement (effort necessary to obtain sucrose). They hypothesized that the effect of nicotine was motivational in nature – nicotine increased the motivation to obtain rewards which would be observed by higher rates of lever responding ("effort"). Thus, the effect of nicotine should be largest when rats respond for larger rewards (e.g., 0% sucrose vs. 20% sucrose) and easier to observe under schedules of reinforcement requiring more effort (e.g., progressive ratio (PR)) as opposed to schedules requiring less effort (e.g., fixed ratio (FR)). In addition to confirmation of both hypotheses, their findings shed light on the mechanisms by which nicotine might be 'enhancing reinforcement.' Nicotine's ability to enhance the effectiveness of reinforcers depends on the subjects' conditioning history (Palmatier, O'Brien, & Hall, 2012).

When the magnitude of the sucrose reinforcer was manipulated in the same rats (e.g., within-subject design), nicotine enhanced responding for all concentrations of sucrose equally – which suggested one of two possibilities – first, the psychomotor stimulant effects of nicotine increased nonspecific behavior (Frenk & Dar, 2004). Second, all the reward-predictive stimuli were congruent in these tests – the same lever predicted all magnitudes of reward delivery. In a follow-up experiment, researchers used a between-subject manipulation of reward magnitude (0, 5, or 20% sucrose) to ensure conditioning history coincided with one sucrose concentration (Palmatier et al., 2012). The enhancing effects of nicotine directly correlated with reward magnitude – nicotine had no effect on responding for rats tested with 0% sucrose, moderately increased motivation in rats tested with 5% sucrose, and robustly increased motivation in rats responding for 20% sucrose. This was interpreted as an increase in 'incentive salience' of the reward-predictive cues. When those cues were homogeneous (within-subject manipulation of sucrose) nicotine enhanced motivation equally across sucrose concentrations. However, when cues predicted one outcome (a strong reward, a moderate reward, or no reward) the enhancing

effect of nicotine increased relative as the conditional strength of the predictors increased. Taken together, the reinforcement enhancing effects of nicotine is dependent upon on reward-predictive nature of cues (incentives) rather than the strength of the actual outcome (sucrose).

In order to confirm this, a follow-up study (Palmatier et al. 2013) used a Pavlovian conditioned approach (PCA) paradigm to directly test if nicotine increased the ability of an incentive to evoke approach behavior. In PCA paradigms brief presentations of a conditioned stimulus (CS) are immediately followed by the presentation of an appetitive unconditioned stimulus (US). The CS is usually presented for 8-10 seconds at which time approach to the CS (sign tracking), or the location where the US will be delivered (goal tracking), can be measured. Although approaching or manipulating the CS does not result in a contingent delivery of the US, rats are more likely to approach the cue when pretreated with nicotine (Olausson, Jentsch, & Taylor, 2003, 2004; Palmatier, Peterson, Wilkinson, & Bevins, 2004; Palmatier et al., 2013; Raiff & Dallery, 2008). In congruence with previous research, nicotine enhanced sign-tracking behavior, thus indicating nicotine increased the incentive salience of the reward-predictive CS. In other words, the stimulus that predicts upcoming reward presentation is more salient to the animal, and therefore elicits more approach behavior. This enhanced saliency of CSs provides an explanatory framework for how nicotine functions as a ‘reinforcement enhancer’ even though perception of the reward (sucrose) is unchanged.

Like nicotine, there is evidence that caffeine enhances motivation for a variety of reinforcers. Caffeine enhances the motivation for primary reinforcing drugs including cocaine, amphetamine, alcohol, synthetic cathinones, and nicotine (Cauli, Pinna, Valentini, & Morelli, 2003; Gannon, Galindo, Mesmin, Rice, & Collins, 2017; Green & Schenk, 2002; Kuzmin, Johansson, Semenova, & Fredholm, 2000; Prieto et al., 2016; Shoaib, Swanner, Yasar, &

Goldberg, 1999), intracranial self-stimulation (Lazenka, Moeller, & Negus, 2015), and non-drug reinforcers (Sheppard et al., 2012). In rats, a bolus dose of 12.5 mg/kg caffeine, a moderate psychoactive dose, enhances operant responding for two reinforcing stimuli - sucrose (20% w/v) and a visual stimulus (30s extinguished house light) (Sheppard et al., 2012). Unlike nicotine, the reinforcement enhancing effect of caffeine declined after repeated daily dosing over fifteen days and partially returned following four and eight days of abstinence, suggesting that tolerance after chronic caffeine exposure attenuates drug-induced reinforcement enhancement. Even with the difference in tolerance between these stimulants, there is strong evidence that caffeine and nicotine enhance motivation for unconditioned and conditioned reinforcers.

The discovery that caffeine has robust reinforcement enhancing effects may help to explain the paradox between widespread human caffeine consumption and the sparse evidence for primary reinforcement in preclinical paradigms. For humans, caffeine is most commonly consumed in complex oral vehicles (coffee, energy drinks, soft drinks) that are replete with both unconditioned reinforcers (sugars, sweeteners, and fats) and conditioned reinforcers (coffee, fruit, and cola flavors). A critical difference between human and pre-clinical caffeine self-administration may be the co-presentation of non-drug reinforcers in one paradigm (human) and the lack of non-drug reinforcers in the other (non-human). In preclinical self-administration the co-presentation of non-drug reinforcers with caffeine has gone untested, but in human studies it may have potentiated substantial caffeine consumption without much discussion from researchers. A preclinical model of caffeine self-administration comprised of simultaneous caffeine and gustatory reinforcer delivery could result in similar results of caffeine intake. This model could utilize caffeine's ability to alter motivation for incentive stimuli, via enhanced salience, while using these incentive stimuli to elicit behavior that perpetuates caffeine

consumption. If this preclinical model was successful, this would be similar to nicotine self-administration paradigms. Furthermore, successful results would indicate that these incentive stimuli, and possibly the cues that temporally predict them, are perpetuating caffeine use via activation of dopaminergic activity in the reward areas of the brain.

The Mesolimbic Dopamine System, Incentive Salience, and Caffeine

Mesolimbic Dopamine System

The mesolimbic dopamine system is a substrate composed of structures located in the midbrain, basal forebrain, and cortices of the forebrain (Koob & Volkow, 2010). The Ventral Tegmental Area (VTA), located in the midbrain, projects dopaminergic signals via the medial forebrain bundle to the nucleus accumbens (NAc) located in the basal forebrain. The NAc also receives limbic input from the amygdala, frontal cortex, and hippocampus, thus making it a highly important and well-studied area for understanding reinforcement and drug addiction (Koob & Volkow, 2010). Furthermore, mesolimbic dopamine, most notably in the NAc, increase as a direct result of environmental presentation of rewarding stimuli and the cues that predict these stimuli (Robinson & Berridge, 1993). For example, regional dopamine activation occurs more when a reward is expected compared to when a reward is received unexpectedly (Volkow et al., 2003). In addition, the midbrain dopamine system promotes performance of goal-directed behavior (Salamone, Correa, Farrar, & Mingote, 2007) as well as activation in general (Le Moal & Simon, 1991).

While dopamine D_1 receptors are found throughout the brain, the majority of dopamine D_2 receptors are found in the striatum (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). These dopamine receptors form a heteromer with the receptors for the endogenous ligand

adenosine (de Lera Ruiz, Lim, & Zheng, 2014). Adenosine mostly plays a regulatory role in the brain. A byproduct of energy use via ATP, adenosine binds to endogenous receptors which causes a down regulation in downstream activity (Ferré, 2016). Adenosine A_1 and A_{2A} receptors form heteromers with dopamine D_1 and D_2 receptors, respectively, and share an antagonistic relationship. In other words, activation of adenosine receptors results in reduced activation of the corresponding dopamine receptor (Ferré, 2016). On the other hand, caffeine indirectly increases dopaminergic activity via antagonism of adenosine A_1 and A_{2A} receptors (Ferré, 2016). If caffeine antagonizes adenosine, then there should be an observable increase in dopaminergic activity in areas receiving dopaminergic input such as the NAc. To the contrary, there are mixed findings on the dopamine-enhancing effect of caffeine within the ventral striatum. An acute bolus dose of caffeine (30 mg/kg) weakly enhances dopaminergic activity in the NAc shell (Quarta, Borycz, et al., 2004; Solinas et al., 2002). Conversely, other studies have found no observable increases in NAc shell dopamine efflux with similar injection procedures (Acquas, Tanda, & Di Chiara, 2002; De Luca, Bassareo, Bauer, & Di Chiara, 2007). No changes in accumbal dopamine efflux after researcher-administered caffeine may explain why contingent caffeine presentations are unable to sustain reliable, robust self-administration in preclinical models (Acquas et al., 2002; De Luca et al., 2007). However, caffeine dose-dependently reinstates extinguished cocaine seeking and can be attenuated through non-selective D_1/D_2 antagonists, therefore suggesting caffeine is increasing NAc dopamine efflux (Green & Schenk, 2002). In other words, when paired with a primary reinforcer, caffeine promotes dopamine release in the NAc. Extracellular dopamine in the NAc is directly related to incentive salience and is hypothesized to play a causal role in substance dependence (De Mei, Ramos, Iitaka, & Borrelli, 2009; Robinson & Berridge, 1993). Accordingly, caffeine may promote goal-directed

behavior towards primary reinforcers and the cues that predict them by enhancing the dopamine response elicited by these stimuli.

Caffeine and Goal-Directed Behavior

Caffeine, like nicotine, has multiple effects on goal-oriented behavior: enhancing operant responding for primary reinforcers (as previously discussed), changing novel stimuli to conditioned reinforcers via pairing with the drug (Yeomans, Durlach, & Tinley, 2005; Yeomans, Mobini, & Chambers, 2007), and possibly as a primary reinforcer itself as suggested in some research with human volunteers (Evans et al., 1994; Griffiths & Chausmer, 2000; Schuh & Griffiths, 1997). In addition to the drug's ability to enhance responding for reinforcers, caffeine alters sensitivity to other illicit drugs as seen by the enhancement of psychomotor effects of stimulants such as amphetamine, cocaine, nicotine, as well as promoting the reinstatement of cocaine-seeking after extinction (Green & Schenk, 2002; Horger, Wellman, Morien, Davies, & Schenk, 1991; Magill et al., 2003; Palmatier & Bevins, 2001; Spealman, Barrett-Larimore, Rowlett, Platt, & Khroyan, 1999; Worley, Valadez, & Schenk, 1994). Caffeine enhances motivation for other incentive stimuli in a way that could perpetuate drug use or elicit caffeine consumption congruent to reported public use.

Other than illicit drugs, incentive stimuli (primary and conditioned reinforcers) acquire the ability to control behavior, specifically goal-directed behavior, through multiple pairings with caffeine. This produces a conditioned motivation state that perpetuates caffeine consumption (Fedorchak, Mesita, Plater, & Brougham, 2002; Myers & Izbicki, 2006; Yeomans, Javaherian, Tovey, & Stafford, 2005). For example, researchers performed a study looking at the difference in attentional bias to caffeine-related words between high, moderate, and low caffeine

consumers. Following overnight abstinence, volunteers participated in a modified dot-probe task with a mix of words associated with caffeine and neutral control words. Only high caffeine users showed an attentional-bias to caffeine-related words which correlated with self-reported typical caffeine consumption and caffeine craving (Yeomans, Javaherian, et al., 2005).

Novel Flavors

In addition to enhancing preference for drug-associated environments and words, caffeine enhances preference for novel olfactogustatory stimuli. Flavors previously paired with caffeine are later preferred in a choice task over unpaired flavors (Fedorchak et al., 2002; Kendler, Myers, & Gardner 2006; Yeomans, et al., 2005; Yeomans, Spetch, & Rogers 1998). Specifically, caffeine associated flavors are preferred, perceived as more palatable, and chosen more often than non-associated flavors (Fedorchak et al., 2002; Yeomans, Durlach, et al., 2005; Yeomans et al., 1998). Caffeine, like nicotine, is altering the preference, and motivation for both novel and rewarding stimuli. Although nicotine and caffeine target different receptors in the brain, both psychostimulants affect reward learning and goal-directed behavior in a similar fashion.

Although researchers have established that caffeine enhances the reinforcing effects of drug (Horger et al., 1991) and non-drug reinforcers (Schenk, Worley, McNamara, & Valadez, 1996; Sheppard et al., 2012), it is unclear if caffeine is enhancing the motivational properties of rewards or their associated cues in these paradigms. If caffeine is priming the incentive system, and therefore increasing the effect of incentive stimuli to promote NAc dopamine release, then caffeine should enhance sign-tracking in a PCA paradigm. To test this hypothesis, we examined the effects caffeine on sign- and goal-tracking behavior. Furthermore, we hypothesized that the enhancement of incentive salience by caffeine will be associated with increased extracellular

dopamine in the NAc. In order to observe co-occurring dopamine efflux, rats will be instrumented with microdialysis probes in the NAc shell to measure extracellular dopamine following the acquisition phase of conditioning.

Research Questions Addressed in this Dissertation

1. Is consistent caffeine self-administration possible?

Caffeine enhances motivation for a diverse range of primary reinforcing stimuli including gustatory stimuli, visual stimuli, and drugs of abuse (O'Neill et al., 2016; Prieto et al., 2016; Sheppard et al., 2012; Sweeney, Levack, Watters, Xu, & Yang, 2016). If caffeine is delivered contingently with a primary reinforcer, as is common in human caffeine consumption, animals may achieve a psychoactive dose of the drug which may promote self-administration. We will test the hypothesis that consistent caffeine self-administration is possible when delivered contingently with a primary reinforcer, saccharin, and will result in higher levels of responding and reinforcers earned compared to saccharin alone or caffeine alone reinforcers. In addition, we hypothesize that consistent caffeine self-administration will generalize to different routes of drug administration.

2. Does caffeine administration increase approach to incentives (i.e., 'sign-tracking')?

Although caffeine functions as a reinforcer, no previous study has established whether caffeine's reinforcement enhancing effects are due to the drug's ability to enhance the salience of incentive stimuli or inflate the rewarding or pleasurable properties of a reward. We will test the hypothesis that caffeine increases the salience of an incentive stimulus by recording sign- and goal-tracking behavior in a PCA paradigm.

