Evaluating the Role of Dopamine in Caffeine-Induced Increases in Alcohol Consumption

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by
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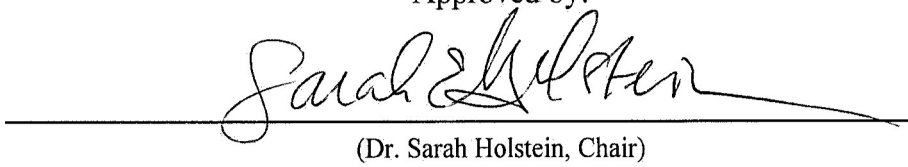

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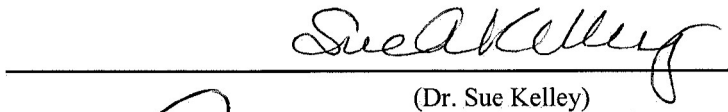

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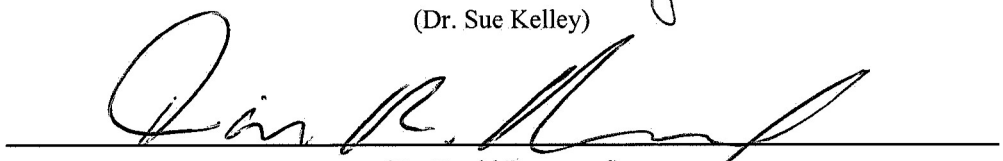

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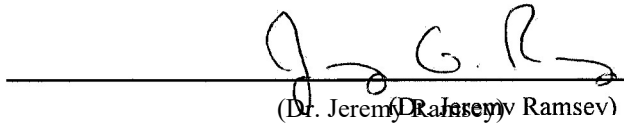

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Abstract

The high prevalence of alcohol mixed energy drink (AmED) use among young adults is concerning, given studies that demonstrate a greater alcohol intake when caffeine is combined with alcohol, as compared to alcohol alone. Previous research suggests caffeine may enhance the sense of reward experienced with alcohol, leading to an increase in alcohol intake. One possible mechanism of action by which caffeine increases alcohol intake is by enhancing dopamine signaling through its actions at the A2A-D2 receptor complex located on striatopallidal neurons. Caffeine's antagonistic actions at the adenosine A2A receptor may increase signaling at the dopamine D2 receptor, decreasing the activity of striatopallidal neurons, thereby increasing drug-seeking behavior. The purpose of the current study was to investigate the possible role of the A2A-D2 receptor complex in caffeine-induced increases in alcohol consumption. In a series of four experiments, male Long Evans rats (n = 8) completed operant self-administration sessions for a sweetened alcohol solution on both fixed and progressive ratio schedules following treatment with caffeine, eticlopride (a selective D2 receptor antagonist), or both. Moderate doses of caffeine (5 and 10 mg/kg) significantly increased operant responding for a sweetened alcohol solution on both fixed and progressive ratio schedules of reinforcement, suggesting that caffeine increases alcohol intake and the reinforcing efficacy of alcohol. However, there was no significant interaction of caffeine and eticlopride, suggesting that altered signaling at the A2A-D2 receptor complex may not be a significant contributor to caffeine's ability to increase alcohol intake.

Evaluating the Role of Dopamine in Caffeine-Induced Increases in Alcohol Consumption

The combined use of caffeine and alcohol has become a public health concern in recent years. Particularly in western culture, there is a high rate of consumption of alcohol mixed with energy drinks (AmED), especially among college-aged students (Attwood, Rogers, Ataya, Adams, & Munafò, 2012; Marczinski, 2014). Results of the national Monitoring the Future study found that prevalence of AmED use was highest among those 21 to 22 years old, with 43.5% of that age group reporting the use of AmED at least once within the past year (Schulenberg et al., 2017). These results support the finding that AmED are most likely to be consumed by younger, less experienced, or underage drinkers (Marczinski, 2015). The dangers of AmED use became apparent following the investigations of caffeine- or energy drink-related emergency room visits, which showed a small but notable percentage of cases (6.3%) involved alcohol use (Nordt et al., 2012). In response to this public health danger, the FDA declared in 2010 that it was illegal to sell pre-mixed drinks containing alcohol and caffeine because caffeine was considered an unsafe additive (Attwood, 2012). However, despite this ban on premixed drinks, AmED use continues to rise among young adult drinkers, with a 2.2% increase in rate of use between 2016 and 2017 for those 19 to 28 years old. (Schulenberg et al., 2017). Since energy drinks and alcohol can still be obtained separately and then mixed, this public health issue appears to be perpetuated (Marczinski, 2015).

Consuming AmED is also a concern because it can be considered a type of high-risk drinking. Because a large portion of AmED drinkers are younger, Marczinski (2015) argues that this population is more susceptible to the combined effects of caffeine and alcohol since they are already at a higher risk for negative effects from alcohol alone due to their inexperience with drinking. Several studies have found that AmED use is associated with alcohol-related risks.

Participants who reported drinking AmED for "hedonistic," or pleasure-seeking, motives had a higher risk for alcohol dependence, injury, and aggressive behavior (Droste et al., 2014). Self-reports among college students indicate that those who consumed AmED reported consuming significantly more drinks per drinking session and a greater number of days on which they drank (Arria et al., 2010; O'Brien, McCoy, Rhodes, Wagoner, & Wolfson, 2008). Likewise, bar patrons who consumed AmED were three times more likely than non-AmED drinkers to leave the bar with a breath alcohol consumption greater than 0.08%, the legal driving limit in the United States (Thombs et al., 2010). According to Marczinski (2015), the use of AmED and its association with heavy drinking may potentially be a risk factor for later alcohol dependence or addiction.

In addition to promoting a higher level of alcohol consumption, several studies have found that AmED use is directly associated with high-risk behaviors. For example, Thombs et al. (2010) found that bar patrons who consumed AmED were significantly more likely to indicate that they planned on driving home after leaving the bar as compared to those who did not drink AmED. Additionally, it has been found that college students who reported drinking AmED also reported an increased number of alcohol-related consequences, such as injury, getting taken advantage of sexually, or driving while intoxicated (O'Brien et al., 2008).

The high prevalence of AmED use and its association with risk behaviors is of particular importance when considering public health, especially that of young adults. However, in order to understand the full scope of this public health issue, one must first investigate why it occurs. Because it has been observed that moderate to high doses of caffeine consumed with alcohol are associated with higher levels of alcohol intake and emergency medical visits (Arria et al., 2010, O'Brien et al., 2008; Marczinski, 2014; Thombs et al., 2010), the next logical area of research is

the mechanisms by which caffeine increases alcohol intake. There are currently two hypotheses that may explain why caffeine increases alcohol drinking – that caffeine increases alcohol drinking by masking the sedative effects of alcohol, or that it enhances the reinforcing efficacy of alcohol. A more complete understanding of how caffeine affects alcohol intake and alcohol-related behavior is necessary to comprehend and combat the public health issue associated with AmED use.

Caffeine Potentially Masks the Sedative Effects of Alcohol

Caffeine is primarily conceptualized as a stimulant drug. However, caffeine has a biphasic dose-response curve, meaning that low doses of caffeine cause stimulation while higher doses have depressant-like or aversive effects (Daly & Fredholm, 1998). Stimulatory doses of caffeine can elicit many behavioral and physiological effects, including locomotor stimulation, tachycardia, and increased feelings of being awake and alert (Cappelletti, Daria, Sani, & Aromatario, 2015; Daly & Fredholm, 1998). These stimulant actions might promote alcohol drinking behavior.

There has been some support for the hypothesis that caffeine, due to its stimulant properties, obscures the depressant or sedative effects of alcohol experienced by alcohol drinkers (Marczinski, 2011). This masking effect essentially keeps the drinker less aware of his or her alcohol intake because the sedative effects of alcohol often act as a cue to stop drinking (Martin, Earleywine, Musty, Perrine, & Swift, 1993; Newlin & Thomson, 1990). Without a cue to stop, the caffeinated drinker continues consuming without realizing how intoxicated they are, potentially leading to higher or dangerous levels of alcohol intake (King, de Wit, McNamara, & Cao, 2011). The hypothesis that caffeine may diminish alcohol's sedative effects is supported by previous research. O'Brien et al. (2008) found that participants self-reported mixing energy

drinks with alcohol so that they would not feel as drunk. Additionally, Ferreira, de Mello, Pompéia, and de Souza-Formigoni (2006) found that participants who consumed AmED had decreased subjective ratings of intoxication. Both these findings imply that caffeine is preventing the awareness of the sedative effects that usually accompany high doses of alcohol.

The possibility that caffeine may mask the sedative effects of alcohol is also supported by research that demonstrates caffeine's ability to attenuate alcohol-related deficits in performance. Animal studies have found that doses of caffeine below 20 mg/kg decrease alcohol-induced motor incoordination on an accelerating rotorod task, a measure of endurance and motor coordination (Connole, Harkin, & Maginn, 2004; Dar, 1988). Marczinski, Fillmore, Bardgett, and Howard (2011) found that participants who consumed AmED versus alcohol alone experienced a reversal of an alcohol-related decrease in performance on a go/no-go task. This task measured inhibitory control of motor reaction time by requiring participants to press a key on a keyboard in response to specified visual cues (in this case, a green rectangle), while refraining from responding to other visual cues (a blue rectangle). The depressant effects of alcohol decreased performance on this task by slowing reaction time, but the group of participants who consumed AmED were less affected by alcohol's slowing of reaction time. The results of both human and animal research suggest that some of the depressant effects of alcohol may be counteracted by caffeine. Marczinski et al. (2011) propose that caffeine may broadly counteract or mask the depressant effects of alcohol to include aspects such as task performance, in addition to the perceived decrease in sedation found in previous studies (e.g., Ferreira et al., 2006; O'Brien et al., 2008). By masking the interoceptive sedative cues experienced with alcohol intake, caffeine may promote continued drinking behavior beyond the typical point at which the drinker would cease consumption (King et al., 2011; Marczinski et al., 2011).

The drinker's perceived stimulation may also be an important factor contributing to caffeine's increase of alcohol drinking, as several studies have found that perceived stimulation increases after the two drugs are combined. For instance, Attwood et al. (2012) found that self-reported sedation increased across both AmED and alcohol-only conditions, but that sedation was lower for the combination of caffeine and alcohol. Furthermore, Peacock, Bruno, Martin, and Carr (2013) found that participants reported greater perceived stimulation when drinking AmED as compared to alcohol alone, a result also reported by Attwood et al. (2012). The authors proposed that their findings may be due to actions of caffeine decreasing or masking the sedative effects of alcohol, while also enhancing stimulatory effects of alcohol.

Because caffeine may be masking the sedation experienced with alcohol, the cue of sedation that helps stop drinking becomes less salient and therefore may extend the drinking behavior, resulting in increased alcohol intake (King et al., 2011). This hypothesis centers on the idea that the drinker is relying primarily on the interoceptive cues of sedation to consider whether or not to continue drinking. However, this masking effect is not the only possible explanation for caffeine's increase of alcohol intake. Alcohol is a drug of abuse and, therefore, is considered rewarding to the user (Charlet, Beck, & Heinz, 2013; Gonzales, Job, & Doyon, 2004). It is possible that caffeine increases alcohol intake, not just because it masks the sedative effects of alcohol, but also because alcohol is more rewarding when combined with caffeine. Previous research has suggested that caffeine may increase the sense of reward drinkers experience when consuming alcohol, and that this enhanced reward may be what leads to greater alcohol consumption.

Caffeine Potentially Enhances Reward

In order to test whether caffeine is in fact enhancing the sense of reward associated with alcohol, the subjective sense of reward must be quantified. Reward in human participants can be measured in several ways, including ratings of desire for or "wanting" of a drug. Participants in one study reported a greater liking of and desire for alcohol after consuming alcohol and caffeine together (Marczinski, Fillmore, Stamates & Maloney, 2016). Likewise, Marczinski, Fillmore, Henges, Ramsey, and Young (2013) found that participants who drank AmED rated their desire for alcohol higher than participants who drank only alcohol, and that these ratings of desire lasted for longer periods of time. Based on self-reports from human participants, it appears that caffeine increases the subjective feeling of reward experienced with alcohol, which may promote increased drinking behavior.

Animals cannot self-report liking or wanting a drug, so other methods must be used to measure the closest concept to a sense of reward in animals, which is likely the concept of reinforcement. Reinforcing efficacy is a measurement of how effective a reinforcer, such as a drug, is at maintaining a given behavior (Bickel, Marsch, & Carroll, 2000). If effort and work are put forth into maintaining a behavior that results in the obtaining of a drug, it may be possible to gain a sense of reinforcing efficacy (Salamone, Correa, Yang, Rotolo, & Presby, 2018). For instance, if an animal subject puts more work into obtaining a certain drug over another, the first drug that elicited more work and effort can be considered more reinforcing, or to have a higher reinforcing efficacy, than the second drug. Reinforcing efficacy and the involvement of effort are likely to be indicative of motivation-related processes (Bickel et al., 2000). Several preclinical studies with rodent models have shown that caffeine may be enhancing the reinforcing efficacy of both non-drug and drug stimuli, including alcohol.

One common method of examining alcohol drinking is home-cage drinking studies, in which alcohol solutions are placed on the cage of the animal and the quantity of solution consumed over a determined period of time is measured (Eisenhardt, Leixner, Spanagel, & Bilbao, 2015). Multiple studies have found that a moderate dose of caffeine increased alcohol drinking in rats using a home-cage drinking model (Kunin et al., 2000; Rezvani et al., 2013). Likewise, Fritz, Quoilin, Kasten, Smoker, and Boehm (2016) found that caffeine significantly increased alcohol intake in both adolescent and adult mice, but that adolescent mice were particularly influenced by the additive effects of the combined drugs. These studies support the hypothesis that caffeine increases alcohol consumption; however, they cannot reliably indicate by which mechanism caffeine increases alcohol drinking.

Home-cage drinking studies have the ability to demonstrate how much the animal drinks (Samson & Czachowski, 2003). However, the fact that an animal consumes alcohol does not necessarily mean that it finds the alcohol reinforcing, and therein lies the problem with home-cage drinking studies. Operant conditioning studies, which most commonly require lever-pressing behavior to earn a drug reinforcer, are a more valid model of drug reinforcement and motivation (Samson & Czachowski, 2003). The lever-pressing behavior included in operant procedures requires work and effort by the animal subject in order to obtain the drug reinforcer. The exertion of effort and involvement of work is considered to be a necessary component of modeling reinforcement in animal subjects (Lester & Freed, 1973; Salamone et al., 2018). Therefore, operant procedures, which require the behavioral output of animal subjects are a better model of drug reinforcement, than home-cage drinking, which only has the capability to measure the quantity of drug consumed (Samson & Czachowski, 2003).