3. Are the putative incentive-promoting effects of caffeine associated with increased NAc dopamine release?

All drugs of abuse alter the incentive systems of the brain via facilitation of mesolimbic dopamine signaling (Robinson & Berridge, 1993). Caffeine enhances motivation to obtain primary reinforcers which may promote caffeine self-administration when the drug and another primary reinforcer are presented together (Sheppard et al., 2012). One hypothesis for the effect of caffeine on goal-directed behavior is that caffeine primes the mesolimbic dopamine system and enhances the response to another reward. However, this hypothesis has not been tested. Previous research has shown only weak or no increases in NAc shell dopamine efflux after an acute caffeine dose (Acquas et al., 2002; De Luca et al., 2007; Quarta, Ferré, et al., 2004; Solinas et al., 2002). We will test the hypothesis that a moderate dose of caffeine will elicit an elevation in NAc dopamine efflux when exposed to an incentive stimulus that has previously predicted sucrose presentation.

CHAPTER 2

METHODS

Subjects

Male Sprague-Dawley rats (Charles River) weighing approximately 350g upon arrival were used in this study. Subjects were individually housed in a temperature and humidity controlled environment with a reverse 12:12 h dark:light cycle. All behavioral testing was conducted during the dark part of the light cycle. Subjects were maintained on a restricted diet of approximately 17g of rat chow (LabDiet, St.Louis, MO) throughout the experiment to ensure motivation for an aqueous sucrose or saccharin solution. Access to diet was provided immediately following behavioral testing and tap water was provided ad libitum in the home cage throughout the experiments. Methods were approved by the Institutional Animal Care and Use Committees at East Tennessee State University (Animal Welfare Assurance #: A3203-01).

Apparatus

Conditioning Chambers

Experimental sessions were conducted in ten Med-Associates (Georgia, VT) operant chambers (ENV-008CT) measuring 30.5 × 25.4 × 30.5 (w×d×h) individually housed in a ventilated, sound and light attenuated enclosure. Chambers were equipped with two retractable levers (ENV-112CM) and stimulus lights located 15 cm above the grid flooring on the same instrument panel where a liquid dipper receptacle was located. Levers were located on either side of the receptacle, approximately 2 cm in from the outside of the instrument panel. A liquid dipper with a 0.1 ml cup attached to a motorized arm delivered a sucrose or saccharin solution

into the receptacle which served as the reinforcer or US in these experiments. For experiment 3 extension of one lever and simultaneous illumination of the right stimulus light for 30s served as the conditioned stimulus stimuli. A Med Associates contact lickometer controller (ENV-250) was attached to the back of the left lever in order to record lever contacts when extended into the operant chamber. An infrared emitter detector unit recorded entries into the receptacle throughout the session. Two houselights were located on both instrument panels approximately 28.5 cm above the floor and 12.2 cm from the outer walls of the operant chamber. Each operant conditioning chamber was also equipped with a drug delivery system with a syringe pump (Med-Associates, model PHM100 – 10 rpm) that was used to deliver intravenous drug during Experiment 1. A computer using MED-PC V software programming controlled the operant chambers and recorded all data.

Microdialysis Collection Assembly

Dopamine dialysate was collected by connecting the microdialysis probe directly to a microdialysis collection assembly. The assembly was constructed with two pieces of tubing. One side connected to a swivel that perfused aCSF towards the probe in the rat's brain. This side was constructed 29 cm of PE20 tubing. The other side collected aCSF coming from the microdialysis probe and was constructed of 29 cm of PE10 tubing with a ball 3 cm from the end. The plastic ball secured an amber vial and cap to the line and the amber vial was exchanged every 20 minutes.

High Performance Liquid Chromatography (HPLC)

Dopamine dialysate was analyzed through electrochemical detection via graphite electrode using a EiCOM HTEC-510 HPLC machine (San Diego, CA). Dialysate samples were

analyzed manually by placing 5-20 µl of sample into a 50 µl loop. Alternatively, 20 µl dialysate samples could be run using a EiCOM AS-700 autosampler (San Diego, CA). A computer using Envision software programming controlled EiCOM equipment and quantified dialysate sample using an area-under-curve analysis.

Drugs and Solutions

Oral Solutions

Saccharin (Sigma-Aldrich, St. Louis, MO) was dissolved in tap water (0.2%, w/v). Caffeine anhydrous (Sigma-Aldrich, St. Louis, MO) (0, 2.5, 3.5, 5.0, and 7.0 mg/ml) was dissolved in tap water with saccharin (0.2%, w/v), decaffeinated instant coffee (0.5% w/v), or both saccharin (0.2%, w/v) and decaffeinated instant coffee (0.5%, w/v). Sucrose (20% w/v) was comprised of table sugar diluted in tap water. All oral solutions were delivered in 0.1 ml dipper cups.

Intravenous Solutions

Caffeine anhydrous (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% sterile saline and infused at a volume of 0.2 ml/kg/infusion intravenously. Sterile saline solution (0.9% Sodium Chloride) was prepared using sterile water and sodium chloride (Acros Organics, New Jersey) and delivered via Intra peritoneal (IP) injection at a volume of 0.2 ml/kg. Caffeine anhydrous (Sigma Aldrich, St. Louis, MO) was diluted in a sterile saline solution at 5 mg/ml and delivered via IP injection at .2 ml/kg to achieve a dose of 10 mg/kg.

Microdialysis Solutions

Artificial cerebrospinal fluid. Artificial cerebrospinal fluid (aCSF) microdialysis perfusion medium was composed of 145.0 NaCl, 2.7 KCl, 1.0 $MgCl_2$, 2.5 $CaCl_2$, and 2.0 Na_2HPO_4. aCSF was filtered through a sterile filter (pore size, 0.2 µM), and prepared the day of microdialysis sessions. When necessary, the pH was adjusted to 7.4 with 0.1 N acetic acid. aCSF was perfused through 2 mm loop-style probes (described below) at a rate of 1.1 µl/min. Dialysate samples were preserved using 5 µl of DA preservative in each sample collection tube.

Dopamine mobile phase. Dopamine mobile phase used for dopamine dialysate analysis via HPLC was composed of 1400 ml HPLC H_20, 328 µl acetic acid, 10.36 g ammonia acetate, 600 ml methanol, 14.2 g sodium sulfate, and 2 ml of 50 mg/ml EDTA. Mobile phase volume was adjusted to 2000 ml using submicron filtered HPLC H_20 (Fischer Chemical, St.Louis, MO).

Dopamine preservative. Dopamine preservative was used for dialysate sample collection and HPLC analysis. Dopamine preservative consisted of 0.1 mol phosphate buffer with 0.1 mmol EDTA. Preservative was filtered through a sterile filter (pore size, 0.2 µM) before use.

Dopamine standard. Dopamine standard was used for HPLC calibration and dopamine detection. Standard was composed of dopamine hydrochloride (Alfa Aeser, Ward Hill, MA) and diluted to a concentration of 5 nM using dopamine preservative.

Procedures

Experiment 1 and 2

Training. Rats from Experiments 1 and 2 were trained to lever press to gain access to a saccharin solution. Initially, the left lever was inserted into the operant chamber for 15 s and immediately followed by activation of the liquid dipper (lowered into saccharin solution for 0.5 s) to gain access to 0.1 ml of saccharin solution. Any responses recorded during the lever presentation resulted in retraction of the lever and 2 activations of the liquid dipper, separated by 5 s. Successful shaping was operationally defined as earning 60 reinforcers within a 1-h session.

Surgery. Rats were anesthetized with isoflourane and implanted with an indwelling jugular vein catheter. Jugular vein catheters were constructed using a blunt needle (22 G x 1-1/2", EXEL Int., California) bent at a 90° angle approximately 2 mm below the hub. A nylon washer (.09x.25x.062) (Fastenal, Minnesota) was placed at the base of the needle followed by a round patch of polyester fabric (37.5 mm diameter) and another washer. Silastic® tubing (Dow Corning, Michigan) (0.51 ID x 0.94 mm OD) approximately 175 mm in length was attached to the needle up to the bend. The needle, washer, patch, and tubing was secured into place using medical device epoxy adhesive (LOCTITE EA M-21 HP, Henkel, North Carolina). A silicone ball (3 mm diameter) was attached 40 mm from the end of the tubing using 100% RTV silicone (DAP, Maryland). Subcutaneous ketoprofen injections (3 mg/kg) were used to alleviate pain for three days after surgery. Catheters were flushed daily with sterile heparinized saline (30 IU) and Timentin (40 mg/kg, bioWorld, Dublin, OH). Operant testing began 7-10 days after surgery to allow full recovery from the surgical procedure.

Intravenous caffeine self-administration. Following the training procedure, rats (n=60) were habituated to lever press for access to saccharin under a progressive ratio (PR) reinforcement schedule during a 60 min session for four sessions. The PR was exponential, adapted from Richardson and Roberts (1996), and used previously to investigate the reinforcement enhancing effects of nicotine (Palmatier et al., 2007) and caffeine (Sheppard et al., 2012). Rats in the 1.0 mg/kg C+S group did not receive any habituation sessions. This did not seem to lower acquisition rates or motivation for the assigned reinforcer. Following habituation, rats were randomly assigned to one of three groups: saccharin only (S, n=10), caffeine only (C, n=23) or saccharin and caffeine (C+S, n=27). For rats in the S group meeting the schedule of reinforcement on the active lever resulted in presentations of 0.2% saccharin. Rats in the C group were randomly assigned to one of 4 unit doses (mg/kg/infusion) of caffeine, 0.125 (n=6), 0.5 (n=6), 1 (n=5), or 4 mg/kg (n=6) and these infusions replaced saccharin presentations for meeting the schedule of reinforcement on the active lever. For rats in the C+S group both 0.2% saccharin and IV caffeine infusions 0.125 (n=7), 0.5 (n=9), 1.0 (n=5), or 4.0 mg/kg (n=6) were presented for meeting the schedule of reinforcement on the active lever. Caffeine dose was a between subjects factor; each rat received only one unit dose throughout the study. Rats were adapted for self-administration of saccharin and/or IV caffeine via lever pressing under a PR reinforcement schedule. Before the start of the session rats receiving intravenous caffeine had a leash attached to their catheter port located between their shoulders for caffeine administration. The leash was counterbalanced to minimize constriction of movement from the weight of the leash. At the start of each session both the active (left lever) and inactive (right lever) were inserted into the operant conditioning chamber; the inactive lever had never been presented before testing began. A 30 second time-out period occurred in which the house light was

extinguished; lever presses were recorded but were not counted toward the schedule of reinforcement. All sessions ended after 60 min, with breaking points operationally defined as 5, 10, or 20 min periods without earning a reinforcer during the session. Sessions were conducted 48 hr apart to avoid tolerance to caffeine. After five days of testing catheter patency was confirmed with propofol (0.2-0.3 ml infusion); only data from rats displaying immediate loss of muscle tone, heart rate, and righting reflex were included in the analyses.

Oral caffeine self-administration. The purpose of this experiment was to determine whether caffeine would be self-administered orally in a vehicle that included a reinforcing gustatory stimulus (0.2% saccharin). All rats were habituated to a saccharin solution for three days under a PR schedule and one day of an FR1 schedule of reinforcement. Following habituation, rats were assigned to their reinforcer condition and responded under a PR schedule with a 30 minute breakpoint (30 minutes passing without earning a reinforcer). Session length varied between individual rats since sessions did not end until rats reached the 30 minute breakpoint. Forty rats were assigned to one of three vehicles (Water (W), Saccharin (S), or Decaffeinated coffee +Saccharin (DS)) with caffeine (henceforth indicated by a + symbol) or without caffeine (henceforth indicated by a – symbol) for completing the schedule of reinforcement on the active lever. Twenty rats received caffeine in a liquid vehicle that either contained saccharin (S+, n=7, DS+, n=7) or just water (W+, n=6). The remaining rats received water (W-, n=6) or saccharin alone (S-, n=7, DS-, n=7) for meeting the contingency. Rats in the caffeine (+) groups were first exposed to a liquid reinforcer with 2.5 mg/ml caffeine concentration. Sessions continued with this concentration until responding stabilized – an informal assessment of group-wise responding was used to determine stability (no linear trend across the last 3 days of testing). Caffeine concentration was increased after group stability in the

following order: 2.5, 3.5, 5, and 7 mg/ml. Rats in the caffeine group responded for 2.5 mg/ml for six days, 3.5 mg/ml for three days, 5.0 mg/ml for five days, and 7.0 mg/ml for four days. Unlike Experiment 1, each concentration of caffeine was experienced by all caffeine (+) groups.

Experiment 3

Group assignment. Rats (n=60) were randomly assigned to one of three drug conditions: 10 mg/kg caffeine (10 mg/kg, n=16), 25 mg/kg caffeine (25 mg/kg, n=20) or saline (0 mg/kg, n=24). Assigned drug injections were delivered via intraperitoneal injection (IP) fifteen minutes prior to the start of each conditioning session. All sessions occurred on alternate days to avoid the development of caffeine tolerance. Following conditioning, rats were randomly assigned to one of two microdialysis testing groups (CS or No-CS; see Conditioning).

Conditioning. Following pretreatment, rats were placed in operant chambers for one hour conditioning sessions. During conditioning sessions rats were exposed to 15 pairings between the CS and US. Since the 'goal' location (receptacle where US was delivered) was continuously available, approach was recorded during Pre-CS intervals (the 15s preceding each CS presentation). During the CS intervals the left lever was extended into the chamber and the stimulus light above the lever was illuminated for 15 seconds. US intervals began once the dipper was cycled to deliver a 0.1 ml dipper presentation of sucrose solution (US) that was available until the next US delivery. Conditioning trials did not occur in the first or last eight minutes of the session and were separated by inter-trial intervals that averaged 120 s (90-150 s). Lever contacts, presses, and dipper receptacle entries were recorded throughout the session. Lever contacts and presses during CS intervals are considered measures of 'sign-tracking.' Dipper receptacle entries during the CS intervals are measures of 'goal-tracking.' There were 8 total

conditioning sessions – the first five lasted approximately 1 h. Ten rats received an 8th conditioning session because of experimenter error (4 rats in the 10 group and 6 rats in the 0 group); during the 6th testing session the dipper receptacles were left in a locked position and CS presentations were not followed by US presentations.

Sessions 6 and 7 served as microdialysis habituation; rats were placed in operant chambers for 2 h prior to the start of the conditioning session and left in the apparatus for 1 h after the session was completed to simulate the microdialysis sample collection. The door to the sound-attenuating chambers was left open to mimic conditions during sample collection. Microdialysis samples were collected on the 8th test day and tests were conducted in 'extinction', meaning that the US was never presented. Rats in the CS groups were exposed to CS presentations during the microdialysis session. Rats in the No-CS group did not experience any CS presentations during microdialysis to investigate the effects of caffeine alone on extracellular DA. Sample collection lasted 4 h, with a washout (60 min), baseline (60 min), testing session (60 min) and post-test samples (60 min).

Cannula and probe implantation. Following the 7th conditioning session all animals underwent stereotaxic surgery to implant a cannula for microdialysis. Rats were anesthetized using 2% isoflurane, placed in a stereotaxic instrument in which ear and incisor bars were adjusted to ensure a flat skull position (level lambda and bregma height), and implanted ipsilaterally with one 18 gauge cannula (Plastics One Inc., Roanoke, VA) approximately 1 mm above the targeted brain region. The targeted region was the shell of the Nucleus Accumbens using the following stereotaxic parameters: + 1.7 mm Anterior from bregma, +2.3 Lateral to midline, and -5.4 Ventral from the dura mater at a 10° angle. Dummy stylets were placed into the cannula following surgery and remained until the microdialysis collection session. Rats rested

for a minimum of 5 days in their home cage for recovery prior to dialysate collection. On the final day of recovery, a loop style microdialysis probe was inserted into the shell of the nucleus accumbens following the 7th habituation session.

Microdialysis. Approximately 16 hours after microdialysis probe implantation, rats were placed into operant boxes after connecting probes to a collection assembly using 23 G 1 ¼ needles (Becton Dickinson, Franklin Lanes, NJ). The collection assembly was connected to a swivel (type) located on a Gimbal ring attached to a drug delivery arm (Med Associates, PHM-110-SAI). The swivel was connected to a Harvard pump using P20 tubing (Braintree Scientific, Braintree, MA). Microdialysis aCSF was perfused at a flow rate of 5µl/min until aCSF was continuously flowing through the probe. Once confirmed for all rats, flow rate was changed to 1.1 µl/min for the remainder of the session. Following a one hour washout period, four 20-min baseline samples were taken. Rats were given an IP injection of their assigned drug at the start of the fourth sample collection. After baseline, rats in the CS group were exposed to 15 CS presentations over a one hour period. Rats in the No-CS group received no CS presentations during this hour. Following CS/No-CS presentations, three 20-min post dialysate samples were collected. All dialysate samples were collected in a labeled microfuge vial containing 5 µl of dopamine preservative, then immediately frozen using dry ice and stored at -80° C.

Histology. Immediately following microdialysis testing, rats were euthanized with an overdose of CO_2 inhalation. Bromophenol blue (1%; 0.5µl) was perfused into the shell of the nucleus accumbens via dialysis probe. Brains were then extracted, frozen on dry ice, and stored at -80° C. Brains were later sectioned (40 µm) using a cryostat microtome. Brain sections containing bromophenol blue stain were placed on glass slides. Sections were examined to verify

probe placement using the rat brain atlas of Paxinos and Watson (2005). Animals with a probe location outside the NAc shell were excluded.

Dialysate analysis. All samples were analyzed using high-pressure chromatography (HPLC) with electrochemical detection using instruments described above. Samples were removed from the -80 freezer and 25 µl of each sample was pipetted into a 96 well sample plate. Plates were placed into an autosampler where each well was sampled individually. Samples containing less than 25 µl were filled with dopamine preservative to reach an overall volume of 25 µl. Sample amount was noted and dopamine sample was corrected after analysis. Dopamine concentration was determined using the area-under-curve analysis for each histogram. Samples were analyzed over a 14 min time span to allow analysis of entire sample without contaminating analysis of upcoming sample.

Data Analyses

Caffeine Self-Administration

In Experiment 1, data were analyzed with a mixed-factors ANOVA on active lever presses, inactive lever presses, and reinforcers earned, including Group (C+S vs. S vs. C) and Session (1-5, repeated) as the independent factors. This was done to ensure reliable caffeine self-administration over multiple days. For Experiment 2, the concentration of caffeine was the critical manipulation so the mixed factors ANOVA on lever presses and reinforcers earned including Group (DS+, DS-, S+, S-, W+, W-) and Dose (2.5-7 mg/ml, repeated) as the independent factors. Lever presses and reinforcers earned at the 20 min break point were analyzed due to the increase in inactive lever presses in caffeine groups during the final half hour of each session. Final two day averages of lever presses and reinforcers were used in the analysis

to compare caffeine concentrations. These two day averages provided stable rates of self-administration that were representative of each group at each caffeine concentration. For Experiments 1 and 2, second-order contrasts were used to compare groups on individual sessions (Experiment 1) and individual doses (Experiment 2). Break points (Experiments 1 and 2) were not included in the analysis because the PR schedule increased at an exponential rate (0.12), therefore violating the assumption of linearity and thus increasing the susceptibility of a Type II error.

Pavlovian Conditioned Approach

PCAI. The extent to which a rat emitted more sign- or goal-tracking behaviors during sessions was quantified using a compound index. The index incorporated three measurements of Pavlovian conditioned approach which occurred during the 15s CS interval: Probability difference score, Response bias score, and a Latency difference score. The Probability difference score [P(sign) - P(goal)] is the difference in probability of entry into the dipper well (P (goal)) subtracted from the probability of a lever contact or press (P(sign)). Probability of sign- and goal-tracking for each CS trial is scored as a 1 or 0 dependent upon whether a rat entered a receptacle or contacted the lever (1=yes, 0=no). For example, if a rat contacted the lever during all 15 CS presentations, the P(sign) would be scored as a 15. The response bias score reflects the difference between lever contacts and receptacle entries during each CS period [(total lever presses - total receptacle entries)/(total lever presses + total receptacle entries)]. The Latency difference score [(lever contact latency - receptacle entry latency)/15] is the difference in latency to contact the lever and latency to enter the dipper well. Together, these measurements were formulated to form the Pavlovian Conditioned Approach Index (PCAI) [(Probability difference score + Response bias score + Latency difference score)/3]. Scores range from -1.0 to +1.0

indicating a strong bias to the dipper receptacle entry (-1.0) or a strong bias to contact the lever (+1.0).

Acquisition. Separate 2-way (dose x session) Mixed Factors ANOVAs were conducted for the acquisition portion of the experiment on PCAI, Probability difference score, P(sign), P(goal), Response bias score, lever contacts, dipper receptacle entries, Latency difference score, lever contact latency, and receptacle entry latency with Dose (0, 10, 25 mg/kg) and Session (1-7) as independent factors. Session was treated as a within-subject variable during acquisition. Caffeine dose was treated as a between-subjects variable since individual rats only received repeated administration of the same dose throughout the experiment. Significant main effects on dose were probed using Dunnett's test with the 0 mg/kg group serving as the reference group for comparison with the 10 mg/kg and 25 mg/kg groups. Significant interactions were followed up with simple effects analyses and t-tests where appropriate.

Goal-Tracking. For conditioning and microdialysis sessions head entries into the dipper receptacle were recorded in 15 s bins during the Pre-CS and CS intervals. Pre-CS intervals were used to measure baseline head-entry behavior in the dipper well. Head-entries during CS presentations were measured in order to determine the elevation score (Palmatier et al., 2004). Elevation scores were calculated by subtracting head-entries during the Pre-CS bins from head-entries during the CS bins. Elevation scores from each bin were averaged for each session and used as the primary dependent variable for goal tracking behavior. Scores above a theoretical mean of zero indicate that a rat spent more time entering the dipper well during CS presentation. In addition, latency to enter the dipper receptacle during CS trial was recorded and reported as an average at the end of each session. Dipper receptacle entries and receptacle latency were used to

determine receptacle entry score, receptacle latency score, and P(goal). These components are needed to determine PCAI and indicate goal-tracking behaviors.

Sign-Tracking. For conditioning and the microdialysis session lever contacts and presses were recorded in 15 s bins during each CS interval and reported as the cumulative total at the end of each session. Latency to contact the lever during each CS trial was recorded and reported as an average at the end of each session. Lever contacts, presses, and latency to lever contact were used to determine lever contact, contact latency score, and P(sign). These components are needed to determine PCAI and indicate sign-tracking behaviors.

Microdialysis

Dopamine. Dopamine efflux was normalized by averaging the first three baseline microdialysis samples collected after the initial 1 h washout period. Remaining samples were calculated as a percentage of baseline value. Only samples representative of dopamine efflux during CS exposure and afterwards were analyzed for group comparisons. Samples 6-8 represented dopamine efflux during CS exposure (Test Interval) and samples 9-11 represented dopamine efflux after CS exposure (Post Test Interval). Percent change from baseline dopamine samples collected during Test and Post Test intervals were transformed for Area Under the Curve (AUC) analysis. Group AUC averages during the Test Interval and Post Test Interval were used to analyze the main effects of Dose and Interval. Separate analyses were conducted for groups exposed to the CS and groups not exposed to the CS. Final statistical analyses include only rats with confirmed accumbal shell probe placement. Changes in dopamine using AUC calculated data was analyzed by separate 2 x 3 (Interval x Dose) ANOVA for groups exposed to

the CS and not exposed to the CS. Pairwise comparisons using Bonferroni's adjustment was used to examine interactions between caffeine dose and intervals.

CHAPTER 3

RESULTS

Caffeine Self-Administration

Experiment 1

Acquisition. Caffeine dose-dependently promoted self-administration when contingently paired with a saccharin reinforcer (C+S) relative to control groups that received caffeine (C) infusions or saccharin alone (S). This conclusion was supported by omnibus 2 x 4 x 5 (Group x Dose x Session) ANOVA on the reinforcers earned dependent measure. There was a significant main effect of Group, $F(1,43)=67.712$, $p<.001$, as well as significant Session x Group, $F(3.943,169.551)=2.344$, $p<.001$, Dose x Group, $F(3,43)=3.831$, $p=.016$, and Session x Dose x Group, $F(11.829,169.551)=2.62$, $p=.003$, interactions. Pairwise comparisons revealed C+S groups earned more reinforcers than C groups at the .125 mg/kg/infusion dose on sessions 1-5 (*p's*<.05, Fig. 1A), 0.5 mg/kg/infusion dose on sessions 2-5 (*p's*<.05, Fig.1B), 1.0 mg/kg/infusion on sessions 2-5 (*p's*<.05, Fig.1C), and 4.0 mg/kg/infusion dose on session 2 ($p<.05$, Fig. 1D). An omnibus 2 x 4 x 5 (Group x Dose x Session) ANOVA on active lever responses revealed a significant Session x Group interaction, $F(2.207, 94.891)=5.711$, $p=.003$. Posthoc pairwise comparisons with Bonferroni adjustment revealed C+S groups had more active lever responses than C groups at the .125 (Sessions 1-4, Fig. 1E), 0.5 (Sessions 2-5, Fig.1F), and 1.0 doses (Sessions 2-5, Fig. 1G). A 2 x 4 x 5 (Group x Dose x Session) ANOVA was conducted on inactive lever presses and revealed no main effects or interactions. Taken together, these

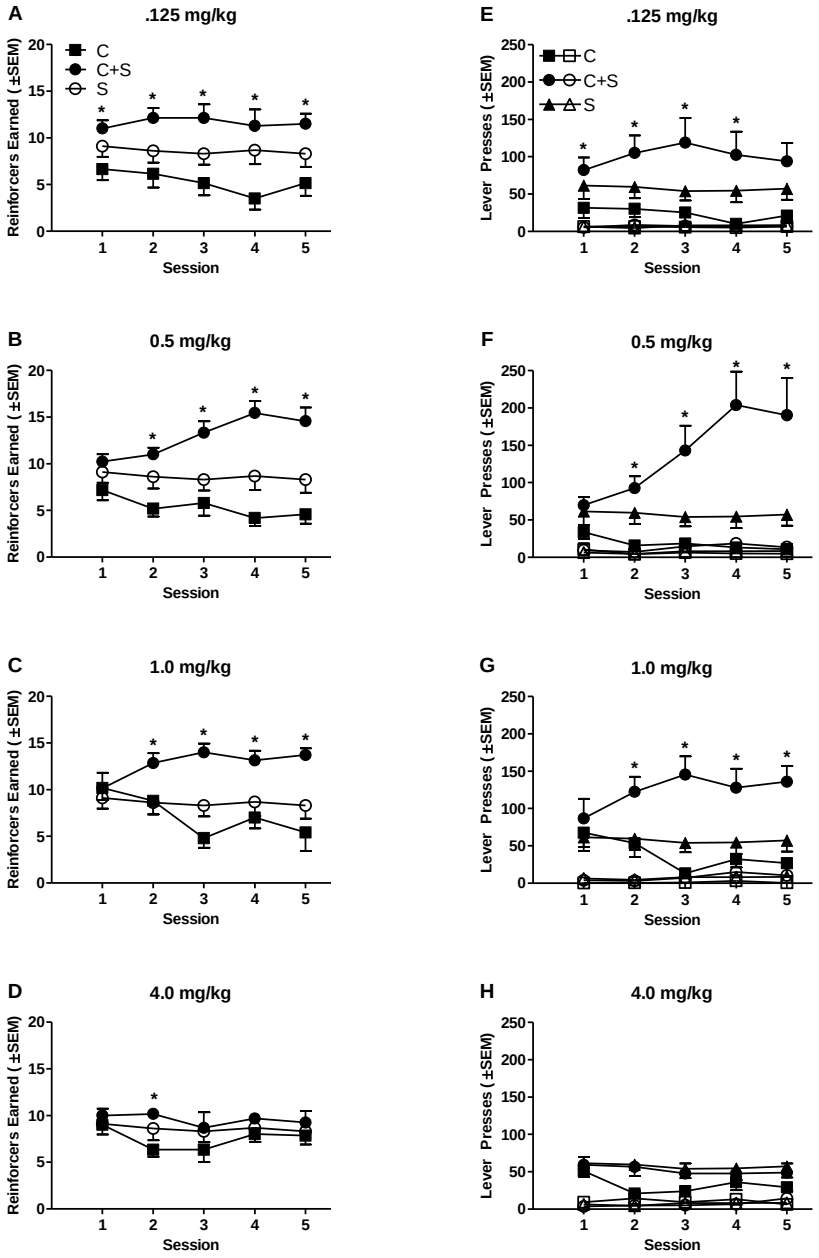

Figure 1: Low-to-moderate doses of caffeine enhance acquisition of self-administration when paired with dipper presentations of liquid saccharin. Figures A-D represent reinforcers earned for each group on each day of caffeine self-administration. Figures E-H represent active and inactive lever presses for each group on each day of caffeine self-administration. Asterisks (*) represent C+S being significantly higher than C groups as determined through pairwise comparisons using a Bonferroni adjustment (*p's*<.05).

analyses reveal that caffeine, when paired with saccharin, can promote consistent self-administration at moderate to high doses. Furthermore, group differences were not caused by a nonspecific psychomotor stimulant effect of caffeine as seen by the lack of significant differences of inactive lever presses between groups.