In relation to operant procedures, there are two types of behavior that can be classified – appetitive and consummatory. Appetitive behaviors refer to those involved with seeking out the drug, whereas consummatory behaviors refer to the consumption of the drug (Gonzales et al., 2004). While home-cage drinking can reliably measure consummatory behavior, operant conditioning paradigms are most often used because they can reliably measure consummatory behaviors as well as appetitive behaviors (Samson & Czachowski, 2003). Operant procedures can also measure reinforcing efficacy. By requiring behavioral output on the part of the animal subject, the maintenance of that behavior and the extent to which a subject will work to gain a reinforcer can be measured (Bickel et al., 2000; Salamone et al., 2018). Because operant procedures measure both consummatory and appetitive behaviors, they indicate the reinforcement the animal subject experiences from drinking better than home-cage drinking procedures.

Several preclinical studies have used operant procedures to assess the effects of caffeine on behavior. Caffeine has been shown to increase operant responses for non-drug stimuli, including sucrose, in rats (Sheppard, Gross, Pavelka, Hall, & Palmatier, 2012). Retzbach, Dholakia, and Duncan-Vaidya (2014) also found that a moderate dose of caffeine (5 mg/kg) significantly increased responding for sucrose. Caffeine's enhancement of reinforcement appears to extend to drug stimuli as well. Rezvani et al. (2013) found that caffeine significantly increased self-administration of nicotine. When combined with methylone, one of the primary ingredients in the drug mixture known as "bath salts," caffeine also increased self-administration of the drug mixture (Gannon, Mesmin, Sulima, Rice, & Collins, 2018). Several studies have indicated that caffeine increases reinforcement and motivation for cocaine as well (O'Neill et al., 2015; Prieto

et al., 2016). Since caffeine appears to be enhancing the reinforcing efficacy for other drugs of abuse, it is possible that it does the same for alcohol.

In our previous research, we have found that a moderate dose of caffeine (10 mg/kg) significantly increased operant responding for alcohol (see Figure 1), as well as increased reinforcing efficacy in the form of increased breaking points on a progressive ratio schedule of reinforcement for alcohol in rats (see Figure 2). The increased number of responses on a fixed ratio schedule indicates that caffeine is increasing the amount of alcohol obtained by the subjects. The increased breaking point on a progressive ratio schedule indicates that caffeine is increasing the amount of work performed to obtain the alcohol, which can be interpreted as an increased level of motivation and reinforcement. Likewise, a recent publication by Roldán et al. (2017) found that the addition of a Red Bull energy drink to an alcohol solution increased operant responding for alcohol, especially as the alcohol content of the solution increased. These results can partially support our own findings in that they indicate that moderate doses of caffeine, such as those found in energy drinks, increase operant responding for alcohol. However, the Roldán et al. (2017) study cannot fully support caffeine's enhancement of operant responding for alcohol since Red Bull energy drink contains ingredients other than caffeine that have the potential to affect operant responding. Therefore, further investigation of caffeine's effects on operant-self administration and the reinforcing efficacy of alcohol is needed. Since our results are supported by previous literature showing that caffeine may increase the reinforcement that accompanies alcohol, it is natural to next investigate the neurobiological mechanisms of how this reinforcer enhancement might occur.

Modeling Drinking Behavior in Rats

The current study will use Long Evans rats to investigate the effects of caffeine on alcohol consumption. Using animals to model human behavior is common in the fields of neuroscience and psychology, particularly because it allows for a greater ability to control the environment, as well as an ability to perform drug testing that might not be ethical when using human participants (Lester & Freed, 1973; Mello, 1976). Mello (1976) defines two classes of animal model experiments – behavioral, in which the subjects work at a task to obtain the drug or may freely ingest it, and pharmacological, in which the drug is directly administered to the animal. While the pharmacological model is advantageous because it allows for more direct control of dosage, it only reveals the immediate effects of a drug (Mello, 1976). The behavioral model assesses the use of a drug over time, as well as dependence (Mello, 1976). Additionally, the behavioral model, which will be used in the current study, is more advantageous because it allows researchers to determine whether the drug is reinforcing. Mello (1976) argues this is a necessity to studying drug abuse and addiction because the animal model must demonstrate some sort of desire and reinforcement, as is common for humans with substance use disorders to experience.

The type of behavioral model used in the current study to examine alcohol intake will be an operant conditioning model. Subjects will be required to learn to press a lever to receive a sweetened alcohol reinforcer. By requiring this lever-pressing behavior, operant procedures allow for the examination of the reinforcing properties of alcohol by showing that subjects will learn to press a lever and continue to work for a reinforcer (Gonzales et al., 2004; Samson & Czachowski, 2003). This idea of working to obtain the drug is a main criterion for the development of an accurate animal model of drug abuse (Lester & Freed, 1973). Operant procedures have the ability to measure consummatory behaviors, appetitive behaviors, and the

amount of work the subject is willing to perform to obtain the drug (Samson & Czachowski, 2003). Other methods, such as home-cage drinking, only have the ability to clarify whether subjects are consuming the drug and, if so, how much they are consuming (Samson & Czachowski, 2003). This method is less preferable to operant procedures because operant procedures can reveal that the behavior to obtain the drug requires effort, and therefore, motivation. The willingness to work for the drug reinforcer by pressing a lever and continuing to maintain that lever responding to obtain it also implies that the drug is reinforcing (Salamone et al., 2018).

Operant procedures can measure motivation and reinforcement with different schedules of reinforcement. Two common schedules of reinforcement are fixed ratio (FR), in which the response requirement to obtain the reinforcer remains consistent, and progressive ratio (PR), in which the response requirement increases with the number of reinforcers obtained. Fixed ratio schedules can indicate whether the reinforcer is effective or not, because continued responses indicate that more reinforcers are worth the effort. If a substance was not reinforcing, effort would not be exerted. Conversely, if a substance is reinforcing then more exertion of effort would occur, demonstrated by increased operant responding. Progressive ratio schedules extend that analysis of reinforcement to quantify exactly how reinforcing the drug is, with higher breaking points, or the maximum number of responses produced to obtain the reinforcer, indicating a greater sense of reinforcing efficacy and motivation due to more work being performed to obtain the reinforcer (Katz, 1990; Salamone et al., 2018). In order to determine whether caffeine is enhancing the reinforcement associated with alcohol and to measure whether an alcohol solution is reinforcing at all, it is necessary for the current study to use an operant conditioning paradigm because operant responding for alcohol can be used as a measure of

reinforcement and motivation. Because our previous research, as well as that of others (e.g., Roldán et al., 2017), has suggested that caffeine increases the reinforcing efficacy of alcohol using an operant conditioning model, the neurobiological mechanisms of this possible reinforcer- or motivation-enhancement still remain unclear.

Mesolimbic Dopamine Pathway

Drugs of abuse, including alcohol, have generally been associated with a rise in dopamine levels (Charlet et al., 2013; Gonzales et al., 2004), a neurotransmitter important for reward and pleasure (Li, McCall, Lopez, & Kash, 2013; Pierce & Kumaresan, 2006). The mesolimbic dopamine pathway is considered to have one of the largest roles in drug reinforcement, with nearly all drugs of abuse leading to an increased level of dopamine in the striatum (Charlet et al., 2013; Gonzales et al., 2004). Specifically, this pathway primarily consists of dopamine neurons from the ventral tegmental area (VTA) projecting to the ventral striatum, which includes the nucleus accumbens (NAc), a prime brain area involved in reward and motivation, as well as in associative learning (Charlet et al., 2013; Salamone & Correa, 2012). It has been found that the shell of the NAc is the primary area of that nucleus involved in drug-seeking as well as associative learning, which connects drugs with related external stimuli (Gonzales et al., 2004). For example, Bassareo, Cucca, Frau, and Di Chiara (2017) found that self-administered sucrose increased dopamine in only the shell of the NAc, while a combination of alcohol and sucrose increased dopamine in both the shell and core of the NAc. Because sucrose only activated the shell of the NAc, both alone and with alcohol, it lends support to the findings of Gonzales et al. (2004) that the shell of the NAc is important for stimuli associated with alcohol. Alcohol alone also activated both the shell and core of the NAc, suggesting that, on

its own, alcohol produces a less specific dopamine response in the NAc than its associated stimuli (Bassareo et al., 2017).

Several studies have investigated the effects of alcohol in the mesolimbic dopamine pathway. Brodie and Appel (1998) found that alcohol increased the firing rate and depolarization of VTA neurons, suggesting that alcohol increases some aspects of overall activity of VTA neurons. Brodie, Pesold, and Appel (1999) further investigated these results and showed that alcohol directly excites VTA neurons because VTA neurons that were isolated from other neurons were still able to be excited by administration of alcohol. This suggests that alcohol directly excites VTA dopamine neurons, rather than that other neuronal systems are necessary for stimulation of the VTA to release dopamine. Intraperitoneal injections of alcohol in mice led to higher extracellular dopamine levels in the NAc, suggesting an increase in dopamine release in this brain area as well (Tang, George, Randall, & Gonzales, 2003). Additionally, Gonzales et al. (2004) also found that dopamine in the NAc is able to promote plasticity, or flexibility, in the mesolimbic pathway in response to alcohol by strengthening neural connections associated with alcohol reward. This increase in plasticity is significant because experience with alcohol can lead to acquired learning about the drug and assigning reinforcing value to it through changes in dopamine signaling in the NAc (Gonzales et al., 2004).

Dopamine activity in the various parts of the striatum occurs at two different types of neurons projecting out of the NAc – striatonigral, which activate the direct pathway, and striatopallidal, which activate the indirect pathway (Ferré et al., 2018). Striatopallidal neurons are particularly important to consider when trying to understand the relationship of caffeine to dopamine signaling and subsequent reinforcement or motivation.

Adenosine and Dopamine Systems and their Relation to Caffeine

The neurotransmitter adenosine is an important neuromodulator of dopamine systems. Adenosine can modulate neuronal activity by "tuning," a process in which adenosine activates its own receptors to control other neurotransmitter systems by affecting calcium channels and neurotransmitter release (Ribeiro & Sebastião, 2010). Adenosine's actions are particularly of interest when studying caffeine, as caffeine's primary actions result from its antagonism, or blockade, of adenosine receptors (Daly & Fredholm, 1998; Ribeiro & Sebastião, 2010).

Adenosine has a profound effect on dopamine signaling. Broadly, when adenosine receptors are inhibited, they inhibit dopamine release from presynaptic terminals (Garrett & Holtzman, 1994). Therefore, caffeine's actions as an adenosine antagonist should result in the same consequence of inhibited dopamine release. Adenosine often modulates dopamine signaling at the neuronal membrane and through enzymatic cascades (Fuxe, Ferré, Genedani, Franco, & Agnati, 2007). The effects of adenosine on dopamine are most prominent in interactions between the dopamine D2 receptor and the adenosine A2A receptor. A2A and D2 receptors are often colocalized in the form of a heterotetramic complex (two A2A and two D2 receptors) on striatopallidal neurons (Ferré, Fredholm, Morelli, Popoli, & Fuxe, 1997; Fuxe et al., 2007; Yacoubi et al., 2000). These striatopallidal neurons are primarily responsible for modulating the indirect pathway, which is especially important in controlling responses in situations with aversive or non-rewarding stimuli (Gallo et al., 2018; Ferré et al., 2018).

When A2A receptors are activated, they increase activity of the striatopallidal neurons. This is accomplished through the activation of adenylyl cyclase 5 (AC5) protein (Ferré et al., 2018). This protein initiates the cyclic AMP (cAMP) and protein kinase A (PKA) cycles within the cell, causing the phosphorylation of voltage-gated calcium channels, NMDA receptors, and AMPA receptors (Ferré et al., 2016). Overall, these actions increase the excitability of the

striatopallidal neuron. When the striatopallidal neuron is more excitable and more active, its signals activate the indirect pathway, resulting in the inhibition of locomotor and drug-seeking behavior (Ferré et al., 2018; Gallo et al., 2018).

A2A receptor activation also has an allosteric counteractive effect at the dopamine D2 receptor (Bonaventura at el., 2015; Ferré et al., 2016). Essentially, dopamine, or other ligands that might bind to the D2 receptor, have decreased affinity for and effectiveness at the D2 receptor (Bonaventura et al., 2015). This is supported by the findings of Mingote et al. (2008), who found that A2A receptor agonism produced effects similar to dopamine depletion. These results indicate that active A2A receptors, which inhibit signaling at the D2 receptor, should produce behavioral effects similar to those that would occur in the absence of D2 signaling.

When D2 receptors are activated, they decrease the overall activity of the striatopallidal neuron by opposing the actions of A2A receptors in two ways. First, D2 receptor activation leads to the activation of an intracellular pathway that produces protein phosphatase 2B (PP2B; Ferré et al., 2018). PP2B targets phosphorylated voltage-gated calcium channels, NMDA, and AMPA receptors and inactivates them through dephosphorylation, thus decreasing excitability of the neuron (Ferré et al., 2018). This dephosphorylation effect reverses the effect of A2A receptors by affecting the same targets. Secondly, D2 activity inhibits the activation of the AC5 protein, thereby preventing the cAMP/PKA pathway from being activated, and stopping the resulting phosphorylation of calcium channels, NMDA receptors, and AMPA receptors. These two actions combined will decrease the excitability of the striatopallidal neuron. When these striatopallidal neurons are less active, the indirect pathway is inhibited. This manifests behaviorally as an increase in locomotor activity and drug-seeking behavior (Ferré et al., 2018).

When caffeine is administered, its actions as an A2A adenosine receptor antagonist should look very similar to that of D2 receptor activation. A2A receptor inhibition would eliminate the allosteric counteraction of ligand binding at D2 receptors, allowing dopamine to more readily bind to the D2 receptor. Previous research supports this idea by showing that when caffeine blocks A2A receptors, dopamine appears to have increased activity and potency at the dopamine D2 receptor (Daly & Fredholm, 1998). Additionally, because A2A is inhibited, AC5 activation, and subsequent cAMP/PKA activation, does not occur and therefore the excitability of the striatopallidal neuron is decreased. This would result in less activation of the indirect pathway, leading to increased drug-seeking behavior. When caffeine and alcohol are used together, alcohol would increase extracellular dopamine in the NAc, where the striatopallidal neurons are located (Tang et al., 2003). Caffeine's antagonism of adenosine A2A receptors would promote this extracellular dopamine to more readily bind at the disinhibited dopamine D2 receptors, thereby decreasing activity of the striatopallidal neuron and the subsequent indirect pathway activation. It is possible that this may be a process by which caffeine enhances dopamine signaling when alcohol is used, and how it may contribute to increased alcohol use by promoting seeking behavior.

Additionally, Solinas et al. (2002) found that moderate to high doses of caffeine (10-30 mg/kg) significantly increased dopamine release in the shell of the NAc. Because caffeine may be increasing dopamine signaling in parts of the mesolimbic dopamine pathway, it is possible that caffeine enhances a sense of reinforcement in the drug user. However, caffeine is likely not reinforcing to the user on its own. It has been found that caffeine and A2A antagonists injected alone did not significantly increase extracellular dopamine levels in the NAc (Acquas, Tanda, & Di Chiara, 2002). Additionally, operant responding for caffeine alone does not differ

significantly from control conditions, suggesting that caffeine alone is not reinforcing (Bradley, Sanders, Williams, & Palmatier, 2017; Prieto et al., 2016). Since caffeine does not produce an enhancement of operant responding and does not directly affect dopamine receptors, it likely does not directly produce its own sense of reward. Rather, caffeine likely plays a modulatory role in dopamine signaling, and its resulting sense of reward and reinforcement, in combination with other drugs by way of its antagonistic actions at adenosine receptors.