Dose response curve. To further assess the ability of caffeine to promote self-administration when contingently paired with a saccharin reinforcer, C+S groups were compared to S groups during the final two days of acquisition at each dose. Only the final two days were main effect of Dose, $F(4,70)=9.395$, $p<.001$, on reinforcers earned (Fig.2A). Pairwise comparisons with Bonferroni adjustment found that the C+S groups receiving 0.5 and 1.0 mg/kg/infusion earned more reinforcers than S groups, p's$\leq.002$. Additionally, the C+S group receiving 0.5 mg/ml earned more reinforcers than the C+S group receiving 4.0 mg/kg/infusion, $p=.003$ (Fig. 2A). Furthermore, a One-Way ANOVA revealed a significant main effect of Dose, $F(4,70)=8.968$, $p<.001$, on active lever presses (Fig. 2B). Pairwise comparisons with Bonferroni adjustment revealed that the C+S group receiving 0.5 mg/kg/infusion responded significantly more than the S group, the C+S group receiving.125 mg/kg/infusions, and 4.0 mg/kg/infusions, p's$\leq.015$ (Fig. 2B). Caffeine's ability to promote self-administration, when contingently paired with saccharin, at moderate doses was not caused by a nonspecific psychomotor stimulant effect of caffeine as seen by a non-significant effect of Dose on inactive lever presses ($p>.05$, Fig. 2B). The C group was not included in this analysis because there was no control group receiving 0 mg/kg caffeine infusions alone.

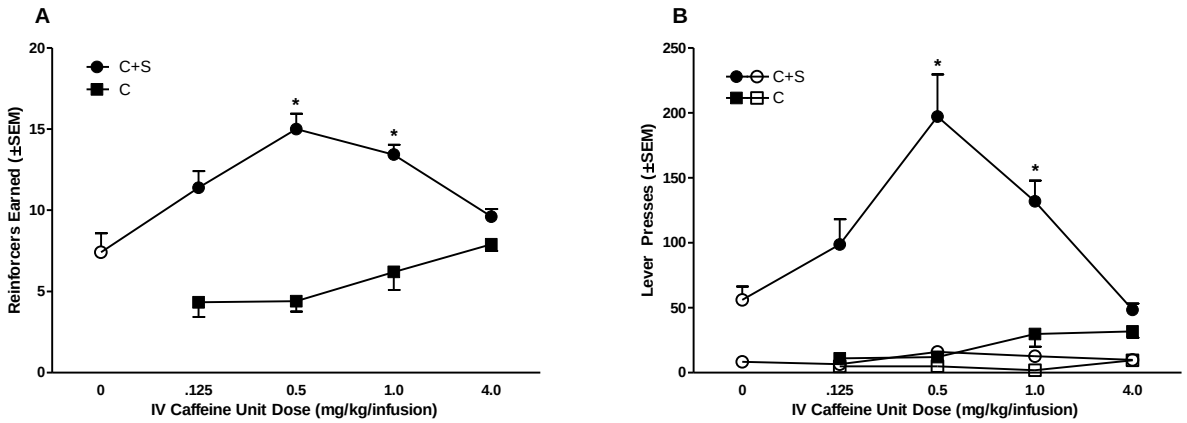

Figure 2: Intravenous delivery of caffeine dose-dependently increases self-administration when paired with dipper presentations of liquid saccharin. Figure A illustrates the final two day average reinforcers earned across doses of caffeine. One-Way Between Groups ANOVA revealed a significant main effect of Dose, $p<.001$, on reinforcers earned. Figure B illustrates final two day averages of active and inactive lever presses across doses of caffeine. One-Way Between Groups ANOVA revealed a significant main effect of Dose, $p<.001$, on active lever presses. Asterisks (*) represent C+S being significantly higher than the S group as determined through pairwise comparisons using a Bonferroni adjustment (*p's*<.05).

Experiment 2

Acquisition. Oral administration of caffeine paired with saccharin increased motivation under the PR schedule of reinforcement. During acquisition, a vehicle containing saccharin and 2.5 mg/ml of caffeine significantly increased motivation compared to groups receiving caffeine alone. This was confirmed by a 3 x 6 (Vehicle x Session) ANOVA that revealed significant main effect of Vehicle, $F(2,17)=5.766$, $p=.012$, and approaching significance for Session, $F(2.708,40.035)=2.605$, $p=.069$, on reinforcers earned. Planned pairwise comparisons revealed rats working for vehicles containing saccharin and caffeine earned significantly more reinforcers than rats receiving vehicles containing caffeine alone, p's$\leq.019$ (Fig. 3A). As expected, there was also a significant main effect of Vehicle, $F(2,17)=4.748$, $p=.023$, and approaching significance for Session, $F(2.657,45.164)=2.596$, $p=.071$, on active lever presses (Fig. 3B). Notably, there

was no main effects of Vehicle or Session on inactive lever presses, *p's*>.05 (Fig. 3B). Vehicle-alone groups (S-, DS-, W-) were not statistically different across acquisition. This was confirmed using separate 3 x 6 (Vehicle x Session) ANOVA on reinforcers earned, active, and inactive lever presses. There were no significant main effect of Vehicle, Session or interaction across acquisition, *p's*>.05 (Fig. 3A, 3B).

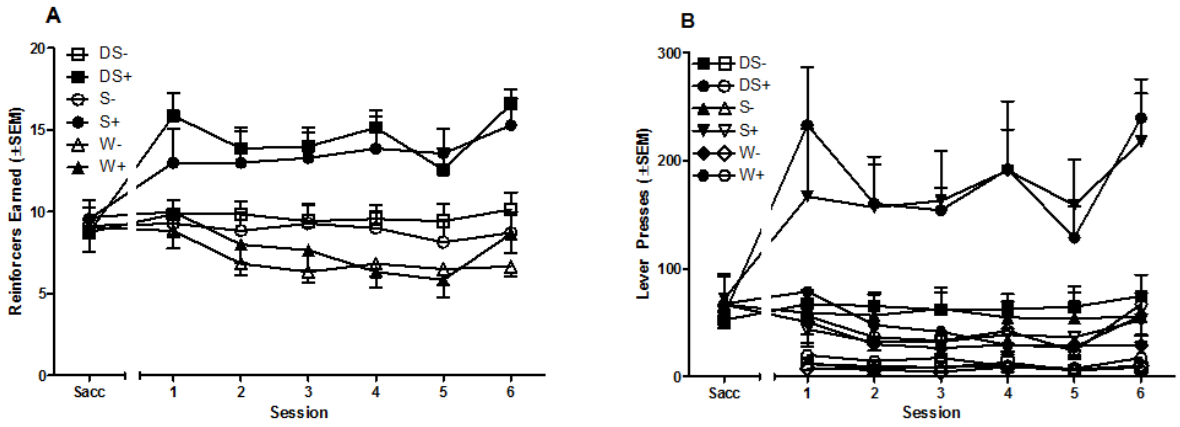

Figure 3: Caffeine delivered in a vehicle containing saccharin increases self-administration across sessions. Figure A illustrates average reinforcers earned throughout each session of acquisition. A concentration of 2.5 mg/ml caffeine was used for acquisition for groups receiving caffeine. Sacc session represents data from baseline procedures in which rats responded for a saccharin solution only. Figure B illustrates average lever presses throughout each session of acquisition. Separate Repeated Measures Mixed ANOVAs revealed significant main effects of Drug and Vehicle on all dependent measures, *p's*<.05. Second-order contrasts revealed that DS+ and S+ groups responded more on the active and inactive lever and earned more reinforcers than the S- group.

Concentration response curve. Caffeine increased responding for the saccharin-containing vehicles across much of concentrations tested. This conclusion was supported by a 3 x 4 (Vehicle x Concentration) ANOVA which revealed a significant main effect of Vehicle, $F(2,37)=26.263$, $p=.001$, on reinforcers earned. The Vehicle x Concentration interaction did not reach statistical significance, $p=.063$ (Fig.4A). As expected, an identical pattern was observed for active lever responses (Fig. 4B). Planned pairwise comparisons using Bonferroni correction

revealed that caffeine, when paired with a vehicle containing saccharin, increased the number of reinforcers earned at every concentration (*p's*≤.008) except 7 mg/ml (*p's*=.102), when compared to water. Planned pairwise comparisons revealed similar effects on active lever presses, *p's*≤.005 (Fig. 4B). Lastly, a 3 x 4 (Vehicle x Concentration) ANOVA revealed a significant main effect of Vehicle, $F(2,37)=6.017$, $p=.005$, on inactive lever presses. Planned pairwise comparisons revealed no differences between vehicle groups on inactive lever presses, apart from S+ at the 7 mg/ml concentration, $p=.007$ (Fig. 4B).

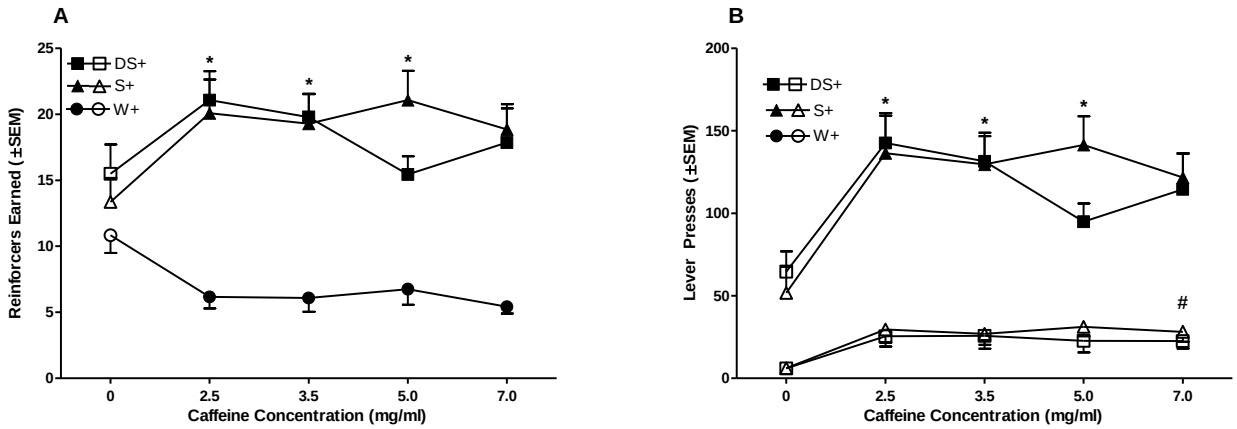

Figure 4: Caffeinated vehicles containing saccharin increase self-administration at multiple concentrations. Figure A illustrates the final two day average reinforcers earned across concentrations of caffeine. A 3 x 4 Repeated Measures ANOVA revealed significant a main effect of Vehicle and interaction of Vehicle x Concentration approaching significance, p=.063, on reinforcers earned. Figure B illustrates final two day averages of active and inactive lever presses across concentrations of caffeine. A 3 x 4 Repeated Measures ANOVA revealed significant main effect of Vehicle on active and inactive lever presses, *p's*<.05. Asterisks (*) denote significant differences of active lever presses and reinforcers earned between the S+/DS+ and vehicle controls, *p's*<.05. The hash mark (#) denotes a significant increase in inactive lever presses for the S+ group compared to water control, *p's*<.05.

Pavlovian Conditioned Approach

Acquisition

PCAI. Caffeine dose-dependently increased sign-tracking (approach to the CS) and reduced goal-tracking (approach to the location of US delivery) in the Pavlovian conditioned approach (PCA) paradigm. Figure 5 illustrates the average PCA index (PCAI) across conditioning sessions for rats assigned to the 0 (n=14), 10 (n=14), and 25 (n=13) mg/kg caffeine groups. The 10 and 25 mg/kg caffeine doses increased PCAI compared to saline controls (0 mg/kg) and the bias toward sign-tracking increased across conditioning sessions.

This was supported by two-way (Dose x Session) ANOVA with significant main effects of Session, $F(6,324)=8.735$, $p<.001$, and Dose, $F(2,54)=13.778$, $p<.001$ on PCAI scores. The Session x Dose interaction approached significance, $F(12,324)=1.544$, $p=.107$. Dunnett's test was used to further assess the main effect of Dose revealed that 10 mg/kg, $M=0.025$, $SEM=0.057$, and 25 mg/kg groups, $M=-0.014$, $SEM=0.071$, had significantly higher PCAI than the 0 mg/kg group, $M=-0.351$, $SEM=0.052$, $p's\leq.001$. While the PCAI is a good

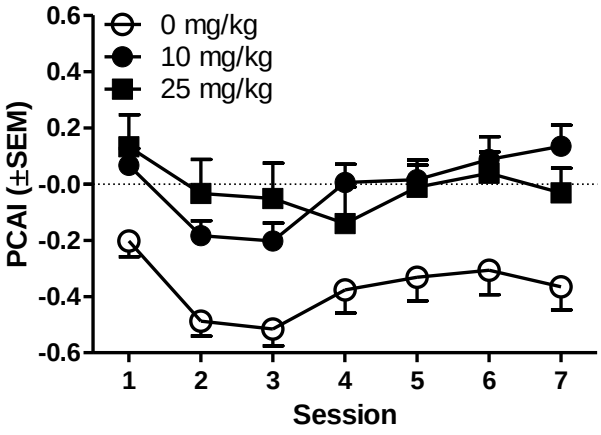

Figure 5: Caffeine increases PCAI when compared to a saline control group. A Mixed ANOVA revealed a main effect for Dose and Session on PCAI, $p's<.001$. A Dunnett's post hoc analysis revealed saline controls had significantly lower PCAI than subjects treated with 10 mg/kg caffeine and 25 mg/kg caffeine bolus doses prior to the start of each session, $p's\leq.001$. Positive scores indicate a tendency to be attracted to the lever during CS presentations. Negative scores indicate a tendency to emit more behavior towards the receptacle during CS presentations.

indicator of behavior 'topography' during CS presentations, the scores are not sensitive to acquisition of conditioned responding. For example, a rat that does not acquire an association and approaches neither the CS nor the US during a test session will receive a score of 0. Similarly, a rat who equivalently allocates large amounts of behavior to the sign and goal (in terms of timing, probability, and frequency) will have a PCAI of 0. For this reason, it is imperative to analyze the individual components that comprise the PCAI in order to get a clearer picture of how caffeine dose affects Pavlovian conditioning.