There is some inconsistency in the literature concerning the role of caffeine and adenosine and how they affect dopamine signaling. For example, Quarta et al. (2004) found that A2A receptor activity stimulated striatal glutamate and dopamine release in the NAc, which is the opposite of what would be expected since previous studies have found that A2A receptors antagonize dopamine activity (Garrett & Holtzman, 1994; Mingote et al., 2008). Malave and Broderick (2014) found that caffeine blocked cocaine-induced release of dopamine due to adenosine receptor antagonism, whereas previous research has suggested that caffeine enhances dopamine signaling and subsequent reinforcement, specifically with cocaine (O'Neill et al., 2015; Prieto et al., 2016). This may have been due to caffeine's biphasic dose response, where higher doses of caffeine have a depressant effect as compared to the stimulatory effects of moderate doses. Nevertheless, these inconsistencies in the literature surrounding caffeine and adenosine systems and their subsequent effects on dopamine signaling and the resulting sense of reinforcement gained from drugs, including alcohol, prompt further investigation.

This increased dopamine signaling elicited by the neurobiological effects of caffeine may promote an enhanced sense of reinforcement in the user. When the dopamine D2 receptor is disinhibited by caffeine's actions at the adenosine A2A receptor, the high levels of extracellular dopamine caused by alcohol intake can bind to the D2 receptor at an increased rate (Ferré et al.,

2018; Tang et al., 2003). If caffeine is increasing drinking behavior by affecting dopamine signaling, it may be that enhanced reinforcement promotes drinking behavior. However, it remains undetermined whether this enhancement of dopamine signaling at the dopamine D2 receptor and reinforcer enhancement by caffeine is a cause of increased drinking behavior associated with moderate doses of caffeine combined with alcohol.

The Current Study

The current study seeks first to replicate our previous findings that a moderate dose (10 mg/kg) of caffeine increases operant responding for alcohol, both on a fixed and progressive ratio schedule of reinforcement. This finding is supported by previous research which has found that caffeine at small to moderate doses increased both drinking and operant responding for alcohol in rodent models (Fritz et al., 2016; Kunin et al., 2000; Roldán et al., 2017). Because operant responding is a reliable measure of drug-seeking behavior and can be interpreted as representative of reinforcement and motivation, we have suggested that caffeine is increasing the reinforcing efficacy of alcohol, as demonstrated by an increased number of responses on a fixed ratio schedule of reinforcement and increased breaking points on a progressive ratio schedule of reinforcement to receive the alcohol reinforcer. Based on these prior results and the literature, I hypothesize that a moderate dose of caffeine will increase operant responding for a sweetened alcohol reinforcer.

The second aim of the current study is to investigate how caffeine is leading to an increase in reinforcement. There is evidence to suggest that caffeine enhances a sense of reward when drinking alcohol (Marczinski et al., 2013; Marczinski et al., 2016). If caffeine is increasing the sense of reward associated with alcohol, caffeine may be enhancing dopamine signaling through its effects at adenosine receptors. By blocking A2A receptors inhibitory effects on D2

receptor signaling, caffeine should increase dopamine signaling at D2 receptors. It has been shown that alcohol increases extracellular levels of dopamine in the striatum, and this dopamine would now be able to bind more effectively at D2 receptors (Brodie & Appell, 1998; Tang et al., 2003). It has also been shown that dopamine D2 receptor activity is necessary for alcohol reinforcement, as high doses of various D2 antagonist drugs, which block D2 receptor activity, decrease operant responding for alcohol (Arolfo, Yao, Gordon, Diamond, & Janak, 2004; Hodge, Samson, & Chappelle, 1997). Therefore, a caffeine-induced increase in dopamine signaling at the D2 receptor should lead to heightened reinforcement (Pierce & Kumaresan, 2006; Li et al., 2013), demonstrated by increased operant responses for alcohol in the current study. To test whether caffeine is affecting responses toward alcohol due to A2A-D2 receptor interactions, the dopamine antagonist eticlopride will be administered to subjects in conjunction with caffeine.

Eticlopride is a highly selective dopamine D2 receptor antagonist (Hall, Kohler, & Gawell, 1985; Ferrari & Giuliani, 1995; Seeman & Ulpian, 1988). Eticlopride has been found to dose-dependently increase self-administration of drugs, including cocaine and methylphenidate (Hemby, Smith, & Dworkin, 1996; Botly, Burton, Rizos, & Fletcher, 2008). It has also been found to dose-dependently decrease responding for alcohol in rats (Arolfo et al., 2004). Eticlopride has also been shown to have no significant effect on inactive lever presses in some of these studies, indicating that it is affecting motivated behavior rather than causing an overall change in locomotor activity (Arolfo et al., 2004; Botly et al., 2008). Because of eticlopride's high selectivity, low number of side effects, and previous effectiveness at decreasing responses for drugs, including alcohol, it has been chosen as the D2 receptor antagonist to be used in the current study.

By administering caffeine and a dopamine receptor antagonist together, it will clarify whether caffeine is increasing alcohol drinking by increasing the motivation for and reinforcement experienced with alcohol through its antagonism of the A2A receptor, and the resulting increase in D2 receptor activity. I hypothesize that when a dopamine D2 receptor antagonist and caffeine are co-administered, operant responding for alcohol will not significantly differ from baseline responding for alcohol since eticlopride will counteract the dopamine- and reinforcing-enhancing effects of caffeine.

Method

Subjects

Adult male Long Evans rats (n = 8) were used as subjects for this experiment, and arrived at 50-75 g (Envigo, Indianapolis, IN). It has been shown that Long Evans subjects will learn operant responding for alcohol without food deprivation or restriction, making them an excellent strain to use for the current study (Grant & Samson, 1985b; Samson, 1986). Rats were pair-housed in plexiglas cages with wire tops, with food and water available ad libitum (except where noted). Two subjects were singly housed. Subjects were kept on a 12-hr light/dark schedule (lights on at 11:00), and testing occurred during the light phase, beginning at approximately 2 p.m. All experimental procedures regarding animal subjects were approved by the Lycoming College Institutional Animal Care and Use Committee.

Design

A within-subjects, repeated measures design was used in all experiments. This design allowed each subject to serve as its own control, accounting for individual differences in baseline responding. The first experiment examined the effects of four doses of caffeine (0, 5, 10, 20 mg/kg) on fixed-ratio responding for a sweetened alcohol solution. The second experiment

examined the effects of caffeine (5 and 10 mg/kg) on progressive-ratio responding for a sweetened alcohol solution. The third and fourth experiments examined the combined effects of the dopamine D2 receptor antagonist, eticlopride, and a moderate dose of caffeine on fixed ratio and progressive ratio responding for a sweetened alcohol solution.

Drug Preparation

Sucrose solutions and sweetened alcohol solutions were prepared at the beginning of each week using deionized water, sucrose, and a 95% ethanol stock. Caffeine (Sigma-Aldrich, St. Louis, MO) was prepared on the day of testing and dissolved in physiological saline (0.9% NaCl). Eticlopride (Sigma-Aldrich, Milwaukee, WI) was administered at a dose of 0.01 mg/kg. This dose of eticlopride was based on pilot testing using a prior group of animal subjects, as well as on previous research stating that doses of eticlopride within a range of 0.001-0.1 mg/kg can significantly affect operant responding for alcohol without causing motor side effects (Arolfo et al., 2004; Barrett et al., 2004; Fowler & Liou, 1998; Hemby et al., 1996). All injections were performed intraperitoneally and held at a constant volume of 1 mL/kg.

Apparatus

Operant self-administration took place in operant chambers (12.0” w x 9.5” d x 8.25” h; Med Associates, Inc., St. Albans, VT) enclosed within sound-attenuating boxes equipped with fans to reduce external noise. Chambers had polycarbonate front, back, and top walls with two aluminum walls on the left and right sides. Chambers also had a stainless steel bar floor with a pan of bedding underneath for waste. For each chamber, either the left or right wall had a house light located near the top. Both the left and right walls contained a retractable lever with a stimulus light directly above it. One wall contained a recessed liquid receptacle located next to the lever and stimulus light. For each chamber, the lever next to the liquid receptacle was

designated the active lever which, when pressed, resulted in stimulus light activation and delivery of 0.1 mL of solution to the liquid receptacle by a motorized syringe pump located outside the chamber. Delivery of the liquid reinforcer took place over 1.66 seconds and was accompanied by activation of the stimulus light and the sound of the motorized syringe pump for the duration of that time. On the opposite wall, the second lever was designated the inactive lever, which resulted in no programmed consequence when pressed. The location (i.e., left or right side of the chamber) of the active lever and liquid receptacle was counterbalanced across subjects.

Operant Self-Administration Training

Sixteen-hour overnight operant sessions began when rats were approximately 250-300 g. Subjects were deprived of water for 24 hours prior to the study to motivate responding, as consistent with previous literature (Besheer et al., 2008). Rats were then trained to self-administer a 10% w/v sucrose solution on a fixed ratio 1 (FR1) schedule, during which a single press of the active lever within the operant conditioning chamber resulted in the delivery of the sucrose reinforcer (0.1 mL). After a subject received 10 such reinforcers, the reinforcement schedule increased to a fixed ratio 2 (FR2) schedule for the duration of the session, during which two lever presses were required in order to receive the sucrose reinforcer. Each subject completed two of these sessions, until the lever-pressing behavior was learned, with at least 250 reinforcers earned in one session.

Sucrose Fading Procedure

Once rats learned to press a lever for sucrose reinforcement, rats began a sucrose fading procedure, during which the sucrose content of the solution was gradually decreased as the alcohol content of the solution was gradually increased. It has previously been found that

exposing and/or initially reinforcing subjects with sucrose allows for the development of responding for alcohol when it is first introduced (Grant & Samson, 1985b; Samson, 1986). This initial exposure to sucrose allows rats to acquire lever-pressing behaviors, as well as overcome a natural taste aversion to alcohol (Grant & Samson, 1985a). Besheer, Faccidomo, Grondin, and Hodge (2008) have refined these procedures involving operant responding for alcohol in rats by modifying a sucrose fading procedure. Subjects receive solutions in which alcohol concentration is increased gradually while sucrose concentration is gradually decreased (Besheer et al., 2008). This procedure allows for animal subjects to learn the lever-pressing behavior with sucrose and gradually overcome their taste aversion to alcohol while simultaneously establishing a reliable and stable level of responding for alcohol as its concentration increases.

In the current study, the sucrose fading procedures of Samson (1986) and Besheer et al. (2008) were modified to solution concentrations that have successfully produced stable responding in previous experiments in our lab. Rats began on a 10% w/v sucrose solution and ended on a final concentration of 10% v/v ethanol + 2% w/v sucrose (10E2S), which has been shown in our previous research to be the concentration at which rats most stably respond (see Table 1). Subjects responded to each solution in the fading procedure for a minimum of two days, which was increased per individual subject if stable responding did not occur. Additionally, 10% ethanol alone was included in the sucrose fading procedure to encourage stable responding when sucrose was reintroduced into the reinforcer solution. After the sucrose fading procedure was completed, each rat continued to respond for 10E2S solution for a minimum of 39 sessions, or until stable patterns of responding were observed across experimental subjects.

Experiment 1: Effects of Caffeine on the Reinforcing Efficacy of Alcohol

The first experiment investigated whether moderate doses of caffeine affect the reinforcing value of alcohol. After stable responding was established for the 10E2S solution for the minimum of 39 days ($M = 47.125$, $SD = 3.621$), subjects underwent caffeine testing. Subjects were first habituated to handling and injections, with saline injected 30 min prior to the daily operant conditioning session two times. Once per week doses of 0, 5, 10, and 20 mg/kg of caffeine were tested over the course of 4 weeks on an FR2 schedule with 10E2S solution as the reinforcer. Subjects experienced one injection per week, occurring 30 min prior to their daily operant session. The order of caffeine doses was counterbalanced across subjects using a Latin Square design.

Experiment 2: Effects of Caffeine on Progressive Ratio Responding for Alcohol

Following successful completion of the four FR2 sessions preceded by a caffeine injection, subjects had one week to return to baseline levels of responding during standard 30 min FR2 sessions with a 10E2S reinforcer. Subjects then completed a progressive ratio (PR) schedule for the second experiment. The progressive ratio procedure was programmed to increase the response requirement by a factor of 1 for every reinforcer delivered (FR1 for the first reinforcer, FR2 for the next reinforcer, and so on) for a fixed 30 min session. Previous literature shows that this progressive ratio schedule has been successfully used to test subjects on progressive ratio schedule repeatedly (Besheer et al., 2008). Subjects continued to receive 10E2S as the reinforcer and received once-weekly injections of caffeine dose (0, 5, or 10 mg/kg) 30 min prior to the PR session. The 5 and 10 mg/kg doses of caffeine were used, specifically as they had been found to be the doses of caffeine that increased operant responding for alcohol in Experiment 1. The order of caffeine doses tested was counterbalanced across subjects using a

Latin Square design. On non-injection days rats completed standard 30 min FR2 sessions to maintain responding between PR drug test sessions.

Experiment 3: Effects of Dopamine D2 Receptor Antagonism on Caffeine's Effects on FR Responding for Alcohol

Following successful completion of the FR and PR studies with caffeine, subjects were allowed nine to 10 weeks to return to their baseline level of responding at the FR2 schedule with 10E2S reinforcer, due to the time constraint of the break between semesters. After baseline responding was achieved, subjects completed two sessions of saline habituation injections to habituate them to the double injection procedure. Then treatment began with the selective dopamine D2 receptor antagonist eticlopride. Effective doses of eticlopride range from 0.007 to 0.01 mg/kg, with doses below 0.02 mg/kg not producing catalepsy or other negative motor side effects (Ferrari & Giuliani, 1995). The current study used 0.01 mg/kg eticlopride based on this prior research, as well as pilot testing in a separate group of subjects. Each of the subjects in the current experiment received pairs of injections for drug testing: saline and saline, 5 mg/kg caffeine and saline, 0.01 mg/kg eticlopride and saline, and 0.01 mg/kg eticlopride and 5 mg/kg caffeine. The paired double saline injection served as a control. The order of each of the four injection pairs was counterbalanced using a Latin Square design to eliminate order effects or carryover effects of the drug. These paired injections occurred once per week, 30 min prior to the start of the daily 30 min operant conditioning session on the FR2 schedule with the 10E2S reinforcer. One week was allowed between injections to ensure a return to baseline responding.

Experiment 4: Effects of Dopamine D2 Receptor Antagonism on Caffeine's Effects on PR Responding for Alcohol

Following successful completion of all four FR2 sessions, subjects had two weeks without injection to return to baseline responding. Then testing began on a PR schedule. Again, each subject received one of the four injection pairs (saline and saline, 5 mg/kg caffeine and saline, 0.01 mg/kg eticlopride and saline, and 0.01 mg/kg eticlopride and 5 mg/kg caffeine) 30 min prior to the daily 30 min PR operant session with a 10E2S reinforcer. The PR schedule used in Experiment 2 was used in this experiment, where the response requirement increased by a factor of 1 for every reinforcer delivered. Again, one week was allowed between each injection to ensure a return to stable baseline responding.

Data Analysis

Data was analyzed using IBM SPSS statistics software. The levels of the independent variable are the doses of caffeine used in experiments 1 (0, 5, 10, and 20 mg/kg) and in Experiment 2 (0, 5, and 10 mg/kg), as well as the two independent variables of caffeine dose (0 and 5 mg/kg) and eticlopride (0 and 0.01 mg/kg) in Experiments 3 and 4. The dependent variables being analyzed were the number of active lever presses per session, the number of reinforcers received per session, the number of inactive lever presses per session, calculated alcohol intake (g/kg) per session, and breaking point on progressive ratio sessions. To analyze the differences in each of these dependent variables across the levels of the independent variable, a repeated measures ANOVA was used. A factorial repeated measures ANOVA was used for Experiments 3 and 4, as there were two independent variables (caffeine dose and eticlopride dose) involved. Factorial repeated measures ANOVAs were also used to analyze operant responding for the sweetened alcohol solution over time.

Results

Experiment 1: Effects of Caffeine on the Reinforcing Efficacy of Alcohol

As hypothesized, caffeine, at moderate doses, significantly increased operant alcohol self-administration in rats on a fixed ratio schedule of reinforcement. Due to an equipment malfunction on one test day, four subjects were tested again the following week and this new data was used for analysis.

In order to analyze the effects of caffeine on active lever responses over time, the number of alcohol-reinforced lever responses per minute during the 30 min operant session were condensed into 5-min time bins. A 4 (caffeine dose) X 6 (time) repeated measures analysis of variance (RM-ANOVA) revealed a significant main effect of caffeine dose [$F(3, 21) = 5.693$, $p = .013$] and time [$F(5, 35) = 8.838$, $p < .001$]. However, there was no significant interaction of caffeine dose and time on active lever responses [$F(15, 105) = 1.283$, $p = .226$] (see Figure 3). Therefore, subsequent analyses were performed on the total number of responses over the 30 min operant session.

When responses were summed over the 30 min operant session, results of a one-way RM-ANOVA revealed a significant effect of caffeine on alcohol-reinforced lever responses, $F(3, 21) = 7.186$, $p = .002$. Bonferroni-corrected post-hoc comparisons revealed that both the 5 mg/kg ($p = .019$) and 10 mg/kg ($p = .001$) caffeine doses significantly increased active lever responses for the sweetened alcohol solution relative to the saline control condition. There was no significant difference in active lever responses between the 20 mg/kg ($p = .092$) caffeine dose and saline (see Figure 4).

Moderate doses of caffeine also significantly increased the number of reinforcers earned and alcohol intake. As shown in Figures 5 (reinforcers) and 6 (alcohol intake), a one-way RM-ANOVA showed a significant effect of caffeine both on the number of reinforcers obtained, $F(3, 21) = 6.975$, $p = .002$, and alcohol dose consumed (measured as g/kg), $F(3, 21) = 6.556$, $p = .003$.

Bonferroni-corrected post-hoc analysis showed that both the 5 mg/kg (*p*s < .05) and 10 mg/kg (*p*s < .01) doses of caffeine significantly increased the number of reinforcers obtained and alcohol consumed, relative to saline. There was no significant difference between the 20 mg/kg caffeine dose and saline for either reinforcers obtained ($p = .115$) or for alcohol intake ($p = .100$).

Although there was a significant overall effect of caffeine on the number of inactive lever responses [$F(3, 21) = 3.205$, $p = .044$], Bonferroni-corrected post-hoc comparisons revealed no significant increase or decrease in inactive lever responses with any caffeine dose relative to the saline control condition (see Figure 7).

Experiment 2: Effects of Caffeine on Progressive Ratio Responding for Alcohol

Because both the 5 mg/kg and 10 mg/kg dose of caffeine produced significant effects on operant responding and alcohol intake in the first experiment, these doses were used in the second experiment in order to further investigate the effects of moderate doses of caffeine on the reinforcing efficacy of alcohol. The results of the second experiment reveal that moderate doses of caffeine likewise increased operant responding for alcohol on a progressive ratio schedule of reinforcement. To analyze the effects of caffeine on active lever responses over time, the number of active lever responses per minute during the 30 min operant session were condensed into 5-min time bins. A 3 (caffeine dose) X 6 (time) RM-ANOVA revealed a significant main effect of caffeine dose [$F(1.172, 8.205) = 7.649$, $p = .021$] and time [$F(1.745, 12.213) = 12.738$, $p = .001$] on active lever responses. There was also a significant caffeine dose X time interaction, $F(10, 70) = 2.004$, $p = .046$. Follow-up analyses revealed a significant effect of caffeine at the 15 min time point, $F(2, 14) = 6.178$, $p = .012$. However, Bonferroni-corrected post-hoc comparisons revealed no significant difference in active lever responses when comparing the saline condition to the 5 mg/kg or 10 mg/kg caffeine doses (*p*s > .06) (see Figure 8).

Active lever responses, therefore, were summed across the total 30 min operant sessions for subsequent data analysis. A one-way RM-ANOVA revealed a significant effect of caffeine dose on active lever responses over the 30-min progressive ratio session, $F(1.149, 8.041) = 7.751$, $p = .021$ (see Figure 9). Specifically, Bonferroni-corrected post-hoc comparisons revealed that the 5 mg/kg dose of caffeine significantly increased the number of alcohol-reinforced lever responses compared to the saline control ($p = .001$). Although the 10 mg/kg caffeine dose increased alcohol-reinforced responses relative to the saline control, this was not significant though it is approaching statistical significance ($p = .054$).

In addition to active lever responses, breaking points were analyzed in the current study, as they can quantify the amount of work and effort exerted to obtain a drug reinforcer and are a representation of reinforcing efficacy. The breaking point is the point at which an animal stops responding for a reinforcer, and, in the current study, is indicative of how many reinforcers were earned (Besheer et al., 2008). As hypothesized, caffeine significantly increased the breaking point for the sweetened alcohol solution, $F(2, 14) = 7.850$, $p = .005$, with both the 5 mg/kg ($p = .011$) and 10 mg/kg ($p = .04$) doses significantly increased breaking point, as compared to the saline control (see Figure 10). However, there was no effect of caffeine on the number of inactive lever presses during the progressive ratio session, $F(2, 14) = 1.910$, $p = .185$ (see Figure 11).

Experiment 3: Effects of Dopamine D2 Receptor Antagonism on Caffeine's Effects on FR Responding for Alcohol

While 5 mg/kg caffeine continued to significantly increase operant responding for alcohol, 0.01 mg/kg eticlopride decreased responding and there was no significant interaction of caffeine and eticlopride. A 2 (caffeine dose) X 2 (eticlopride dose) X 6 (time) RM-ANOVA

revealed a significant main effect of caffeine dose [$F(1, 7) = 16.616$, $p = .005$], eticlopride dose [$F(1, 7) = 8.573$, $p = .022$] and time [$F(1.674, 11.716) = 9.607$, $p < .001$] on alcohol-reinforced responding on the active lever. Moreover, there was a significant caffeine dose X time interaction, $F(5, 35) = 2.655$, $p = .039$, with 5 mg/kg caffeine significantly increasing active lever responses at the 10 and 15 min time points, relative to saline (ps < .02) (see Figure 12). There was no significant interaction, however, of eticlopride dose and time [$F(5, 35) = .960$, $p = .456$], caffeine dose and eticlopride dose [$F(1, 7) = .051$, $p = .827$], or between caffeine dose, eticlopride dose, and time [$F(5, 35) = .842$, $p = .529$].

In order to further assess the impact of eticlopride and caffeine on alcohol self-administration, total responses over the 30 min operant session were analyzed. The results of a 2 (eticlopride dose) X 2 (caffeine dose) RM-ANOVA revealed a significant main effect of caffeine dose [$F(1, 7) = 16$, $p = .005$] and eticlopride dose [$F(1, 7) = 10.467$, $p = .014$] on alcohol-reinforced responding on the active lever, with 5 mg/kg caffeine significantly increasing responses and 0.01 mg/kg eticlopride significantly decreasing responses relative to saline. However, there was no significant caffeine dose X eticlopride dose interaction on active lever responses [$F(1, 7) = 0.075$, $p = .792$] (see Figure 13).

Likewise, the results of a second 2 (caffeine dose) X 2 (eticlopride dose) RM-ANOVA revealed a significant main effect of caffeine dose [$F(1, 7) = 16.625$, $p = .005$] and eticlopride dose [$F(1, 7) = 10.240$, $p = .015$] on the number of reinforcers obtained. There was, again, no significant caffeine dose X eticlopride dose interaction on reinforcers obtained [$F(1, 7) = .213$, $p = .658$] (see Figure 14). There was also significant main effect of caffeine dose [$F(1, 7) = 14.673$, $p = .006$] and eticlopride dose [$F(1, 7) = 9.107$, $p = .019$] on alcohol dose consumed (measured as g/kg). Again, there was not a significant caffeine dose X eticlopride dose

interaction on alcohol intake (g/kg) [$F(1, 7) = .016$, $p = .904$] (see Figure 15). However, there was no significant main effect of caffeine dose [$F(1, 7) = 1.255$, $p = .300$] or eticlopride dose [$F(1, 7) = 2.558$, $p = .154$] or caffeine dose X eticlopride dose interaction [$F(1, 7) = .006$, $p = .941$] on inactive lever presses (see Figure 16).

Experiment 4: Effects of Dopamine D2 Receptor Antagonism on Caffeine's Effects on PR Responding for Alcohol

Overall, a 5 mg/kg dose of caffeine continued to increase operant responding for alcohol, while 0.01 mg/kg eticlopride continued to decrease that responding. There was again no significant interaction between caffeine dose and eticlopride dose. One subject's data for one test session was eliminated due to a lack of any lever responses. The subject was retested and this new data was used for analysis.

A 2 (caffeine dose) X 2 (eticlopride dose) X 6 (time) RM-ANOVA revealed a significant main effect of caffeine dose [$F(1, 7) = 40.231$, $p < .001$], eticlopride dose [$F(1, 7) = 6.942$, $p = .034$] and time [$F(1.295, 9.065) = 15.775$, $p < .001$] on alcohol-reinforced responding on the active lever. Moreover, there was a significant caffeine dose X time interaction [$F(5, 35) = 3.261$, $p = .016$], with 5 mg/kg caffeine significantly increasing active lever responses at the 10 ($p = .002$) and 30 min ($p = .007$) time points, relative to saline (see Figure 17). There was also a significant interaction of eticlopride dose and time [$F(5, 35) = 3.851$, $p = .007$], with 0.01 mg/kg eticlopride significantly decreasing active lever responses at 5 min ($p = .004$) relative to saline (see Figure 18). There was, however, no significant interaction of caffeine dose and eticlopride dose [$F(1, 7) = 1.723$, $p = .231$], or between caffeine dose, eticlopride dose, and time [$F(5, 35) = 2.202$, $p = .076$].

To further analyze the effects of caffeine and eticlopride on progressive ratio alcohol self-administration, responses over the 30 min operant session were summed. The results of a 2 (eticlopride dose) X 2 (caffeine dose) RM-ANOVA revealed a significant main effect of caffeine [$F(1, 7) = 40.535$, $p < .001$] and eticlopride [$F(1, 7) = 7.023$, $p = .033$] on alcohol-reinforced responding on the active lever, with 5 mg/kg caffeine significantly increasing responses and 0.01 mg/kg eticlopride significantly decreasing responses relative to saline. However, there was no significant caffeine dose X eticlopride dose interaction on active lever responses [$F(1, 7) = 1.701$, $p = .233$] (see Figure 19). Likewise, there was a significant main effect of caffeine [$F(1, 7) = 43.707$, $p < .001$] and eticlopride [$F(1, 7) = 9.145$, $p = .019$] on breaking point. There was no significant caffeine X eticlopride interaction on breaking point [$F(1, 7) = 5.147$, $p = .058$] (see Figure 20).

The results of a 2 (caffeine dose) x 2 (eticlopride dose) factorial repeated measures ANOVA revealed no significant main effect of caffeine [$F(1, 7) = 4.365$, $p = .075$] or eticlopride [$F(1, 7) = 1.074$, $p = .334$] or caffeine X eticlopride interaction [$F(1, 7) = 2.096$, $p = .191$] on inactive lever presses (see Figure 21).

Discussion

The current study's series of four experiments all demonstrate that caffeine, at moderate doses, increases operant responding for a sweetened alcohol solution in Long Evans rats. This increase in operant responding is indicative of caffeine's ability to increase the reinforcing efficacy of alcohol. However, the results of Experiments 3 and 4 suggest that this caffeine-induced increase in alcohol consumption may not be due to caffeine's actions at the A2A-D2 heterotetramer receptor complex, as the D2 antagonist eticlopride was not able to counteract caffeine's enhancement of operant responding. Rather, the dose of eticlopride used in the current

study may have been too small to enact sufficient change to the A2A-D2 receptor complex. Or, it is possible that caffeine enhances operant self-administration behavior through the modulation of other neurotransmitter or receptor systems. Overall, the results of the current study support the hypotheses that caffeine enhances the reinforcing efficacy of alcohol and consummatory behaviors, but does not support the hypothesis that this caffeine-induced enhancement of reinforcement occurs through the enhancement of dopamine signaling at the A2A-D2 receptor complex.

Effect of Caffeine on Alcohol Self-Administration: Fixed Ratio Study

The results of the first experiment showed that caffeine, at both a 5 and 10 mg/kg dose, significantly increased the number of active lever responses, number of reinforcers consumed, and alcohol consumed (calculated as g/kg) on a fixed ratio schedule, with no significant change in inactive lever responses. These results are consistent with previous studies that found moderate doses of caffeine significantly increased alcohol intake (Fritz et al., 2016; Kunin et al., 2000; Rezvani et al., 2013). However, the results of the current study expand upon the previous literature by suggesting that caffeine increases the reinforcing efficacy of alcohol. The current study used an operant self-administration model, as compared to prior home-cage drinking studies, and was able to demonstrate that caffeine significantly increased active lever responses for alcohol. In other words, subjects increased their number of attempts to gain a sweetened alcohol reinforcer. Since subjects were exerting additional effort (i.e., additional active lever presses) toward gaining the alcohol reinforcer, it can be inferred that the reinforcing efficacy of alcohol, or its ability to maintain active lever pressing behavior, was enhanced by moderate doses of caffeine (Bickel et al., 2000; Salamone et al., 2018). One way to interpret these results is

that caffeine seems to be enhancing the reinforcement experienced with alcohol intake, and producing a greater subsequent intake.