Latency analyses. The Latency score is the subtraction of total Goal latency during CS presentation from total Sign latency during CS presentation. This composite score reveals the relative speed with which the subject approaches the sign or the goal. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=23.781$, $p<.001$, Dose, $F(2,57)=41.75$, $p<.001$, and an interaction of Session x Dose, $F(12,342)=2.35$, $p=.007$, on Latency Difference score. As seen in Figure 6A, the 0 mg/kg group, $M=23.13$, $SEM=5.453$, has significantly lower Latency Difference scores compared to 25 mg/kg groups, $M=106.425$, $SEM=7.562$, $p<.001$, but not quite the 10 mg/kg group, $M=37.632$, $SEM=5.813$, $p=.135$, thus indicating they learned the relationship between CS and US presentations, as well as a preference to goal-track during CS presentations. Individual analyses of Sign and Goal latency scores are necessary to understand if group averages are the result of approach solely to the receptacle and lever or approaching both with a slight preference to approach the sign or goal first.

Low Sign Latency scores indicate rats are quick to approach the CS when it is presented. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=$, $p<.001$, Dose, $F(2,57)=13.264$, $p<.001$, and interaction of Session x Dose, $F(12,342)=2.186$, $p<.05$, on Sign Latency score (Fig. 6B). A Dunnett's test to further assess the main effect of Dose revealed that

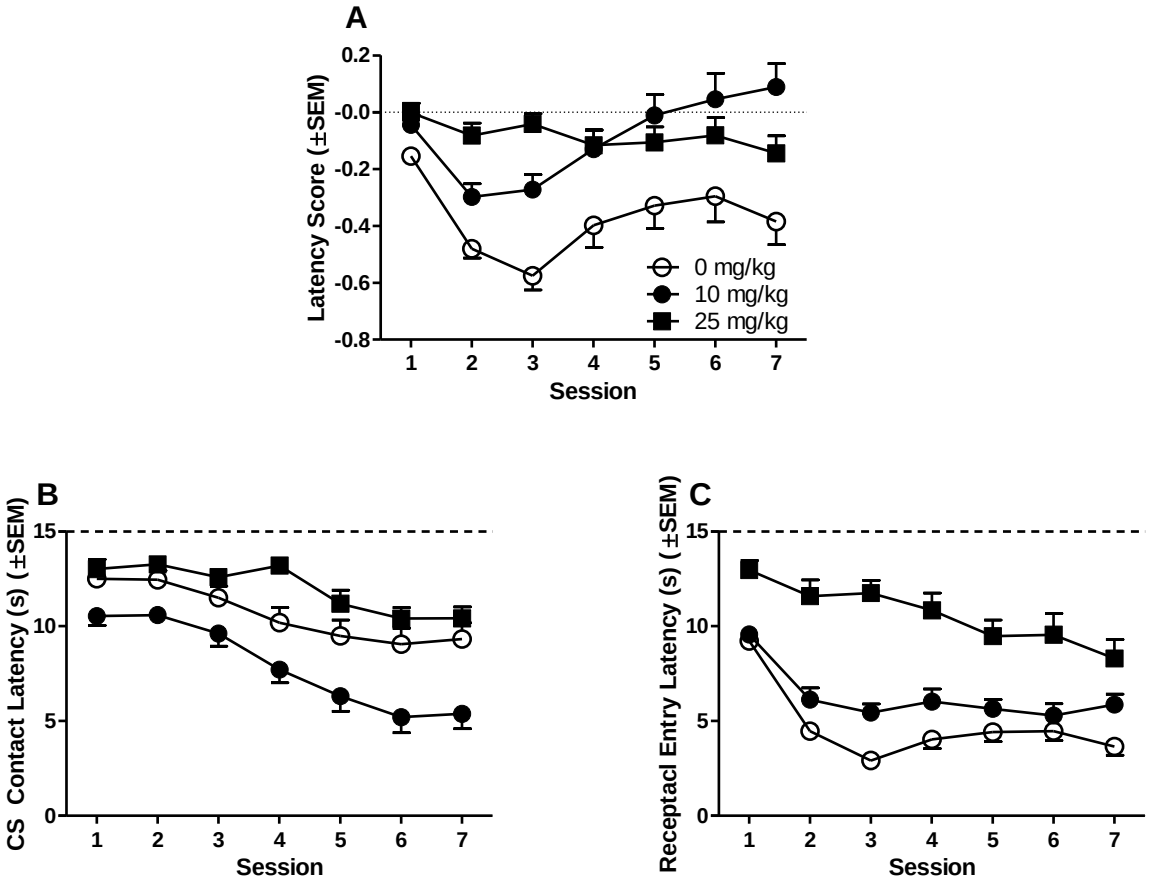

Figure 6: Caffeine exhibits a biphasic effect on latency to approach a conditioned stimulus. Figure A illustrates the Latency Score used to determine PCAI Score. Rats pretreated with 25 mg/kg caffeine had significantly higher Latency Scores than saline controls across conditioning sessions, $p<.001$. Figure B illustrates that rats in the 10 mg/kg group were significantly quicker to approach the sign than the 0 mg/kg group, $p<.001$. Rats in the 25 mg/kg group were not significantly different than the 0 mg/kg group in sign latency, $p=.194$. Figure C illustrates that rats in the 0 mg/kg group were quicker to approach the receptacle during the CS presentation than the 25 mg/kg group, $p<.001$, but no different than rats in the 10 mg/kg group. Taken together, these figures illustrate rats given a moderate dose of caffeine were quicker to sign-track than other groups, but still learned the contingency between CS presentations and the following US presentations. Rats given a high dose of caffeine did not appear motivated to approach the sign or goal during CS presentations.

10 mg/kg, *M*=7.912, *SEM*=0.522, *p*=.001, but not 25 mg/kg, *M*=12.015, *SEM*=0.679, *p*=.194, had significantly lower Sign Latency scores than the 0 mg/kg group, *M*=10.649, *SEM*=0.489. In other words, rats pretreated with 10 mg/kg caffeine were quicker to approach the lever during CS presentation. Rats in the 25 mg/kg group were no different from the 0 mg/kg group, indicating weak or no motivation to approach the lever during CS presentation.

Low Goal Latency scores indicate rats are quick to approach the receptacle when the CS is presented. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=31.239$, $p<.001$, Dose, $F(2,57)=51.846$, $p<.001$, and the interaction of Session x Dose, $F(12,342)=3.568$, $p<.001$, on Goal Latency score (Fig. 6C). Rats in all groups lowered total Goal Latency score across sessions, indicating learning the contingency between CS presentation and sucrose reward. However, the time it took to first approach the receptacle was retarded by caffeine in a dose-dependent manner. A Dunnett's test to further assess the main effect of Dose revealed that 25 mg/kg, *M*=10.643, *SEM*=0.473, *p*<.001, and 10 mg/kg, *M*=6.283, *SEM*=0.363, *p*=.006, had significantly higher Goal latency scores compared to the 0 mg/kg group, *M*=4.741, *SEM*=0.341.

Probability difference score. The Probability difference score [P(sign) - P(goal)] is the difference in probability of entry into the dipper well (P (goal)) subtracted from the probability of contacting the lever (P(sign)). Each CS trial results in a 1 or 0 for sign or goal probability if the rat approaches the lever, receptacle, or not. For example, if a rat contacts the lever in each of the 15 CS trials, the sum of each trial is 15 which is then divided by 15 for a P(sign) of 1. Once the P(goal) is taken and subtracted from P(sign) the outcome is a number ranging from -1 - 1. Low scores represent tendency of a rat to solely goal-track during CS exposure and high scores represent tendency of a rat to solely sign-track during CS exposure. A Mixed ANOVA yielded a

significant main effect for Session, $F(6,342)=4.500$, $p<.001$ (Fig. 7A). No Dose, $F(2,57)=2.312$, $p=.108$, or Session x Dose interaction, $F(12,342)=1.069$, $p=.385$, was detected.

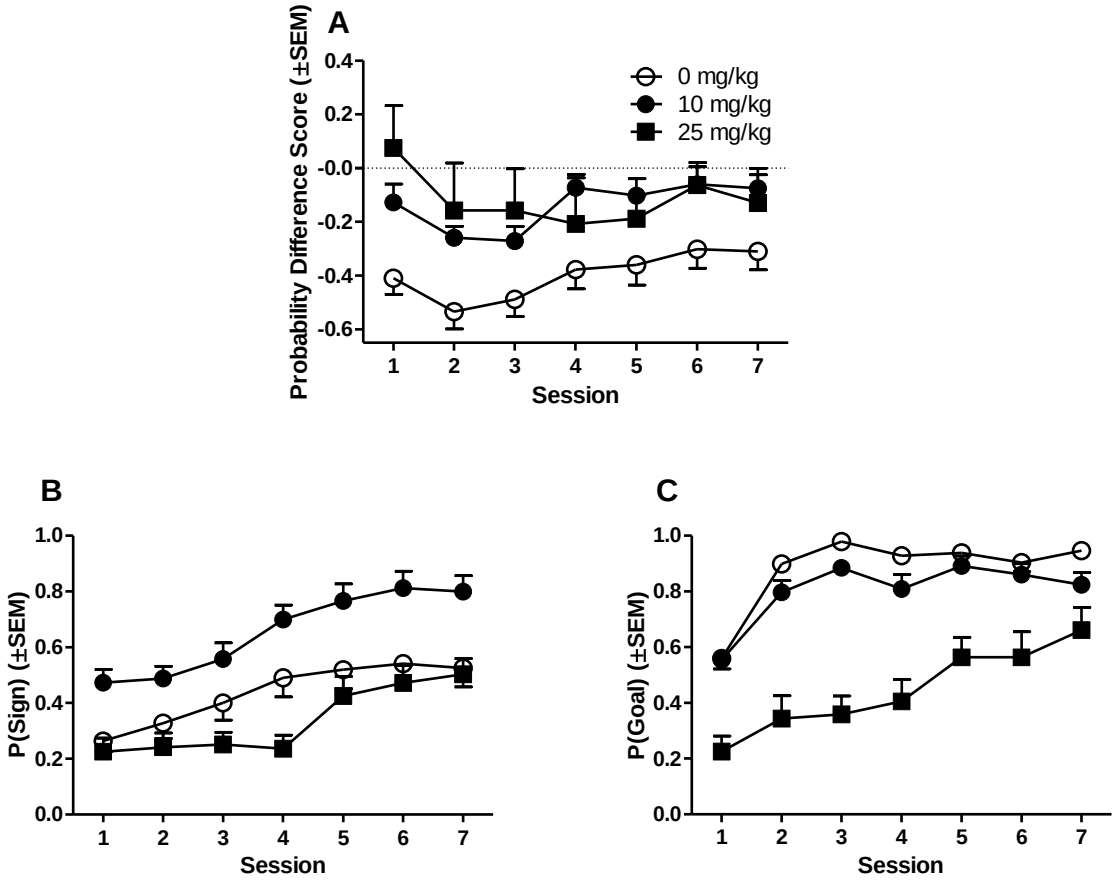

Figure 7: Moderate doses of caffeine increase probability to approach a conditioned stimulus. Figure A illustrates average Probability Difference Scores across all sessions of conditioning. A Mixed ANOVA revealed a main effect for Session, $p<.001$, but not Dose, $p=.108$ on Probability Difference Score. Figure B illustrates average probability of sign contacts across sessions. Dunnett's post hoc analysis revealed 10 mg/kg, $p=.002$, but not 25 mg/kg groups, $p=.288$, had significantly higher P(sign) scores. Zero and 25 mg/kg groups did not approach the sign in more than half of CS trials each session. Rats in the 10 mg/kg group were systematically more likely to contact the lever across sessions, with the highest P(Sign) score during the final session of conditioning. Figure C illustrates average probability of receptacle entries during conditioning. Dunnett's post hoc analysis revealed saline controls were significantly more likely to enter the receptacle than rats treated with 25 mg/kg caffeine, $p<.001$, but no different than rats pretreated with 10 mg/kg caffeine, $p=.091$.

Sign probability scores (P(sign)) range from 0 to 1 indicate how often an animal contacted the lever during CS presentation for each trial. A score of 0 indicates a rat did not contact the lever during any CS presentation, and a score of 1 indicates a rat contacted the lever during every CS presentation. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=25.922$, $p<.001$, and Dose, $F(2,57)=10.84$, $p<.001$, but no interaction between Session and Dose, $F(12,342)=1.279$, $p=.229$, was detected (Fig.7B). A Dunnett's test to further assess the main effect of Dose revealed 10 mg/kg, $M=0.657$, $SEM=0.045$, had significantly higher P(sign) scores ($p=.002$) than the 0 mg/kg group, $M=0.438$, $SEM=0.043$, but there was no difference between 0 mg/kg and 25 mg/kg groups, $M=0.336$, $SEM=0.059$, $p=.288$. As seen in Figure 7B, rats pretreated with 10 mg/kg caffeine were more likely than the 0 mg/kg group to contact the lever during each sessions of conditioning. Although all groups increased lever contacts across sessions, 0 mg/kg and 25 mg/kg groups never contacted the lever in half of CS presentations during any one session.

Goal probability scores (P(goal)) range from 0 to 1 indicating how often a rat entered the receptacle during CS presentation for each trial. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=40.942$, $p<.001$, Dose, $F(2,57)=49.861$, $p<.001$, and a Session x Dose interaction, $F(12,342)=4.172$, $p<.001$), on P(goal) (Fig. 7C). A Dunnett's test to further assess the main effect of Dose revealed that the 0 mg/kg group, $M=0.879$, $SEM=0.026$, had higher P(goal) scores than the 25 mg/kg group, $M=0.446$, $SEM=0.036$, $p<.001$, but were no different from 10 mg/kg group, $M=0.803$, $SEM=0.028$, $p=.091$. As seen in Figure 7C, 0 mg/kg and 10 mg/kg groups were motivated to obtain sucrose with receptacle entries in nearly every CS presentation trial from session 3-7. The 25 mg/kg group learned to approach the receptacle during CS presentations over multiple sessions but never exceeded more than 75% of trials. A

steady but slowed learning curve, in combination with a limit of 0.7 P(goal) indicates that the 25 mg/kg dose reduced motivation to obtain sucrose.