However, caffeine is also a locomotor stimulant drug, which may produce an increase in motor output that could affect lever-pressing behavior (Yacoubi et al., 2000). In order to determine whether the caffeine-induced increase in active lever responding is a specific response pattern, inactive lever responses also need to be considered. Responses on the inactive lever result in no programmed consequence; therefore, subjects should not be expected to press this lever often since there is no reinforcing stimulus (e.g., alcohol) associated with it. If the number of inactive lever responses was significantly increased by caffeine, then it is possible that caffeine's stimulatory effects led to an overall increase in locomotor activity that contributed to non-specific lever-pressing behavior. In the current experiment, there was an overall effect of caffeine on inactive lever responses, but post-hoc analyses revealed no significant difference in inactive lever responses between the control condition and each of the three caffeine doses (5, 10, and 20 mg/kg). Since there was no significant increase of inactive lever responses by caffeine, this result indicates that the increase in active lever-pressing behavior seen at the 5 and 10 mg/kg doses of caffeine was a specific response on the active lever, rather than the result of a stimulatory effect of caffeine which would have increased responding on both levers.

The results of the current study that demonstrate increased operant responding for alcohol following treatment with moderate doses of caffeine are able to support and extend upon the results of previous literature. A prior study by Roldán et al. (2017) also showed compelling evidence for caffeine increasing operant responding; however, that study was not able to demonstrate if caffeine caused the increased responding. The researchers mixed Red Bull energy drink with the alcohol solution for which rats responded, but Red Bull has ingredients other than

caffeine that could have affected operant responding, including sucrose and taurine (Roldán et al., 2017). While the study by Roldán et al. (2017) more closely mimics human AmED consumption (i.e., an energy drink is mixed with alcohol and they are simultaneously orally consumed), the current study's strength lies in the ability to specifically demonstrate that caffeine alone leads to a significant increase in operant responding for a sweetened alcohol solution. This experiment is the first, to my knowledge, to present the finding that caffeine alone significantly increases operant self-administration for a sweetened alcohol solution.

Caffeine Increases Progressive Ratio Responding for Sweetened Alcohol

The results of this experiment again support the hypothesis that caffeine, at moderate doses, increases alcohol self-administration. However, the results of this second experiment expand upon the previous experiment and literature in terms of what kinds of behavior can be measured through operant responding. The first experiment, as well as the study of self-administration of a Red Bull and alcohol mixture by Roldán et al. (2017), used only a fixed ratio schedule of reinforcement during operant sessions. Although fixed ratio responding has been valuable in demonstrating increases in intake of alcohol as well as drug-seeking behaviors associated with caffeine, it cannot accurately assess motivation-related processes. By using progressive ratio responding, those concepts of motivation and reinforcement may be investigated. Breaking points on progressive ratio schedule are able to quantify the exact amount of work and effort that subjects exert to gain the sweetened alcohol reinforcer. This experiment specifically showed that caffeine, at doses of 5 and 10 mg/kg, significantly increased breaking point, though it was a modest effect. These results are consistent with prior studies that showed caffeine's ability to enhances of progressive ratio responding for sucrose and drugs, like cocaine (Prieto et al., 2016; Sheppard et al., 2012). The results of the current study indicate not only that

moderate doses of caffeine increase operant responding for alcohol through an elevated number of active lever presses, but that they are increasing the work and effort the animal is willing to exert in order to obtain alcohol. It is possible that this enhanced effort to obtain alcohol is due to an effect of caffeine enhancing the motivation to seek out or consume alcohol. The results of this second experiment build upon the first by again suggesting that caffeine may be able to enhance drug-seeking or consummatory behavior for alcohol.

Similar to the first experiment, there was no significant effect of caffeine on inactive lever responses, indicating that, yet again, caffeine enhanced only the lever-pressing behavior specific to the active lever and not overall locomotor activity.

Caffeine Increases the Reinforcing Efficacy of Alcohol

The first and second experiments, in combination, provide support that caffeine increases the reinforcing efficacy of a sweetened alcohol solution. Reinforcing efficacy refers to how effective a particular reinforcer is at maintaining a behavior (Bickel et al., 2000). In the current study, lever pressing behavior was maintained by a sweetened alcohol reinforcer. In the first experiment, moderate doses of caffeine increased alcohol self-administration and alcohol intake by increasing the reinforcing efficacy of alcohol, as indicated by enhanced active lever responses for the drug. The second experiment provides more convincing data regarding reinforcing efficacy because progressive ratio schedules are a commonly used model of reinforcing efficacy (Katz, 1990). Breaking points on a progressive ratio schedule can measure the extent to which lever-pressing behavior is maintained, as well as the amount of effort exerted by the subject to gain a reinforcer (Salamone et al., 2018). The second experiment revealed that caffeine, at 5 and 10 mg/kg doses, modestly, but significantly, increased breaking points, indicating an enhanced reinforcing efficacy and enhanced amount of effort put forth to gain the sweetened alcohol

reinforcer. The enhanced effort to obtain alcohol produced by caffeine may imply an increased motivation for alcohol and suggest that when caffeine and alcohol are consumed together, caffeine enhances how alcohol's effectiveness as a reinforcer. The first and second experiments, in combination, provide support for the hypothesis that moderate doses of caffeine increase alcohol intake and show, by use of an operant self-administration model, that reinforcing efficacy of a sweetened alcohol solution is enhanced by caffeine. The results of the second experiment particularly indicate that work and effort, and possibly motivation, may be enhanced by caffeine as well.

Caffeine Influences Responding for Alcohol Over Time

Some of the most notable findings of all four experiments were the results of analyzing active lever responses over the 30 min operant sessions in 5 min time bins. There were significant interactions of caffeine and time in two out of four experiments, with caffeine significantly increasing alcohol-reinforced responses at times ranging from the 10 min time bin to the end of the operant session at 30 min. The times at which caffeine increases operant responding for a sweetened alcohol solution may provide some insight into how caffeine may be increasing alcohol intake.

If the perspective of Samson and Czachowski (2003) is used to interpret this time analysis data, the results suggest that 5 mg/kg caffeine is increasing consummatory behaviors for alcohol. Consummatory behaviors are those that involve the intake of the drug (Samson & Czachowski, 2003). There are also appetitive behaviors involved in the reinforcement process, which encourage the seeking of a drug before it is consumed (Samson & Czachowski, 2003). If appetitive behaviors are those that occur before the drug is introduced within the system, these should be evident within the first 5 min time bin, when subjects are making their initial responses

for the sweetened alcohol reinforcer (Samson & Czachowski, 2003). If caffeine was increasing alcohol intake by increasing drug-seeking behavior, we would expect to see a significant increase in responding at the 5 min time bin in subjects treated with caffeine, relative to those treated with saline. The results of the current study show no significant difference in responding within the first 5 minutes, suggesting that caffeine is not significantly altering alcohol-seeking behavior.

However, the results of Experiments 3 and 4 do show a significant increase in responding for the sweetened alcohol solution at the 10 min, 15 min, and 30 min time bins. This increased responding occurs after alcohol is already "on-board," a time when subjects may be beginning to experience the pharmacological effects of alcohol, making it a consummatory behavior (Samson & Czachowski, 2003). Therefore, the results of the time analysis of the third and fourth experiments indicate that a 5 mg/kg dose of caffeine increases consummatory behaviors for alcohol. Because alcohol is "on-board" at the time caffeine increases alcohol intake, and caffeine does not increase alcohol intake initially, these results support that caffeine and alcohol must interact for caffeine to increase the reinforcing efficacy of alcohol. The following two experiments investigated the possibility of the A2A-D2 receptor complex as a neurobiological mechanism for how this drug interaction leads to increased alcohol intake.

Experiments 3 and 4: Caffeine and Eticlopride on Fixed Ratio and Progressive Ratio Responding

Eticlopride was administered in combination with caffeine to subjects to test the hypothesis that caffeine enhances motivation for and consumption of alcohol through increased dopamine signaling at the A2A-D2 receptor complex. When caffeine antagonizes the A2A receptor, enhanced dopamine signaling at the D2 receptor is permitted, resulting in an increase in striatopallidal neuron activity (Ferré et al., 2018). This increased activity by the striatopallidal

neuron decreases the activity of the indirect pathway, permitting drug-directed behaviors to occur (Ferré et al., 2018). The current study found that eticlopride (0.01 mg/kg) alone significantly decreased the number of active lever presses, reinforcers obtained, and alcohol intake. Although caffeine (5 mg/kg) did significantly increase alcohol self-administration in Long Evans rats, this effect was not reversed by eticlopride co-administration.

It was hypothesized that caffeine's increase in alcohol self-administration might be due to enhanced reinforcement. Enhanced motivation, effort, or reward signaling may have been produced by caffeine due to its effects on the A2A-D2 heterotetramer complex, in which caffeine's blockade of the A2A receptor ultimately leads to an increase in D2 signaling and to an inhibition of the indirect pathway, allowing for increased drug-directed behaviors to occur (Ferré et al., 2018). If caffeine does enhance dopamine signaling through its antagonistic effects at the A2A receptor, the administration of eticlopride, a dopamine D2 receptor antagonist, in conjunction with caffeine should result in operant responding similar to that of the saline control condition. This would indicate that eticlopride had counteracted caffeine's enhancement of D2 receptor signaling and would imply that, at least in part, caffeine's increase in alcohol self-administration and motivation was due to its effects at the A2A-D2 receptor complex, by enhancing D2 signaling and inhibiting striatopallidal neurons (Ferré et al., 2018). However, the results of the third and fourth experiments did not support that prediction. There was no significant interaction of caffeine and eticlopride in the third experiment, and while the fourth experiment did show a significant interaction between caffeine and eticlopride, eticlopride still did not reverse caffeine's effects on alcohol reinforced responding of self-administration. Caffeine increased active lever responses by 48.34% compared to the saline control, while the combination of caffeine and eticlopride increased active lever responses by 55.8% relative to

eticlpride alone. In other words, operant responding for alcohol was still elevated by caffeine, even in the presence of an effective dose of eticlopride that decreased responding on its own. These data strongly support that caffeine is a reinforcement enhancer of alcohol. However, because eticlopride did not reverse the effects of caffeine on alcohol-reinforced responding, we cannot conclude that the A2A-D2 receptor complex has a significant role in caffeine's effects on alcohol intake.

The Role of Eticlopride and Dopamine D2 Receptor Antagonism

It is possible that the A2A-D2 receptor complex does play a role in caffeine-induced increases in alcohol consumption and that an aspect of the current study's design prohibited the finding of such results. The 0.01 mg/kg dose of eticlopride used in the current study was based on a range of eticlopride doses from previous research and pilot testing in our own laboratory (Arolfo et al., 2004; Barrett et al., 2004; Fowler & Liou, 1998; Hemby et al., 1996). Because eticlopride is a D2 receptor antagonist and can have sedative locomotor effects (Arolfo et al., 2004; Fowler & Liou, 1998) it could have decreased responding for the sweetened alcohol solution. Therefore, the goal for the current study was to use a dose that would be large enough to block D2 receptor signaling and counteract caffeine's antagonistic effects at the A2A receptor, but that was also small enough to not significantly affect responding for alcohol alone (Arolfo et al., 2004; Fowler & Liou, 1998). However, the current study found that 0.01 mg/kg eticlopride significantly decreased active lever responses, number of reinforcers obtained, and alcohol intake. This result indicates that the 0.01 mg/kg dose of eticlopride was likely too high of a dose and may have produced sedative locomotor side effects or a decrease in reinforcing efficacy through its blockade of dopamine that led to a decrease in operant responding.

A decrease in the reinforcing efficacy of alcohol may be the more likely explanation, due to the lack of change in overall locomotor activity. If inactive lever responses were significantly decreased with eticlopride as compared to the saline control condition, this might indicate that eticlopride decreased overall locomotor activity in the form of lever-pressing behavior, including both the active and inactive levers. However, there was no significant difference in inactive lever responses between the eticlopride and saline conditions in the current study. This suggests that activity specific to the active lever and directed toward gaining the alcohol reinforcer was decreased, rather than overall locomotor activity. In fact, though it was not significant, the average number of inactive lever responses for eticlopride was greater than the average for saline, suggesting that eticlopride may have decreased the specificity of lever-responding behavior relative to saline. Since eticlopride did not significantly affect overall activity and appeared to be leading to less specific responses for alcohol, its decrease in operant responding for alcohol was likely due to a decrease in the reinforcing efficacy of alcohol rather than due to locomotor depressant effects.

The simplest way to more accurately assess this A2A-D2 receptor interaction using eticlopride might be to change the dose. Perhaps if a smaller dose of eticlopride was used, it would eliminate the possible attenuation of alcohol-reinforced responding that occurred in this study. However, the method of decreasing the dose of eticlopride may not be entirely effective when also considering there was no significant interaction between caffeine and eticlopride dose in the third experiment. This lack of interaction at 0.01 mg/kg dose of eticlopride suggests that a higher dose may be necessary for eticlopride to successfully block D2 receptors and counteract a significant portion of caffeine's D2-enhancing effects. However, if the dose of eticlopride was to be increased, the higher dose would likely reduce operant responding for alcohol on its own, to

an even greater extent than in the current study. Since eticlopride significantly decreased operant responding for alcohol at the current dose of 0.01 mg/kg, raising that dose in an attempt to block caffeine's effects at A2A-D2 complex would likely only lead to a further decrease in operant responding for alcohol. Because adjusting the dose of eticlopride would mainly have effects on operant responding for alcohol, the present data likely supports that an interaction between caffeine and eticlopride is unlikely to occur.

While these results do not support the hypothesis that the A2A-D2 receptor complex has a significant role in caffeine-induced increases in alcohol consumption, the results provide strong evidence in clarifying caffeine's effects on alcohol intake. Even when eticlopride, which was shown to decrease responding for alcohol on its own, was administered, moderate doses of caffeine were still able to increase operant self-administration for an alcohol solution. The results of the third and fourth experiments do not suggest that the A2A-D2 receptor complex has a role in caffeine's enhancement of alcohol intake. Therefore, other neurological mechanisms for caffeine's ability to increase alcohol intake must be explored.

Caffeine's ability to antagonize adenosine receptors is not specific to the A2A receptor investigated in the current study. It also antagonizes adenosine A1 receptors (Ribeiro & Sebastião, 2010). A1 receptors form complexes with and alter the signaling of D1 receptors (Ginés et al., 2000). These A1-D1 receptor complexes are often located on striatonigral neurons which modulate the direct pathway which is involved in mediating behavioral responses in the direction of reward-related stimuli (Ferré et al., 2018). Studies have suggested that, similar to the A2A-D2 receptor relationship, activity at the A1 receptor inhibits D1 activity through allosteric actions, or affecting how well ligands bind to D1 receptors, and through the cAMP second messenger system (Ferré et al., 1998; Ginés et al., 2000). Dopamine signaling at D1 receptors

has been shown to be important for alcohol self-administration. Various D1 receptor antagonists have decreased the self-administration of alcohol, suggesting that D1 activity is important for the reinforcing efficacy of alcohol (Hodge et al., 1997; Price & Middaugh, 2004). Therefore, caffeine's effects at the A1-D1 receptor complex may promote alcohol self-administration.