Response bias score. The response bias score reflects the difference between lever contacts and receptacle entries during each CS trial [(total lever contacts - total receptacle entries)/(total lever contacts + total receptacle entries)]. Response bias scores range from -1 - 1 where -1 scores indicate only goal-tracking behavior and +1 indicating only sign-tracking behavior. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=6.673$, $p<.001$, and Dose, $F(2,57)=12.874$, $p<.001$, on response bias score (Fig. 8A). No Session x Dose, $F(12,342)=0.912$, $p=.535$, was detected. A Dunnett's test to further assess the main effect of Dose revealed that 25 mg/kg, $M=0.159$, $SEM=0.102$, $p<.001$, and 10 mg/kg, $M=0.176$, $SEM=0.079$, $p<.001$, had significantly higher Response Bias scores compared to 0 mg/kg, $M=-0.32$, $SEM=0.074$. The Response Bias Score can make it hard to discern the rates of sign- and goal-tracking sign-tracking over the entire session. Hypothetically, rats that touch the lever and enter the receptacle the same amount of times over the session can have the same Response Bias Score regardless of the total contacts and receptacle entries during the session. For this reason, it is important to look at the Sign and Goal Bias scores alone to better understand overall rates of responding.

Sign Bias Score is the sum of lever contacts during CS presentations within a session. Rats that are more likely to sign-track are expected to have higher total lever contacts over a session. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=6.787$, $p<.001$), Dose, $F(2,57)=10.96$, $p<.001$, and a Session x Dose interaction, $F(12,342)=1.831$, $p=.042$, on CS contacts (Fig. 8B). A Dunnett's test to further assess the main effect of Dose revealed that 0 mg/kg, $M=35.669$, $SEM=6.925$, rats had significantly lower lever contacts than

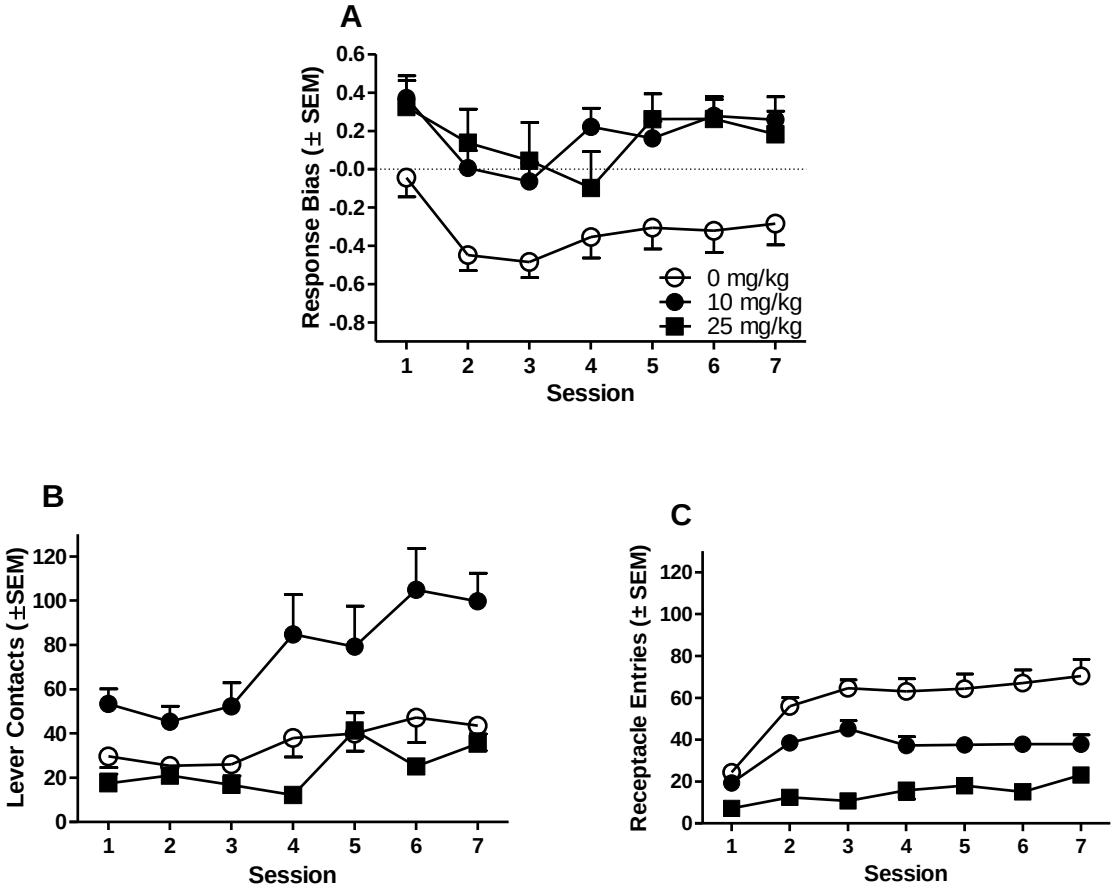

Figure 8: A moderate dose of caffeine elicits bias to a conditioned stimulus. A Mixed ANOVA revealed a main effect for Dose and Session on Response Bias Score, *p's*<.001. A Dunnett's post hoc analysis revealed saline controls had significantly lower Response Bias Scores than subjects treated with 10 mg/kg caffeine and 25 mg/kg caffeine bolus doses prior to the start of each session, *p's*≤.001. Figure B illustrates average lever contacts during CS trials across sessions. Dunnett's post hoc analysis revealed 10 mg/kg, *p*=.001, but not 25 mg/kg, *p*=.537, had significantly more lever contacts. Figure C illustrates average session receptacle entries during conditioning. Dunnett's post hoc analysis revealed saline controls had significantly more receptacle entries than rats treated with 10 and 25 mg/kg caffeine, *p's*≤.001. Taken together, these graphs illustrate caffeine's biphasic effects on sign- and goal-tracking behavior. While 25 mg/kg was not different than 10 mg/kg on Response Bias Scores, the Sign and Goal Bias scores reveal that 25 mg/kg reduced responding towards the sign and goal. These graphs illustrate that a high dose of caffeine may impair motor behavior or reduce motivation to approach a US and the CS. In contrast, 10 mg/kg elicited higher rates of sign and goal-tracking, revealing that rats in this group were motivated to approach both the US and CS.

10 mg/kg, *M*=74.221, *SEM*=7.382, *p*=.001, but was no different from 25 mg/kg, *M*=24.176, *SEM*=9.603, *p*=.537. As seen in Figure 8B, the 10 mg/kg group began with a higher tendency to sign-track which was perpetuated over multiple sessions. Zero and 25 mg/kg groups maintained relatively low rates of CS contacts across sessions.

Goal Bias Score is the sum of receptacle entries during CS presentations in a session. Rats that are more likely to goal-track are expected to have higher total receptacle entries over a session. A Mixed ANOVA yielded significant main effects for Session, $F(6,342)=20.159$, $p<.001$, Dose, $F(2,57)=29.608$, $p<0.001$, and a Session x Dose interaction, $F(12,342)=4.243$, $p<.001$, on receptacle entries (Fig. 8C). A Dunnett's test to further assess the main effect of Dose revealed the 0 mg/kg group, *M*=58.571, *SEM*=3.411, had significantly more receptacle entries than 10 mg/kg, *M*=36.247, *SEM*=3.636, *p*<.001, and 25 mg/kg groups, *M*=14.67, *SEM*=4.731, *p*<.001. As seen in Figure 8C, caffeine retarded receptacle entries across sessions in a dose-dependent manner. Figure 8B and 8C displaying both Sign and Goal Bias Scores illustrate how a high dose of caffeine can disrupt the association between the CS and sucrose presentations, as well as reduce the motivation to obtain sucrose.

Microdialysis Session

PCAI. A One Way ANOVA yielded a non-significant main effect for Dose, *p*>.05, on PCAI during CS presentations (Fig. 9A). Only CS exposed rats were included in the analysis since PCAI could not be calculated for rats that could not sign-track due to the lack of CS presentations.

CS contacts. A 2 x 3 (CS Group x Dose) Two Way ANOVA only found a significant effect of CS Group on CS Contacts, *p*<.05 (Fig. 9B). There was no significant main effect for

Dose or a significant interaction of Dose x CS Group, *p's*>.05. Not surprisingly, CS presentations caused more sign-tracking behavior. When controlling for CS presentations, caffeine dose did not affect sign-tracking behavior when CS-US pairings were extinguished.

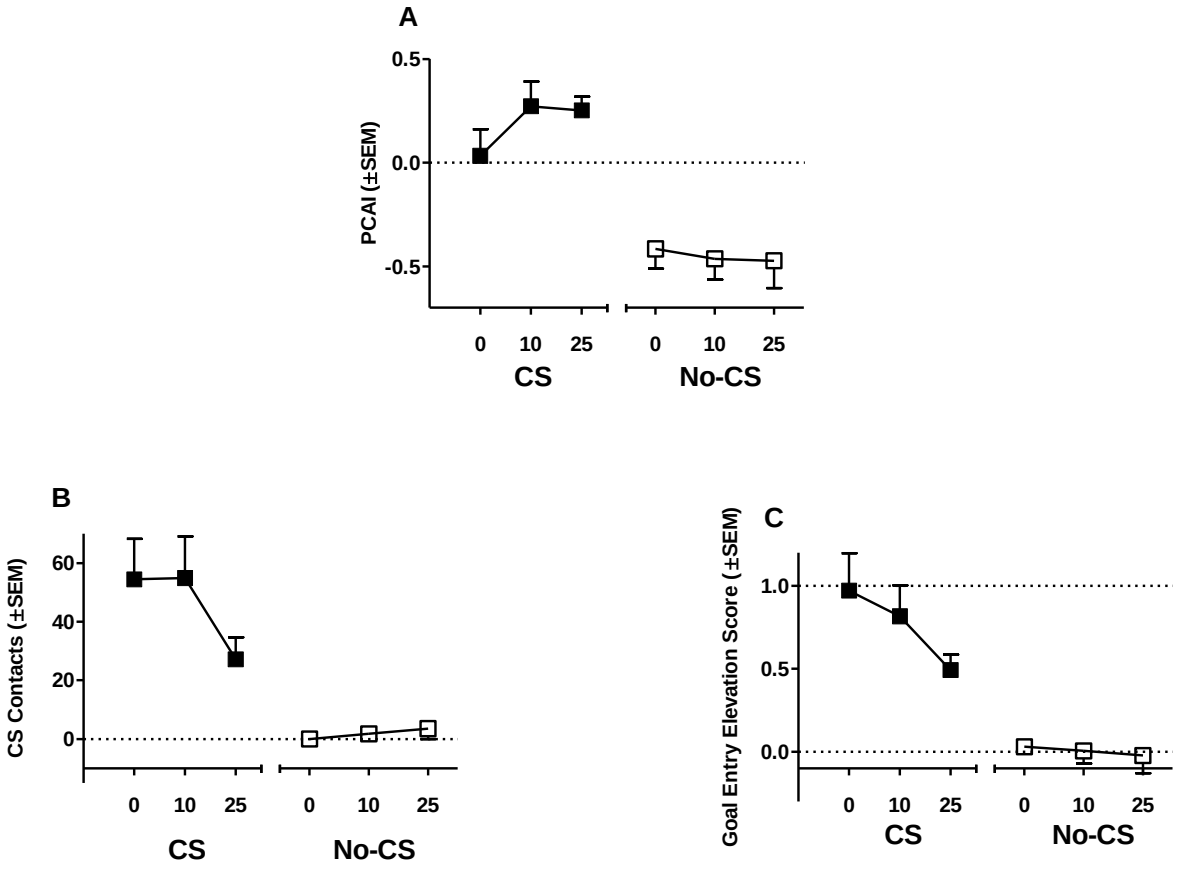

Figure 9: Exposure to a conditioned stimulus evokes sign- and goal-tracking behavior during CS-US extinction. Figure A represents difference in PCAI between CS and No-CS groups during Microdialysis. Filled symbols represent groups who were exposed to CS presentations alone. Open symbols represent groups that received no CS exposure. A One Way ANOVA did not detect an effect of dose on PCAI, *p*>.05. Figure B represents average CS contacts during the CS exposure segment (1 hr) of session. A 2 x 3 (CS Group x Dose) ANOVA revealed a significant effect of CS Group on CS Contacts, *p*<.05. Figure C represents average Goal Entry Elevation Score during CS exposure segment (1 hr) of the session. A 2 x 3 ANOVA revealed a significant effect of CS Group on goal entry elevation score, *p*<.05

Goal entry elevation score. A 2 x 3 (CS Group x Dose) Two Way ANOVA revealed a significant effect of CS Group on goal entry elevation score, $p<.05$ (Fig. 9C). There was no significant main effect for Dose or a significant interaction of Dose x CS Group, p's$>.05$. As expected, CS presentations caused more goal-tracking behavior compared to no CS presentations. However, caffeine did not affect goal-tracking behavior when CS-US pairings were extinguished.

Dopamine dialysate. Caffeine pretreatment increased extracellular dopamine, but only in rats that were tested with the CS. This was confirmed by analyses of Area Under the Curve (AUC) calculated from percent change in DA over 2 sample intervals – samples that were in the brain during the test phase (Test Interval, samples 6-8) and samples that were in the brain during

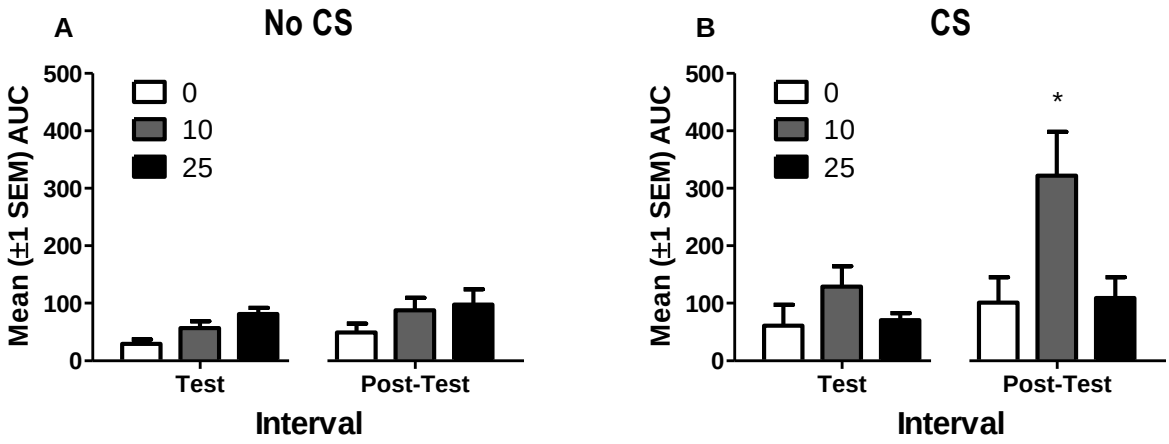

Figure 10: A moderate dose of caffeine enhances sensitivity of accumbal dopamine in response to a conditioned stimulus. Figures A and B represents group average of the area under the curve derived from percent change from baseline dopamine efflux for each sample collected during the Microdialysis session. Figure A represents the group averages for rats in the No-CS groups. Figure B represents the group average for rats in the CS groups. The Test interval represents the average of the three samples collected during CS test exposure (samples 6-8). The Post-Test intervals represent the average of the three samples collected after CS test exposure (samples 9-11). The asterisk (*) represent a significant increase in AUC during the Post-Test compared to the Test interval for rats exposed to 10 mg/kg caffeine, $p<.05$.

the 1 h post test period (Post Test Interval, samples 9-11). A 2 x 3 (Interval x Dose) ANOVA on groups exposed to the CS revealed significant main effects of Interval, $F(1,11)=10.61$, $p=.02$, and Dose, $F(2,11)=4.024$, $p=.048$, as well as an Interval x Dose interaction approaching significance, $F(2,11)=2.68$, $p=.11$ (Fig. 10B). Because there were 3 levels of Dose and a main effect of Interval, pairwise comparisons using Bonferroni's correction examined the main effect of Dose during each interval. The 10 mg/kg group, $M=322$, $SEM=76.1$, showed a significant increase in extracellular DA, relative to the 0 mg/kg group during the Post Test interval, $M=100.9$, $SEM=44.4$, $p<.05$. There were no differences in accumbal dopamine between the 0 mg/kg group and the 25 mg/kg group during the Test and Post Test intervals, p's$>.05$.

The 2 x 3 (Interval x Dose) ANOVA on groups not exposed to the CS revealed a significant effect of Interval, $F(1,18)=6.274$, $p=.02$, a main effect of Dose approaching significance, $F(2,18)=3.248$, $p=.0625$, and no Interval x Dose interaction, $p=.79$, on AUC (Fig. 10A). Pairwise contrasts using Bonferroni's correction did not reveal any significant differences between doses during the Test and Post Test intervals, p's$>.05$.

CHAPTER 4

DISCUSSION

The effects of caffeine on appetitive behaviors are complex. Although it is the most consumed psychoactive substance in the world, it is not a primary reinforcer in preclinical models. This is notable because humans clearly self-administer the drug for the psychoactive effect but no other species will. However, most preclinical models fail to account for the complexity of caffeine self-administration by humans – consumption of the drug in a vehicle containing reinforcing gustatory stimuli. Despite these shortcomings, preclinical research is rife with evidence that caffeine alters motivation for other reinforcers. Caffeine systematically increase behaviors associated with a diverse array of primary rewards including cocaine, amphetamine, sucrose, and visual stimuli (Cauli et al., 2003; Green & Schenk, 2002; Sheppard et al., 2012). As a result, caffeine has been identified as a 'reinforcement enhancer' and this characteristic may play an important role in caffeine use (Sheppard et al., 2012; Sweeney et al., 2016).

Delivery of oral saccharin with intravenous caffeine infusions promoted robust acquisition and maintenance of caffeine self-administration compared to caffeine infusions alone. The peak caffeine doses (0.5-1 mg/kg/infusion) produced approximately three times the amount of active lever presses when they were delivered with saccharin, relative to saccharin alone. Delivering caffeine in an oral vehicle also supported the necessity a primary reinforcer. Rates of caffeine self-administration were highest for groups responding for solutions containing saccharin and low-to-moderate concentrations of caffeine (S+, DS+) when compared to groups receiving solutions containing saccharin alone (S-, DS-), caffeine alone (W+), or neither (W-). Differences between groups for active lever presses and reinforcers earned were observed at a

concentration of 2.5 mg/ml of caffeine. All concentration of caffeine promoted significantly higher responding for saccharin solutions than water alone, with the exception of 7.0 mg/ml.

Caffeine's ability to alter associations between cues and the rewards they predict was examined using a PCA paradigm. A moderate dose of caffeine (10 mg/kg) elicited more sign-tracking across acquisition sessions, as evidenced by faster approach to the CS, higher probability of contacting the CS, and more interaction (contacts) with the CS. Rats who received placebo pretreatments (0 mg/kg) exhibited more 'goal-tracking' relative to the 10 mg/kg caffeine pretreated rats. These rats were quicker to approach the sucrose receptacle and entered the receptacle more often when the CS was presented. A high dose of caffeine (25 mg/kg) appeared to inhibit acquisition of conditioned responding as both sign- and goal-tracking were reduced compared to the 0 and 10 mg/kg caffeine. Although their PCAI did not differ from the 10 mg/kg caffeine group, rats receiving 25 mg/kg were less likely to approach the sign, goal, and had larger latencies to approach the CS and receptacle. Lastly, 10 mg/kg caffeine increased extracellular dopamine evoked by CS presentations during in vivo microdialysis tests. No increase in dopamine efflux was observed at 0 and 25 mg/kg caffeine doses for rats exposed to the CS. These findings are consistent with our hypothesis that caffeine increases the salience of incentives by enhancing or sensitizing the NAc dopamine response to incentive stimuli.

Caffeine Self-Administration

This is the first investigation examining the role of contingently paired primary reinforcers and caffeine in the acquisition and maintenance of caffeine self-administration. The present studies demonstrate caffeine self-administration is caused by the adventitious pairing of the drug with response-contingent vehicle reinforcers. In other words, caffeine does not have to

function as a primary reinforcer to be self-administered, but can promote self-administration by the enhancing the reinforcing effects of non-drug rewards (Sheppard et al., 2012), such as those included in the caffeine vehicle (present studies). It is surprising that no primary reinforcing effect of a drug is necessary to potentiate self-administration to high levels. Instead, the drug strengthens operant behavior by increasing the motivation to obtain saccharin. This matches the majority of human caffeine consumption where the drug is consumed in a beverage, typically coffee and energy drinks, which has created a subset of habitual users (Reissig, Strain, & Griffiths, 2009). One question that arises from this hypothesis is mechanistic - how does caffeine promote vehicle reinforcement without an established primary reinforcing effect? The self-administration data from these studies suggest this effect is achieved through the pharmacokinetic actions of caffeine. The pharmacokinetic effects of caffeine, specifically its long lifespan, make it more suited to a moderating role on non-drug reinforcement.

Pharmacokinetics of caffeine

Traditional 'primary reinforcement' views of drug self-administration posit that a time-sensitive pairing between a well-defined operant response (e.g., pressing a lever) and the pharmacological effect of the drug is necessary for associative learning (Wise, 1987; Wise & Koob, 2014). This theory emphasizes the speed at which the drug reaches its targeted site of action in the brain is important to drug reinforcement and abuse liability (Bouayad-Gervais, Minogianis, Lévesque, & Samaha, 2014). However, drugs which are consumed orally, namely alcohol and caffeine, have a significant delay between initial consumption and the pharmacological effect (Latini, Tognoni, Young, & Garattini, 1984; Sher, 1985). Delays between consumption and subjective drug effect make the pharmacological effects of the drug more contextual and less discrete. Instead, the immediacy of the chemosensory effects of the beverage

is likely reinforcing the operant response while the drug functions as a contextual stimulus or moderator. On one hand this explains most human caffeine consumption and the findings from Experiment 2, yet it does not explain why intravenous caffeine is not self-administered alone in Experiment 1 or previous literature (Atkinson & Enslen, 1976; Griffiths et al., 1979).

Intravenous caffeine rapidly reaches the brain and, therefore, is presumably associated with the operant response (Wise & Koob, 2014). Unlike other intravenously self-administered drugs, caffeine is eliminated slowly, with a half-life between 60-90 minutes in the rat (Latini et al., 1980; Smith, Ma, & Lau, 1999). This lengthy availability of caffeine at targeted receptor sites may make the pharmacological effects more contextual and less discrete than the saccharin presented with each infusion. Intravenous caffeine infusions are only made salient by the drug's interoceptive cues and a slight auditory cue of the syringe pump. On the other hand, saccharin presentations are accompanied by the auditory cue of the dipper, visual cue of dipper movement and eventual sensory cues of the flavor during consumption. Therefore, it is reasonable to assume lever presses are readily associated with presentations of saccharin and the accompanying, salient stimuli. As sessions progress, the association between the lever and saccharin are strengthened by multiple pairings, with caffeine moderating the association. Rapid acquisition of oral and intravenous self-administration provides compelling evidence for this. However, differences between the intravenous dose-response curve and the oral concentration curve demand further scrutiny.

Comparing-Contrasting Intravenous and Oral Caffeine Self-Administration

Experiment 1 provided proof-of-concept for an animal model of caffeine self-administration. Experiment 2 added ecological validity to this paradigm by modeling typical

human caffeine use. A preclinical model of oral caffeine self-administration allows researchers to better understand the factors that influence human caffeine use. Factors such as taste, smell, intestinal drug absorption and corresponding caffeine distribution are all potentially important factors that are unique to an oral self-administration. For instance, caffeine has a potent bitter taste which may negatively influence drug consumption. Despite using a concentration larger than most commercially available caffeinated beverages, rats reliably acquired high rates of responding for caffeinated vehicles containing saccharin. Furthermore, the use of decaffeinated coffee as a masking agent for the bitter taste of caffeine was unnecessary since S+ and DS+ groups acquired self-administration at similar rates. Rats in these groups responded at higher rates across acquisition and concentration exploration. Continued use of caffeine despite the negative consequence of bitter flavor offers compelling evidence that this preclinical model is a useful tool for investigation of caffeine abuse and dependence. Despite validating the intravenous model of caffeine self-administration, Experiment 2 revealed significant differences in the relationship between caffeine dose or, concentration, and the rate of reinforcers earned.

The dose response curve for intravenous caffeine self-administration illustrate the boundaries to which caffeine is effective at promoting self-administration when paired with saccharin compared to saccharin and caffeine control groups. Intravenous infusions of .125 mg/kg are too low to achieve a psychoactive dose and only a few infusions of 4 mg/kg are needed to achieve satiation. The concentration curve for oral self-administration does not provide similar evidence for the lower and upper concentrations of caffeine necessary to reduce motivation to levels equivalent to control groups. In addition to route of administration, the ability to precisely control caffeine dose could be responsible for these differences. In Experiment 2 a liquid dipper delivered 0.1 ml of the assigned caffeinated solution upon meeting

the ratio requirement. The dipper remained available for the rat to freely consume the solution until the next response ratio was met. Free access to solutions allowed rats to titrate their caffeine intake in a manner similar to human caffeine consumption. Hypothetically, rats could be achieving similar doses of caffeine with different concentrated solutions by adjusting how often and how much solution they consume. This ability to precisely control caffeine dose was not available in our intravenous model. Rats could adjust their dose only at the assigned increment of infusion. A quick elevation of dose in which the drug rapidly reaches the targeted receptor site within the CNS could explain why infusions of 4 mg/kg produced low levels of responding. These stark differences between groups receiving saccharin with 4.0 mg/kg intravenous caffeine or 7.0 mg/ml oral caffeine illustrate the importance for an ecologically valid model of human caffeine use. By mimicking human caffeine use, this model integrates factors that may have been previously overlooked by researchers such as precise control of caffeine dose. Animal models of oral caffeine self-administration will prove to be a useful tool for future research investigating the significant factors that influence initiation and maintenance of caffeine use.

Caffeine and Incentive Salience

Experiments 1 and 2 provide empirical evidence that tandem presentations of caffeine with reinforcing gustatory stimuli perpetuate self-administration. These studies explain why caffeine is so readily used and abused, but they do not explain how caffeine increases motivation to obtain reinforcers and perpetuate caffeine use. Based on prior research we know caffeine enhances operant responding for drug and non-drug reinforcers (unconditioned reinforcers), as well as establish non-drug stimuli as conditioned reinforcers (Yeomans, et al., 2005; Yeomans et al., 2007). In other words, caffeine consumption reflects a complex interaction between drug and non-drug stimuli (Fedorchak, Mesita, Plater, & Brougham, 2002; Myers & Izbicki, 2006;

Yeomans, Javaherian, Tovey, & Stafford, 2005). However, it is unclear whether caffeine interacts with the motivational properties of the reward, reward-associated cues, or both.

The PCA paradigm investigates the incentive salience of rewards and associated cues by quantifying approach behavior evoked by both stimuli. In Experiment 3 we discovered a moderate dose of caffeine enhanced sign-tracking behavior. Importantly, 10 mg/kg caffeine reduced goal tracking (approach to the sucrose receptacle, Figure 8C) but increased sign-tracking (contacts with the lever, Figure 8B) across testing sessions. The more the CS predicted the reward, the more that caffeine enhanced the motivational properties of that CS. In other words, caffeine enhanced the incentive salience of the CS with each CS-US pairing and, in turn, elicited more approach behavior.

Another important finding is the effect of the high dose of caffeine on conditioned approach behavior. Surprisingly, 25 mg/kg of caffeine did not enhance incentive salience of the CS more than the 10 mg/kg dose. Instead, the high dose of caffeine inhibited sign and goal-tracking behavior compared to saline and 10 mg/kg caffeine. This high dose was too low to impair motor behavior, but some human research may explain why 25 mg/kg caffeine impaired learning. At high doses caffeine impairs performance in working memory tasks in human volunteers (Kaplan et al., 1997). This may explain why rats receiving a high dose of caffeine were never more than 50% likely to approach the sign during CS trials in the final acquisition sessions. Alternatively, the psychomotor stimulant effects of caffeine could have reduced the likelihood that rats were near the lever and light during acquisition of conditioned approach. However, this explanation seems unlikely because there was no ambient light in the chamber during testing – the illumination of the stimulus light above the lever would have been the only light in the apparatus during CS presentations, and the rats should have been able to detect it

from anywhere in the chamber. Finally, the anorectic effects of caffeine may have reduced the impact of the sucrose US. This seems unlikely because small quantities (0.1 ml), with presumably mild post-ingestive effects, were delivered on each trial. Future research is necessary to confirm the behavioral impairment observed with 25 mg/kg caffeine pretreatments.