By administering caffeine, the A1 receptor's antagonistic effects on the D1 receptor should be removed. Increased D1 activity would follow, which is characterized by increased excitability of striatonigral neurons and increased long-term potentiation of their synapses (Gerfen & Surmeier, 2011). By increasing the excitability of these neurons and laying stronger foundations for their synaptic connections through long-term potentiation, caffeine may be increasing direct pathway behaviors that promote drug-seeking or consumption. Therefore, the A1-D1 receptor complex may also be worth investigating in future research concerning interactions between caffeine and alcohol.

Limitations and Future Directions

There are several ways upon which the current series of experiments can and should be improved in order to continue expanding upon the knowledge of how caffeine affects the reinforcing efficacy of and consummatory behaviors related to alcohol.

First and foremost, the use of a sweetened alcohol solution is of concern. While the animals in this study did consistently respond for this solution and did reach levels of alcohol intake that were pharmacologically relevant (Chappell & Weiner, 2008), the possibility remains that the subjects in the current study were exhibiting lever-pressing behavior due to the two percent sucrose that was mixed with the 10 percent alcohol in the drinking solution. This may be especially concerning, as previous research has shown that caffeine has the ability to increase operant responses for sucrose alone (Sheppard et al., 2012). Currently, we are conducting an

experiment in which a new group of Long Evans rats (n = 8) is self-administering a sucrose solution on a fixed ratio (FR2) and progressive ratio schedule. If caffeine significantly increases responding for a sucrose solution, it may indicate that subjects in the current study may have partially responded for the alcohol solution due to its sucrose content and not the alcohol only. However, this result would not be unexpected due to previous research that has shown caffeine increases responding for sucrose (Sheppard et al., 2012). If caffeine increases responding for sucrose, it would further support that caffeine's general reinforcement properties, that extend to even non-drug stimuli. Conversely, if caffeine does not significantly affect operant responses for a sucrose solution, it would indicate that caffeine's increased responses for a sweetened alcohol solution in the current study are directed toward the alcohol and not the sucrose. This result would provide continued support for caffeine as a strong reinforcement enhancer for alcohol and add to the literature that suggests caffeine enhances the reinforcement of various drug stimuli (Gannon et al., 2018; Prieto et al., 2016; Rezvani et al., 2013; Roldán et al., 2017).

Alternatively, another way to test whether caffeine is enhancing operant responses for only alcohol would be to use a different strain of rat. While the Long Evans rats used in the current study did reach pharmacologically relevant doses of alcohol, they only did this on a sweetened alcohol solution. There is a selectively bred line of alcohol-preferring rats that will consistently exhibit high responses for and drink pharmacologically relevant doses of alcohol alone (Bell, Rodd, Lumeng, Murphy, & McBride, 2006). It may be advantageous for future studies to consider using these lines of animal subjects to replicate these experiments so that we can be sure caffeine's enhancement of operant responding is related to alcohol alone.

There may also be the concern that because caffeine is a stimulatory drug, the moderate doses of caffeine that increase operant responding may be increasing lever-pressing behavior due

to the locomotor stimulant effect of caffeine. While it was explained previously that this was likely not the case in the current study due to the low number of inactive lever presses, it would still be advantageous to ensure that caffeine is not producing behavioral effects due to enhanced locomotor stimulation. It is possible to monitor locomotor activity with the use of infrared technology (Clarke, Smith, & Justesen, 1985). This technology would make it possible to compare measured levels of locomotor activity after the administration of each caffeine dose to ensure that general motor activity is not significantly enhanced by moderate doses of caffeine and that caffeine's enhancement of operant responding is due to its effects on reinforcing efficacy instead.

Eticlopride: Future Directions

It may still be advantageous to replicate this study again using eticlopride because the dose affected responding for alcohol when it was administered alone. To improve upon the current study's design and potentially get more certain answers about the involvement of the A2A-D2 receptor complex, the route of administration of eticlopride could be changed. In the current study, eticlopride was systemically administered through an intraperitoneal injection. Due to this type of injection, eticlopride will have effects throughout the brain. Although dopamine D2 receptors are more predominantly expressed in the striatum, there are other D2 receptors outside the reward circuit that eticlopride may have been antagonizing (Ferré et al., 2018). By using the method of surgically implanting cannulae into the brain, it is possible to pharmacologically target specific brain areas. Several previous studies have been successfully completed by site-specific administration to the NAc (Laviolette, Lauzon, Bishop, Sun, & Tan, 2008; Yawata, Yamaguchi, Danjo, Hikita, & Nakanishi, 2012). By stereotaxically targeting the NAc, from which the striatopallidal neurons containing the A2A-D2 receptor complexes

originate (Ferré et al., 2018), we can obtain an accurate injection site for eticlopride and thereby know for certain in which brain regions dopamine D2 receptors are being inhibited.

Implications for AmED Use

The results of the current study add support to the literature that caffeine increases alcohol intake. These results in an animal model support the reports of human participants who report that when they drink AmED they consume more drinks per session (Arria et al., 2010; O'Brien et al., 2008). Though the results do not support that caffeine increases motivation or reward for alcohol through increased dopamine signaling at the A2A-D2 receptor complex, they provide strong evidence for caffeine as a reinforcement enhancer due to its ability to continue enhancing operant self-administration even with the administration of the D2 antagonist eticlopride. Because of the elevated operant responses, particularly those discovered to be occurring at 10, 15, and 30 min time points, it can be suggested that moderate doses of caffeine in combination with alcohol may be increasing consummatory behaviors. Samson and Czachowski (2003) state that consummatory behaviors are associated with a "loss of control" of drinking behavior. If caffeine significantly increases consummatory behaviors for alcohol as the results of the current study suggest, the use of an energy drink in combination with alcohol may be promoting a loss of controlled drinking behavior, leading to dangerous levels of alcohol intake.

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Table 1

Sucrose Fading Procedure

Percent sucrose	Percent ethanol	Minimum days at concentration
10	0	2
10	2	2
10	5	2
10	10	2
7.5	10	2
5	10	2
2	10	2
0	10	2
2	10	39

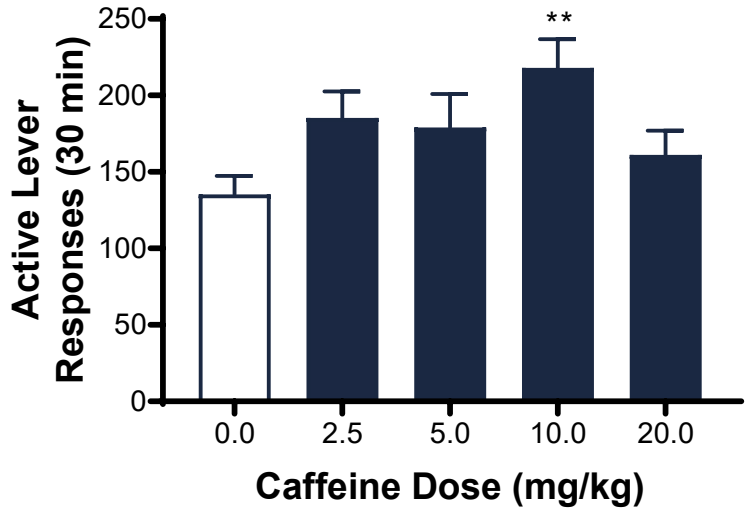

Figure 1. A 10 mg/kg dose of caffeine significantly increased active lever responses on an FR2 schedule of reinforcement for a 10E2S sweetened alcohol solution. Error bars represent standard error. ** indicates a significant difference ($p < .05$) from the saline condition.

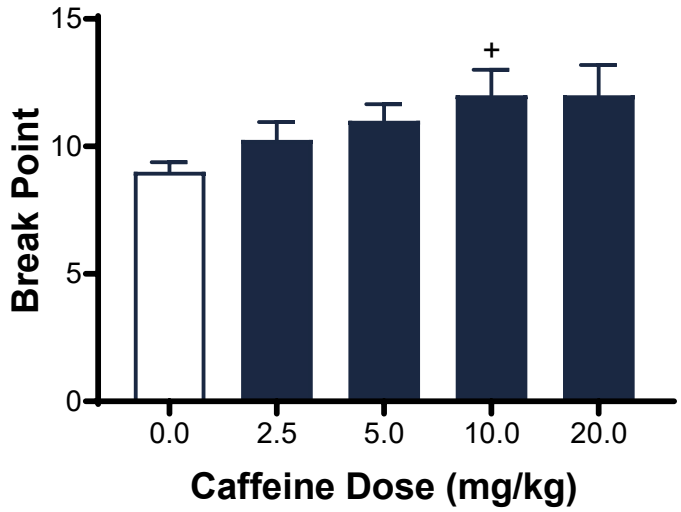

Figure 2. The results of a t-test revealed that a 10 mg/kg dose of caffeine significantly increased breaking point on a PR schedule of responding as compared to the saline control. + indicates a significant difference ($p < .05$) from the control condition.

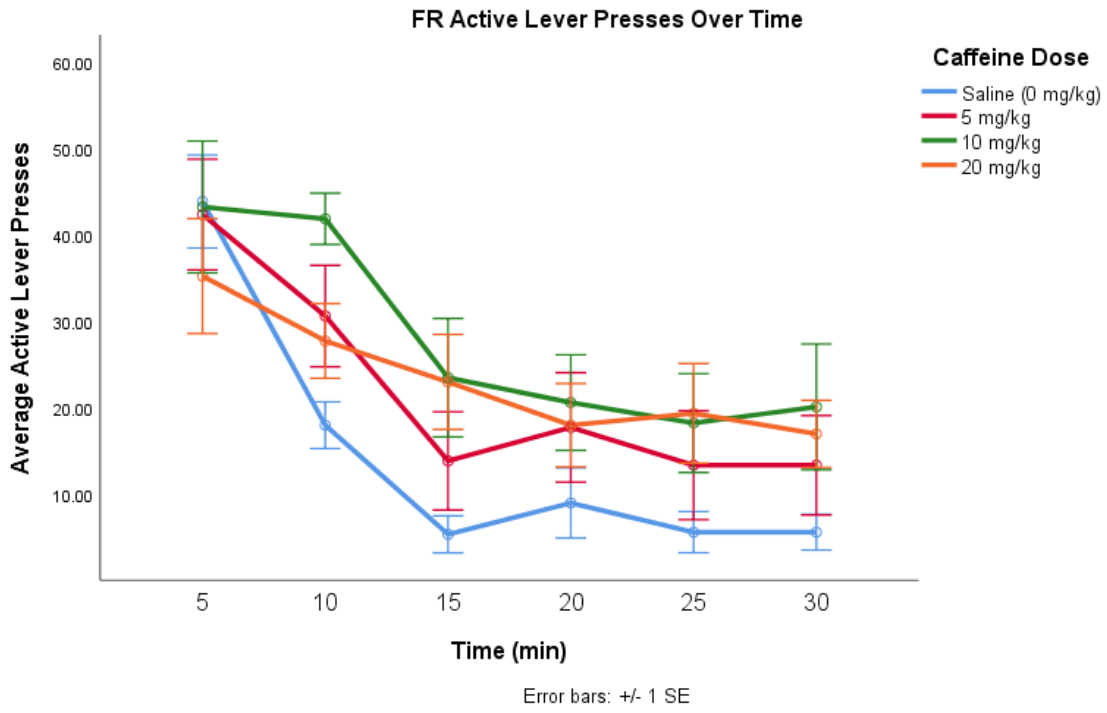

Figure 3. There were no significant differences in average active lever responses between saline and 5, 10, or 20 mg/kg caffeine at each 5 min time bin during 30 min FR2 operant sessions. Error bars represent standard error.

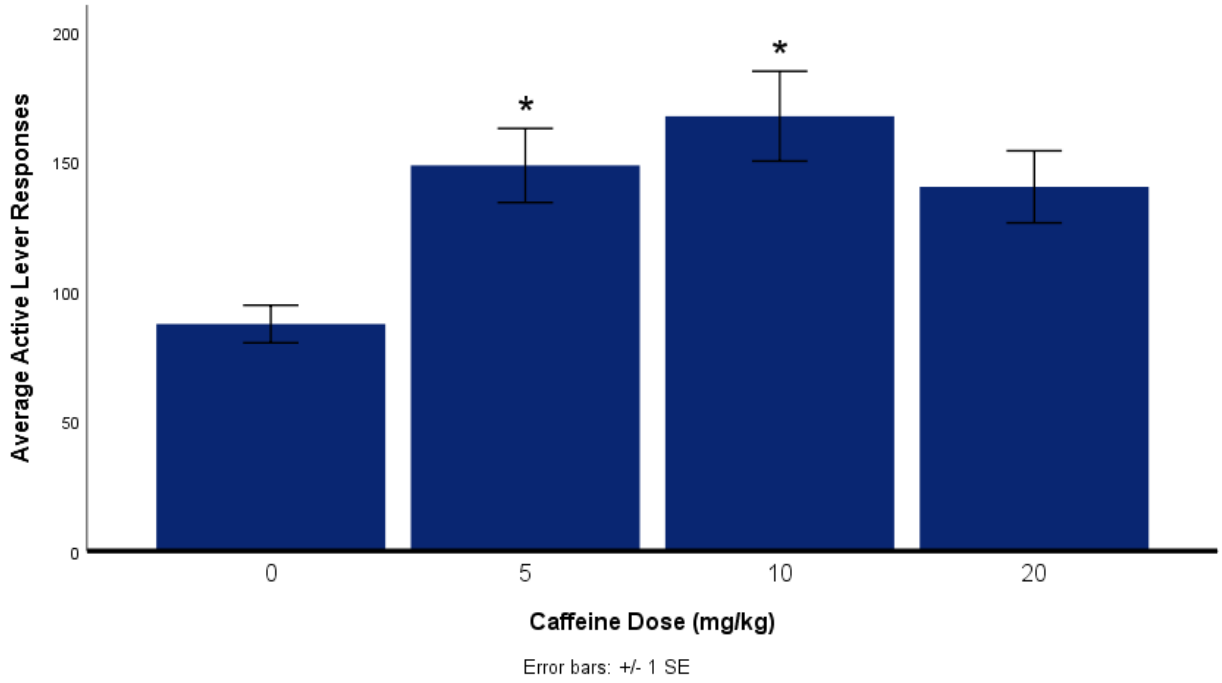

Figure 4. The 5 and 10 mg/kg doses of caffeine significantly increased active lever responses on an FR2 schedule as compared to the saline control. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

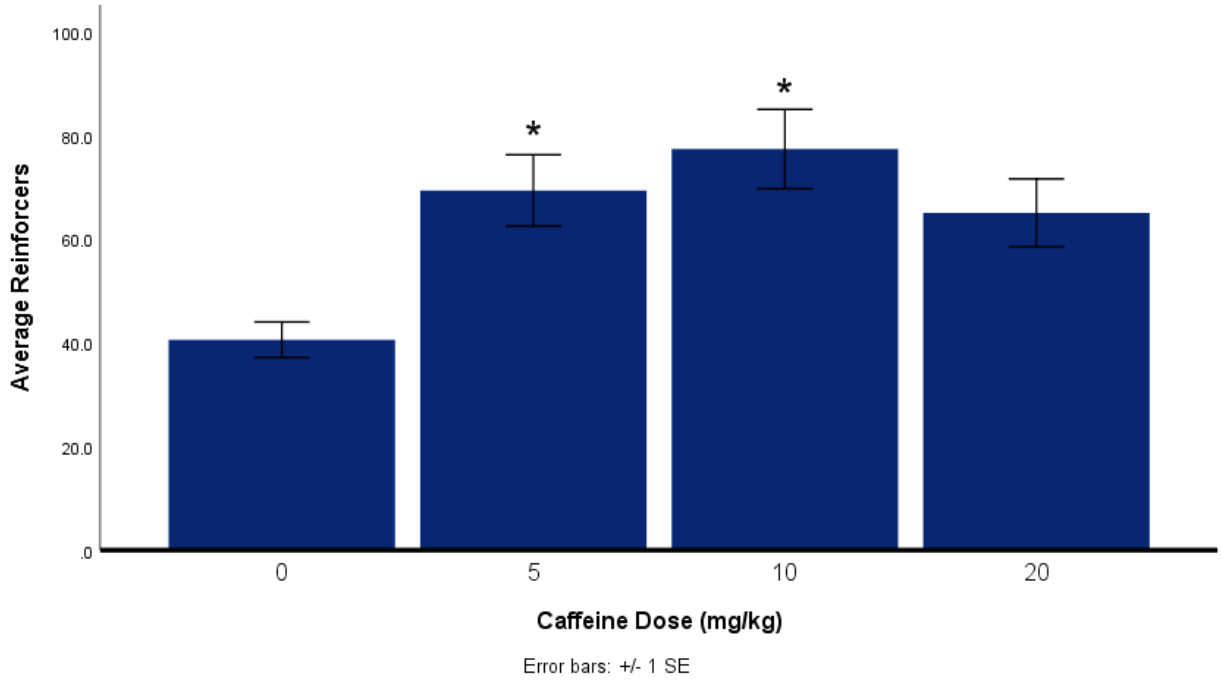

Figure 5. The 5 and 10 mg/kg doses of caffeine significantly increased the number of reinforcers obtained on an FR2 schedule of reinforcement as compared to the saline control. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

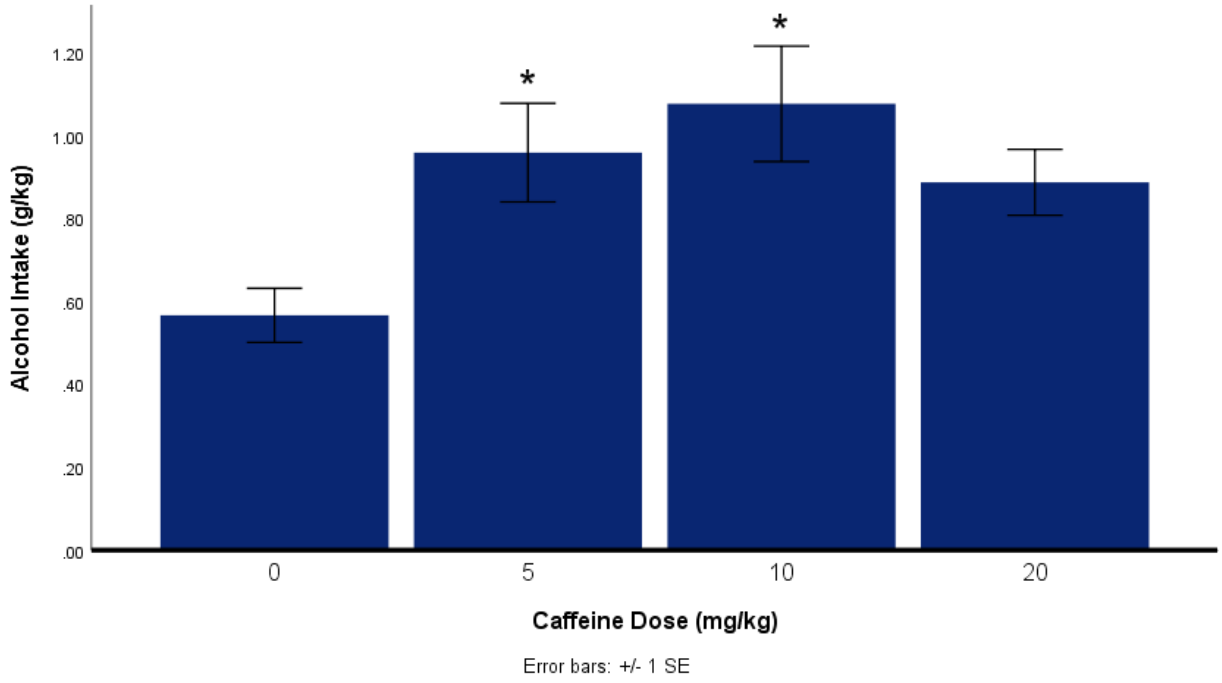

Figure 6. The 5 and 10 mg/kg doses of caffeine significantly increased alcohol intake (g/kg) on an FR2 schedule of reinforcement as compared to the saline control. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

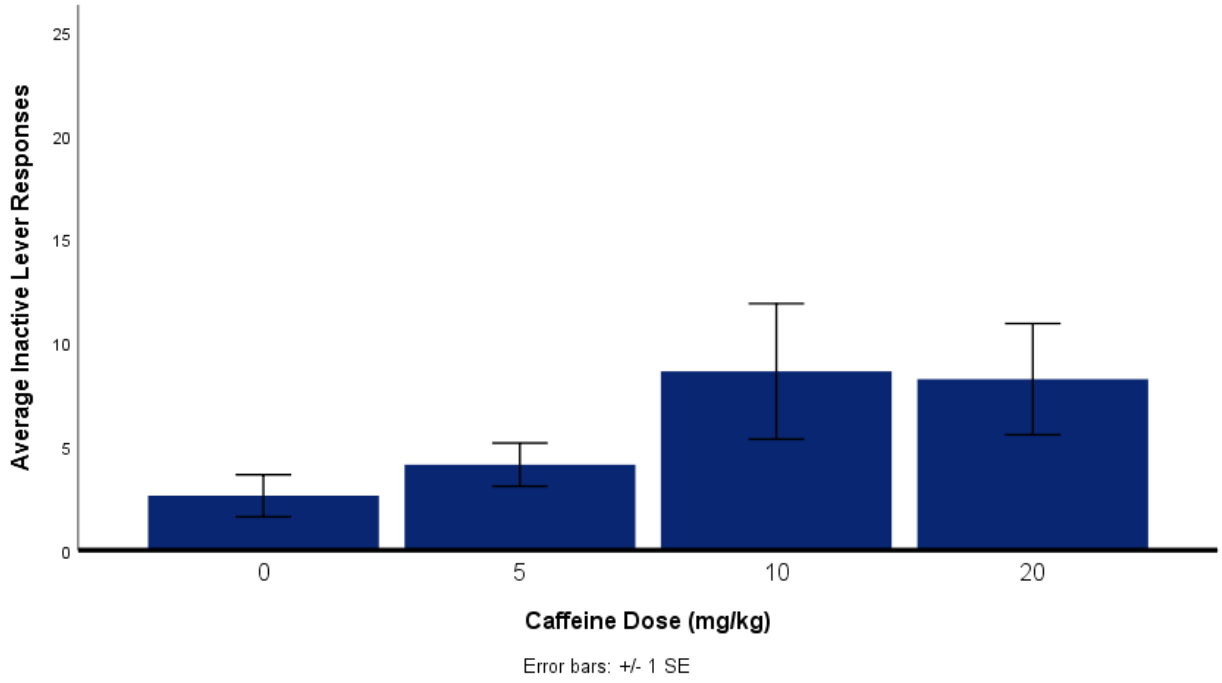

Figure 7. There were no significant differences in inactive lever responses on an FR2 schedule between caffeine doses and the saline control. Error bars represent standard error.

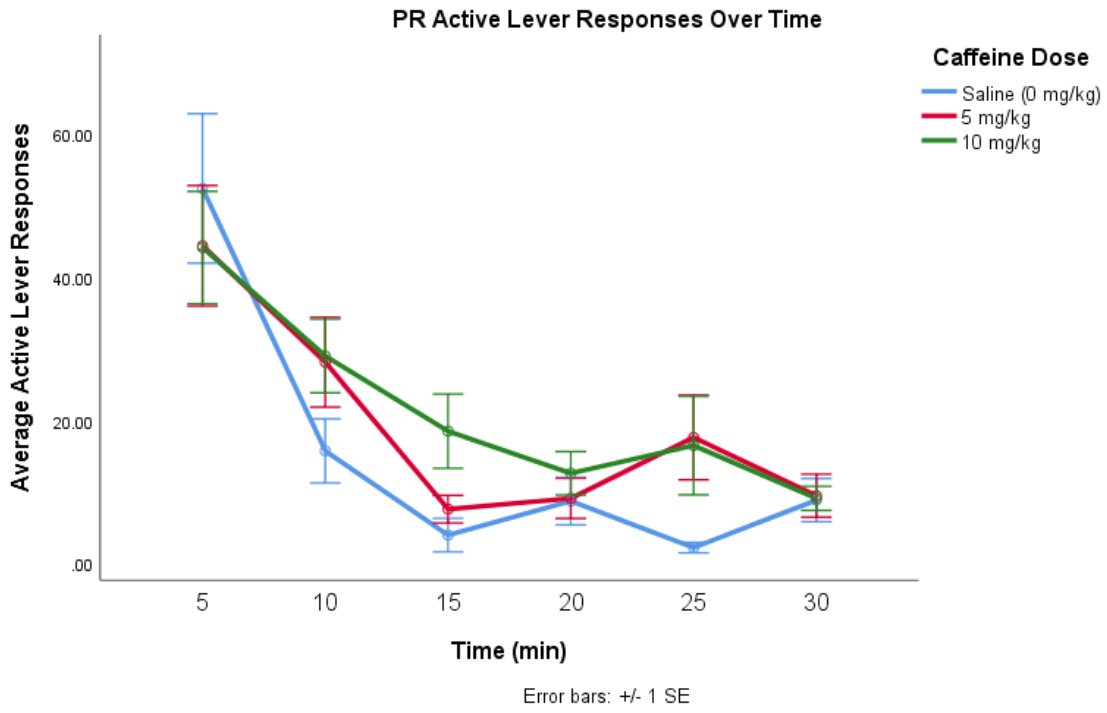

Figure 8. There was no significant difference in active lever responses between saline and 5 or 10 mg/kg caffeine at each 5 min time bin for PR operant self-administration sessions. Error bars represent standard error.

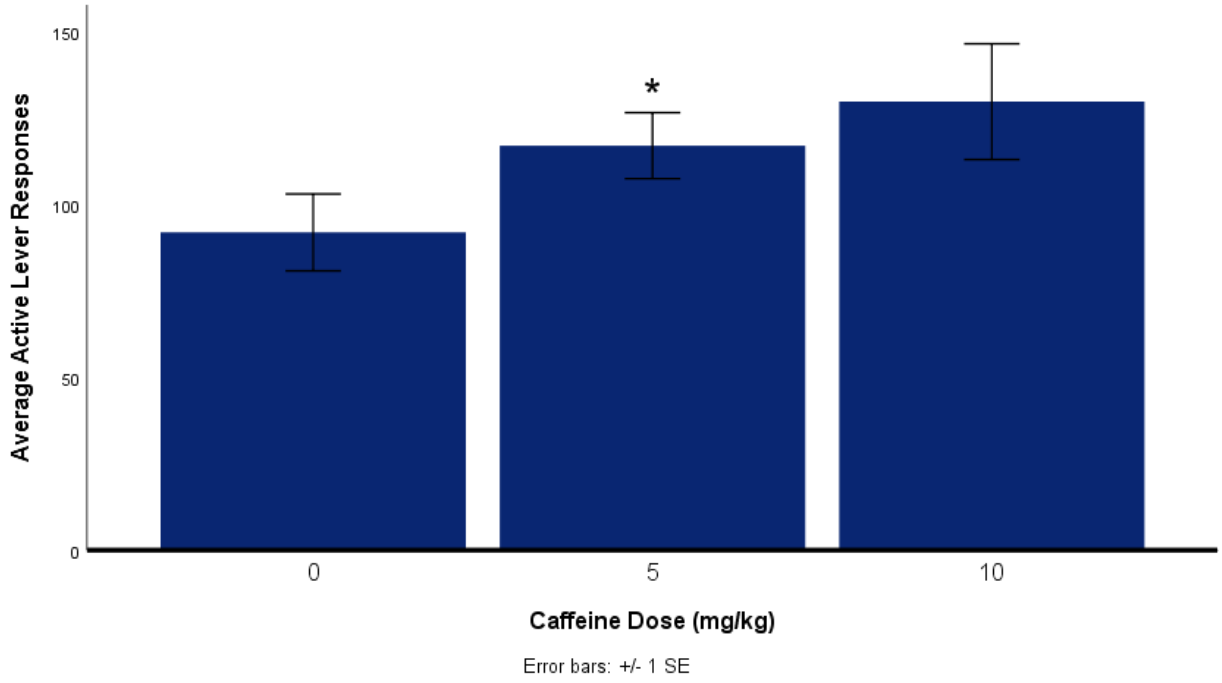

Figure 9. The 5 mg/kg dose of caffeine significantly increased active lever responses on a PR schedule of reinforcement as compared to the saline control. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