The enhanced salience of CSs by a moderate dose of caffeine provides an explanatory framework for how the drug functions as a 'reinforcement enhancer' even though perception of the reward (sucrose) is apparently unchanged. Incentive sensitization is a common feature of popular drugs of abuse. Common illicit substances, such as cocaine and amphetamine, perpetuate substance use by inducing cravings which are triggered by the cues that commonly predict drug use (Robinson & Berridge, 1993). Through multiple pairings with the drug, these cues acquire motivational properties. It is theorized that the mesolimbic dopamine system, the final common pathway for drugs of abuse, becomes sensitive to these drug-predictive cues and is responsible for elicited drug cravings (Robinson & Berridge, 1993). Experiment 3 provides evidence that caffeine enhances approach to cues that predict reward delivery. For example, this may explain why habitual caffeine consumers have beverage preferences (Reissig et al., 2009). Tastes and smells that were once novel, such as coffee or energy drink flavors, now are powerful reinforcers that promote coffee consumption because of previous pairings with sugar, cream, or even social activity.

Caffeine Enhances CS-Elicited Dopamine Release in the Nucleus Accumbens

Past findings indicate that sensitivity of dopamine efflux is responsible for the attribution of incentive salience (Flagel & Robinson, 2017). In support of previous research, our results suggest a moderate dose of caffeine enhances the ability of a reward-predictive cue to elicit

dopamine in the NAc. Aggregate increases in dopamine were only seen after repeated CS exposure for rats treated with an acute dose of 10 mg/kg caffeine. Two critical comparisons were necessary to ensure that dopamine release was not caused by the CS or caffeine alone. First, a saline group exposed to the CS did not exhibit changes in dopamine during CS exposure (Test Interval) or afterwards (Post Test Interval). The CS was not enough to elicit increases in accumbal dopamine efflux during either interval. Next, a 10 mg/kg caffeine group with no CS exposure did not change dopamine efflux during either interval. The moderate dose of caffeine was not enough to elicit increases in accumbal dopamine efflux. Instead, only the 10 mg/kg group exposed to the CS exhibited increases in dopamine efflux during the Post Test Interval. These findings correlate with the behavioral data from PCA acquisition and provide further evidence that NAc dopamine mediates incentive salience.

Previous research using fast scan cyclic voltammetry (FSCV) to record NAc dopamine in real time suggest reward-predictive cues elicit immediate, phasic bursts of dopaminergic activity (Sunsay & Rebec, 2014). Despite these findings, we did not observe significant changes in dopamine efflux until the hour following repeated CS exposure. These seemingly opposing findings can be attributed to differences in methods. First, microdialysis provides low temporal resolution of dopamine efflux. A 20 minute temporal resolution limits the acuity to which we can observe significant changes in dopamine. In other words, low resolution does not allow us to observe changes in dopamine efflux during each CS trial. Instead, aggregate changes in surrounding neurons are necessary to detect differences in dopamine efflux. Noticeable changes may take time for a drug that indirectly promotes dopamine efflux like caffeine. Caffeine does not induce phasic dopamine response alone and may promote tonic rises in dopamine in the

reward pathway during the drug's long half-life. Additionally, the removal of sucrose after CS presentations could have affected the time course of dopamine elevation.

Pavlovian extinction between a CS and US decreases the amplitude of dopamine signal elicited by the CS (Sunsay & Rebec, 2014). For that reason, it is likely that CS-elicited dopamine decreased with each exposure to the CS in our study. Reduction of dopamine signal amplitude may explain why we were not able to observe dopamine changes during the Test interval for the saline-CS and 10 mg/kg-CS groups. While using an extinction procedure may have reduced caffeine's ability to promote dopamine activation elicited by the CS, sucrose presentations may have influenced dopamine efflux in all groups and masked any differences we could observe between groups. Taken together, the low temporal resolution of microdialysis combined with CS-US extinction may explain the delayed dopamine efflux after CS exposure in the 10 mg/kg caffeine group. These data indicate that small elevations in dopamine efflux caused by CS exposure, and perpetuated by caffeine, lead to aggregate elevations in tonic dopamine efflux.

In addition to group differences between saline and 10 mg/kg caffeine groups, dopamine recordings from the 25 mg/kg caffeine groups help explain the disruption in associative learning seen during acquisition. Groups exposed to a high dose of caffeine did not exhibit any overall changes in dopamine between the Test and Post Test Intervals. The inhibition of dopamine sensitivity to CS exposure provides further evidence that a high dose of caffeine inhibits learning. As previously discussed, high doses of caffeine inhibit working memory performance in human volunteers. Our data suggest that disrupted learning caused by high doses of caffeine may be caused by a disruption of the mesolimbic system’s ability to encode associations between stimuli. This may inhibit word recall in humans or inhibit incentive attribution to cues predictive of reward in rodents.

Future Directions

Caffeine Self-Administration

Experiments 1 and 2 provided the proof-of-concept and general parameters for a robust preclinical model of caffeine self-administration. Experiment 1 explored the range of doses at which intravenous caffeine promoted self-administration when paired with the presentation of liquid saccharin. Experiment 2 validated the preclinical model while establishing the parameters at which caffeine concentration promoted oral self-administration for vehicles containing saccharin and caffeine. Due to the novelty of this paradigm, many questions regarding factors influencing caffeine self-administration went unanswered. One important manipulation for future research will be the removal of caffeine. As we saw in Experiment 3 and previous research, caffeine alters incentive salience of reward-related cues. In accordance with our sign-tracking data, it is likely that previous caffeine exposure enhances the incentive salience of the active lever and would likely promote continued approach despite removal of contingent caffeine. Researchers could explore the degree to which groups self-administering caffeine will continue to lever press for saccharin once the drug is removed. This alteration in reward outcome could be a useful indicator for preclinical researchers to explore abuse liability. Continued elevation in lever pressing could be interpreted as sustained drug-seeking despite removal of caffeine.

A second manipulation for future researchers could be the alteration of caffeine's ability to antagonize adenosine receptor sites. Caffeine antagonizes adenosine A_1 and A_{2A} receptors via competitive binding and indirectly enhances constitutive dopamine receptor activity (Ferré, 2008). We have hypothesized that this indirect enhancement of dopamine efflux is responsible for caffeine self-administration. To confirm this hypothesis, future research could systematically

explore the relationship between selective adenosine receptor antagonism and caffeine self-administration. In addition, adenosine agonists and adenosine kinase inhibitors could be used to understand how endogenous adenosine affects caffeine self-administration. Hypothetically, enhanced adenosine could blunt the effect of caffeine by displacing it from the A_1 and A_{2A} receptor site. If this were true, adenosine agonists could potentially shift the dose-response curve to the right or reduce the amplitude of the curve for caffeine self-administration behavior.

Pavlovian Conditioned Approach

Experiment 3 confirmed that a moderate dose of caffeine enhanced the incentive salience of a reward-predictive cue. A dose of 10 mg/kg caffeine enhanced sign-tracking behavior that was not seen at the 0 and 25 mg/kg dose. While we were able to make some general conclusions, further exploration is needed to understand the dose parameters at which caffeine enhances incentive salience, understand why 25 mg/kg reduce performance in this paradigm, and understand what the long-term effects are of caffeine on incentive salience. It appears a dose of 25 mg/kg of caffeine is enough to define the upper bound of caffeine's effectiveness to promote sign-tracking behavior. Nonetheless, our limited dose exploration provides a narrow scope to understanding the relationship between caffeine and incentive salience at lower doses. It is possible that lower doses of caffeine can enhance sign-tracking behavior but research exploring differences in dose between groups of rats is necessary.

There are two possible reasons 25 mg/kg caffeine did not facilitate sign-tracking behavior: the high dose of caffeine interfered with the ability to learn the association between the CS and US, or it impaired motor performance. Based on our dopamine data and research on working memory tasks with human volunteers, it appears that the high dose of caffeine impaired

the ability to learn the predictive relationship between the CS and US (Kaplan et al., 1997). To verify this hypothesis, future studies could conduct a 'probe' session after acquisition. During this 'probe' session, rats previously given 25 mg/kg caffeine could be give sham injections with saline. If caffeine impaired learning, these rats should have conditioned approach behavior comparable to the first day of acquisition for a saline control group. If caffeine impaired performance, conditioned approach to the CS during the probe session should be equivalent to rats assigned to a moderate dose of caffeine.

Another avenue for future research is explorations into the long-term effects of caffeine on incentive salience. Results from similar research with nicotine show persistent sign-track after drug removal suggesting nicotine permanently alters incentive motivation (Palmatier et al., 2012). In other words, once nicotine enhanced the incentive salience of a reward-predictive cue, the drug was no longer needed for the cue to facilitate conditioned approach. Since caffeine and nicotine both promote sign-tracking, we hypothesize that sign-tracking would persist following caffeine removal. Data from habitual users also supports this hypothesis. Habitual consumers of energy drinks have strong beverage preferences which suggests caffeine may permanently alter incentive motivation of reward-associated flavors (Reissig et al., 2009). If preclinical researchers can illustrate the intractable nature of these cues to elicit approach behavior after caffeine exposure, this could inform clinicians on the importance of caffeine-associated to induce use after drug cessation.

Dopamine

Microdialysis data from Experiment 3 suggest that caffeine promotes dopamine efflux in response to conditioned cues. We hypothesize that this alteration in dopamine, while slow, is

evidence to support the mechanism by which caffeine promotes incentive salience to reward-predictive cues. Although we can speculate, empirical evidence is needed to better illustrate the interaction between caffeine, incentive cues, and rewards. For example, we removed sucrose from microdialysis testing to prevent rises in dopamine occurring across all groups and masking any smaller magnitude changes caused by caffeine's interaction with the CS. However, this precaution is only speculative and future research with CS-US pairings is necessary to confirm this hypothesis.

The low temporal resolution of dopamine efflux afforded by microdialysis recordings leaves speculation for the mechanism by which caffeine promotes dopamine in response to the CS. Large, observable changes in dopamine efflux did not occur until the hour following CS presentations which suggest that caffeine either has no immediate impact on CS-evoked dopamine efflux or produces such small changes in dopamine response that more sensitive tools are necessary. Techniques for brain activity recordings with better temporal resolution could provide valuable information to understanding the caffeine-CS-US relationship. One technique, fast scan cyclic voltammetry (FSCV) provides sub-second recordings of transient dopamine signal at the level of individual or small groups of dopamine-firing neurons. In vivo recordings using FSCV would allow researchers to determine if caffeine alters the amplitude of dopamine signal evoked by CS presentations in a PCA paradigm. This data would provide the most compelling evidence that caffeine mediates dopamine sensitivity to incentive stimuli.

Limitations

The current experiments are the first to establish a robust preclinical model for the acquisition and maintenance of caffeine self-administration. Tandem delivery of caffeine with

saccharin, a gustatory reinforcer, enhances motivation to obtain saccharin while perpetuating caffeine self-administration. We hypothesized that this effect is caused by caffeine's ability to enhance incentive salience. While intravenous caffeine self-administration offered the strongest support for this hypothesis, our interpretations of the oral model are limited. First, we are unable to determine caffeine dose since rats were not required to drink any or all of the earned solution in between reinforcer presentations. Analysis of receptacle entries after dipper presentations may offer better indication of consummatory behavior. Second, a clear and systemic relationship was observed between oral caffeine dose and inactive lever presses. Although rats receiving saccharin and caffeine solutions (S+, DS+) were able to distinguish between active and inactive levers, rats were more likely to press the inactive lever when reinforcers had higher concentrations of caffeine. As previously discussed, one reason for this could be the method for session length. Once the ratio requirement was above what rats were willing to work, rats had to wait thirty minutes before the session would end. This extended session length leaves more time for rats to explore the chamber and respond on the inactive lever. Definitive session lengths, like those in Experiment 1, may eliminate this effect.

Dopamine efflux recorded via microdialysis in Experiment 3 increased when rats were pretreated with a moderate dose of caffeine and exposed to a CS. Unexpectedly, this change in dopamine was not observed until the final hour of dialysate collection when nothing was occurring in the operant chamber. In fact, dopamine efflux continued to increase with each sample collected after CS exposure. While we cannot be sure what caused this late surge in dopamine, comparisons to our other groups indicate the experiment-administered caffeine and CS exposure were responsible for this change. Since no sucrose was presented after the CS during microdialysis, it is possible that CS-US extinction was responsible for a lack of dopamine

changed observed earlier in the session (Sunsay & Rebec, 2014). Future research using electrophysiological recording will provide a better understanding of caffeine's influence on dopamine signal in response to rewards and incentive cues.

Conclusion

The current series of experiments are the first to show reliable and robust caffeine self-administration in a preclinical paradigm when the drug is delivered in tandem with a gustatory reinforcer. Moreover, the effects of tandem saccharin-caffeine delivery generalize to both oral and intravenous models of self-administration. In addition, caffeine enhances incentive salience of cues that predict rewards and promotes sign-tracking behavior in a pavlovian conditioned approach paradigm. This promotion of incentive salience and corresponding accumbal dopamine efflux may explain the acquisition and maintenance of caffeine self-administration. In combination with research outlining caffeine's reinforcement enhancing properties (Sheppard et al., 2012), our models of self-administration, pavlovian conditioned approach, and accumbal dopamine efflux indicate that caffeine may be better described as an 'incentive amplifier' like nicotine (Bevins & Palmatier, 2004; Palmatier et al., 2013, 2012). These results suggest that caffeine's incentive amplifying effects may perpetuate self-administration to potentially dangerous levels when heavy concentrations of the drug are placed in vehicles containing salient gustatory reinforcers such as coffee and energy drinks. Finally, these studies highlight the need for additional research to understand caffeine's ability to sensitize the mesolimbic dopamine system, the 'final common pathway' for the reinforcing effect of abused drugs.

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