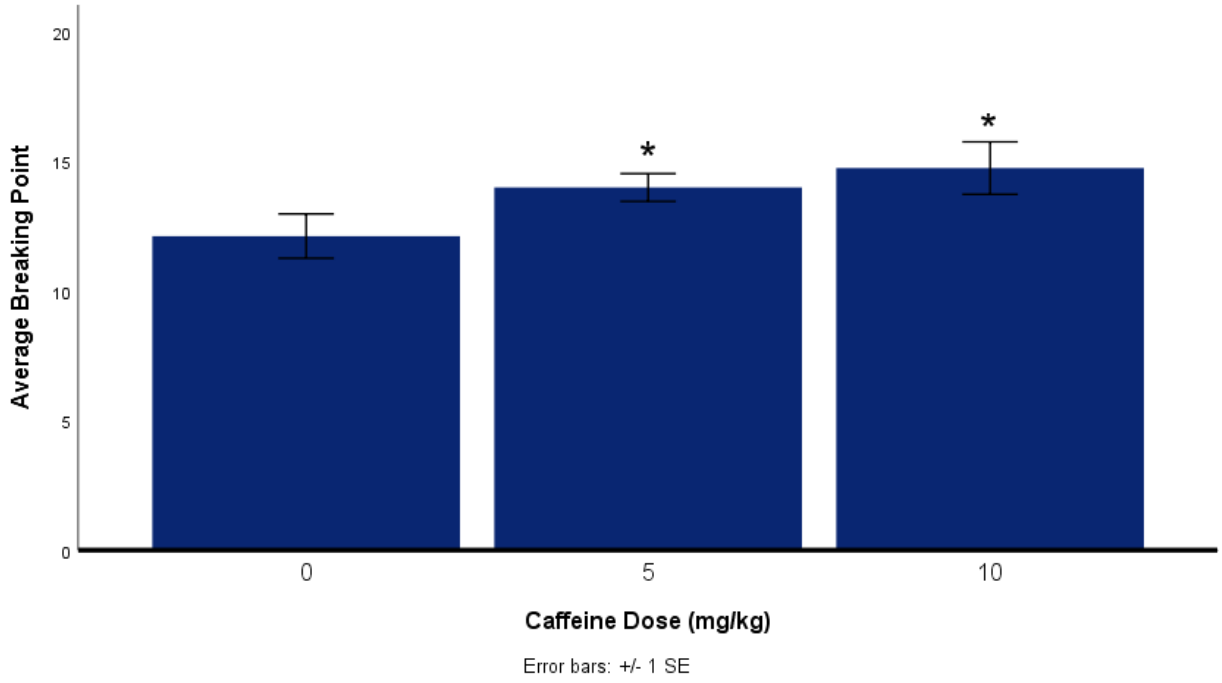

Figure 10. The 5 and 10 mg/kg doses of caffeine significantly increased breaking point on a PR schedule as compared to the saline control. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

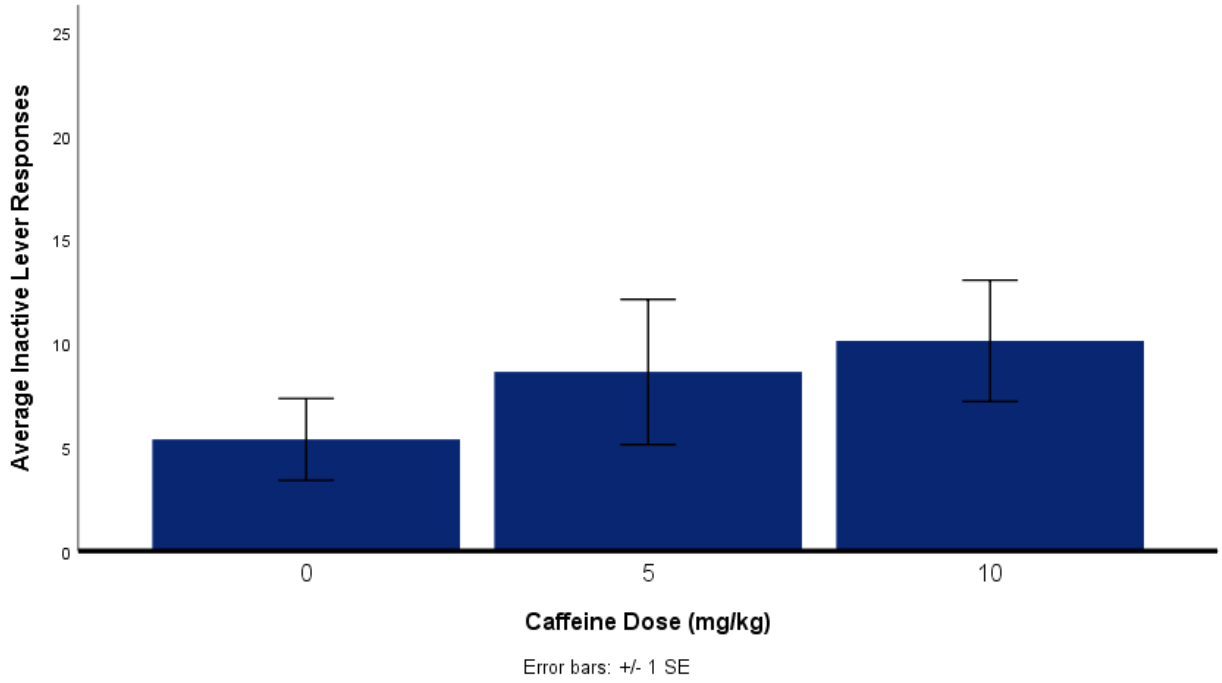

Figure 11. There were no significant differences in inactive lever responses between the caffeine doses and the saline control on a PR schedule of reinforcement. Error bars represent standard error.

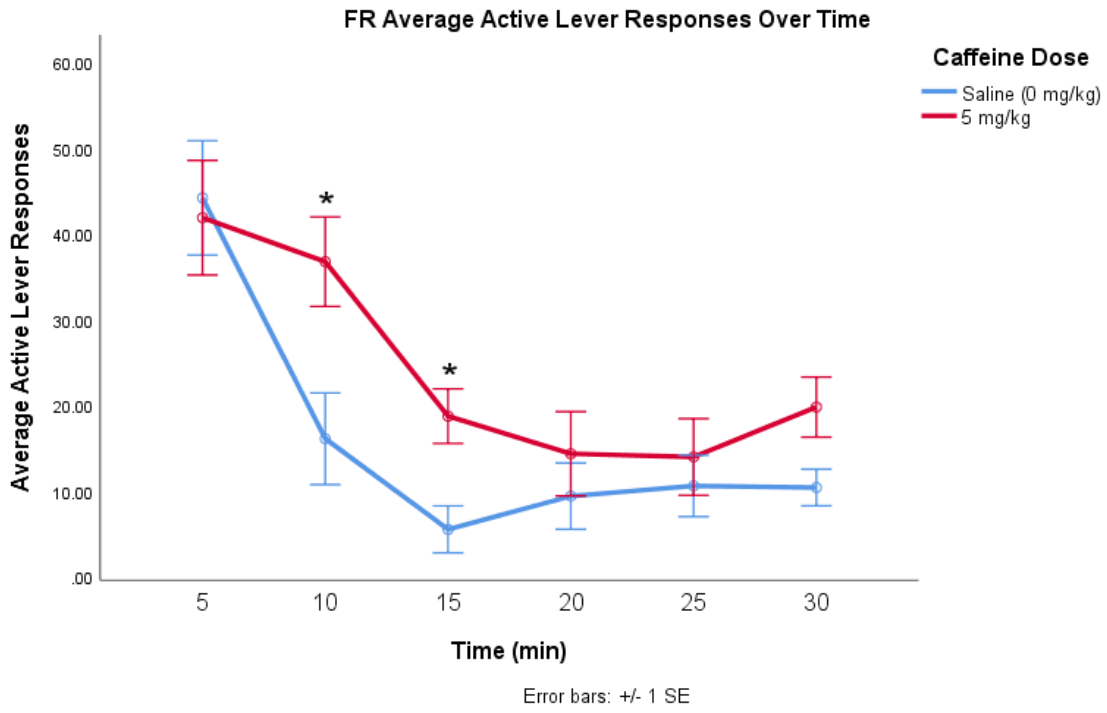

Figure 12. A 5 mg/kg dose of caffeine significantly increased active lever responses at 10 and 15 min time bins on an FR2 schedule of reinforcement. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

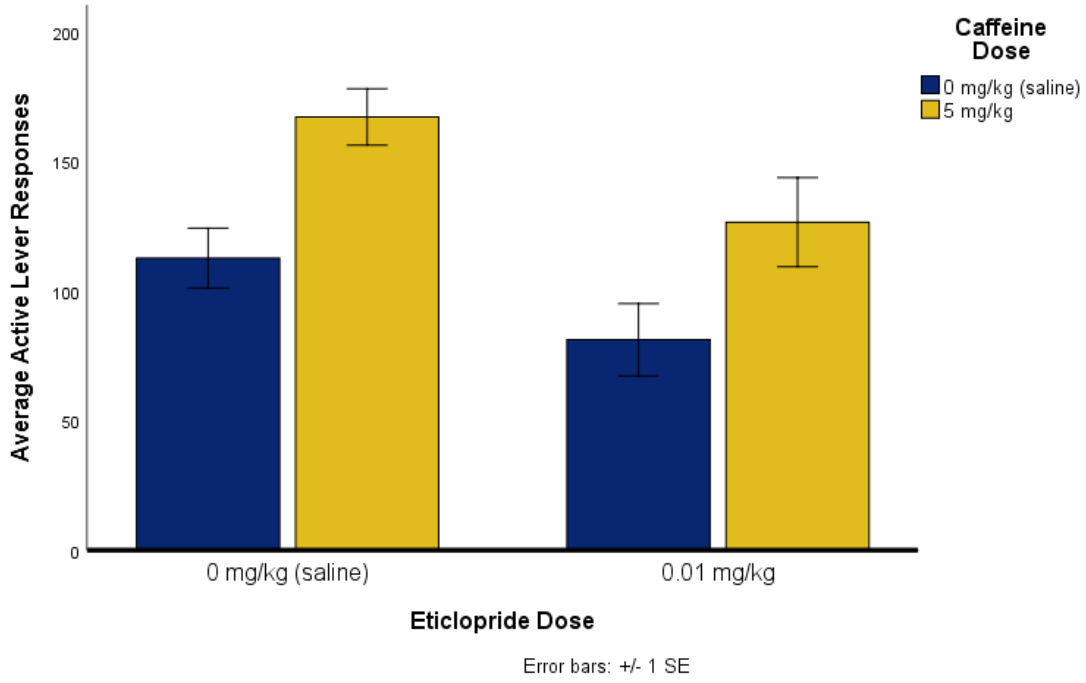

Figure 13. The 5 mg/kg dose of caffeine significantly increased active lever responses as compared to the saline control. The 0.01 mg/kg eticlopride dose significantly decreased active lever responses as compared to the saline control. There was no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.

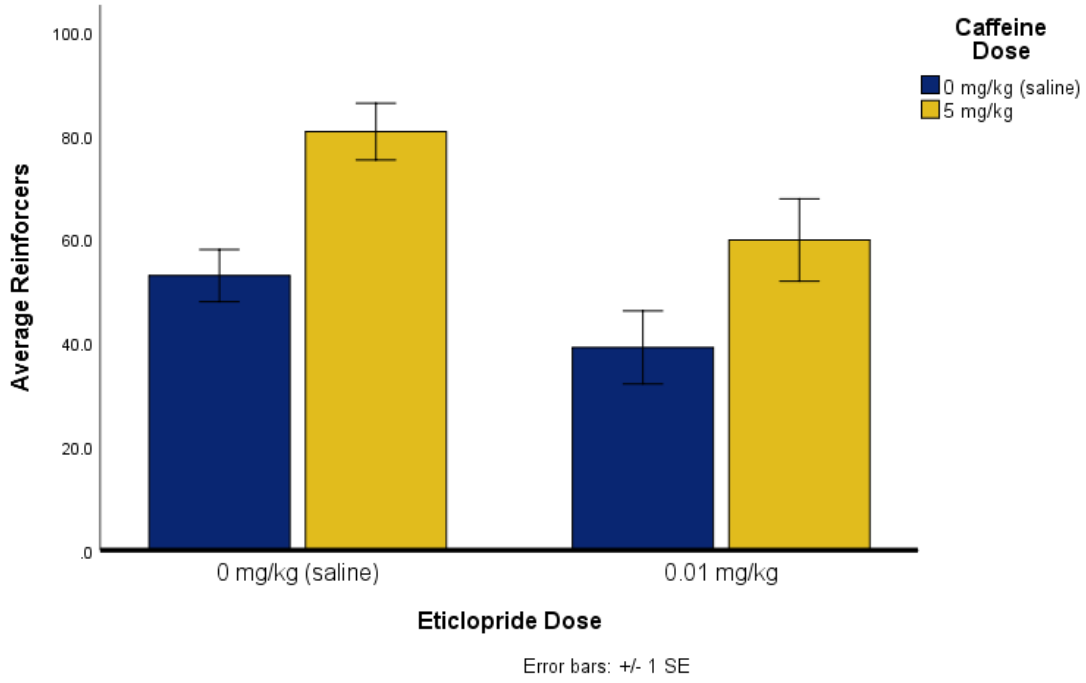

Figure 14. On an FR2 schedule of reinforcement, the 5 mg/kg dose of caffeine significantly increased reinforcers obtained as compared to the saline control. The 0.01 mg/kg eticlopride dose significantly decreased reinforcers obtained as compared to the saline control. There was no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.

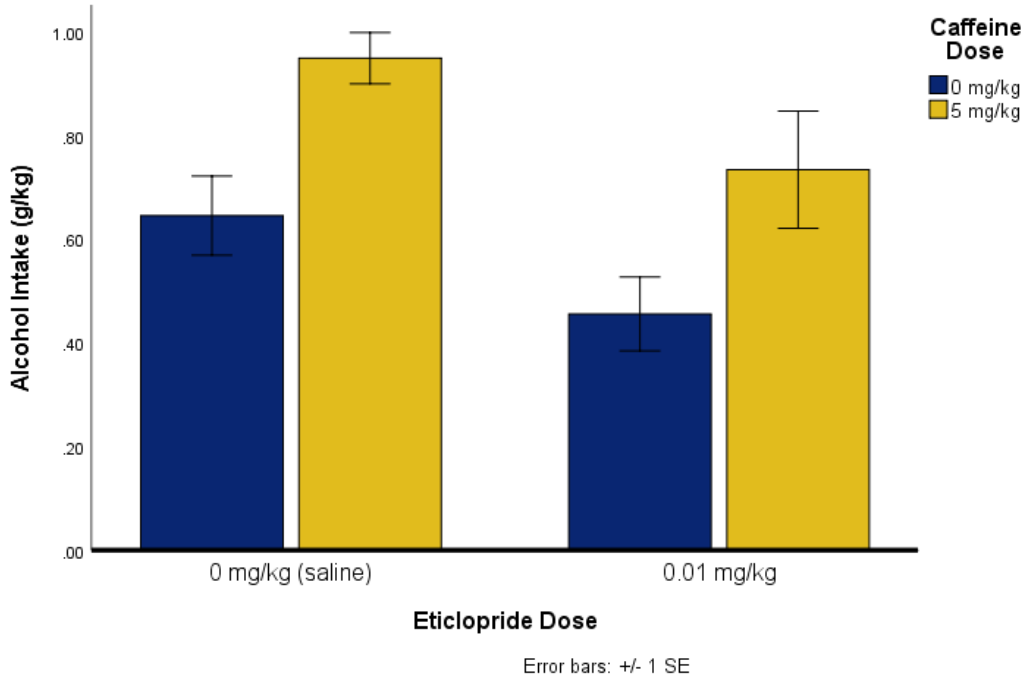

Figure 15. During FR2 operant sessions, a 5 mg/kg dose of caffeine significantly increased alcohol intake as compared to the saline control. The 0.01 mg/kg eticlopride dose significantly decreased alcohol intake as compared to the saline control. There was no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.

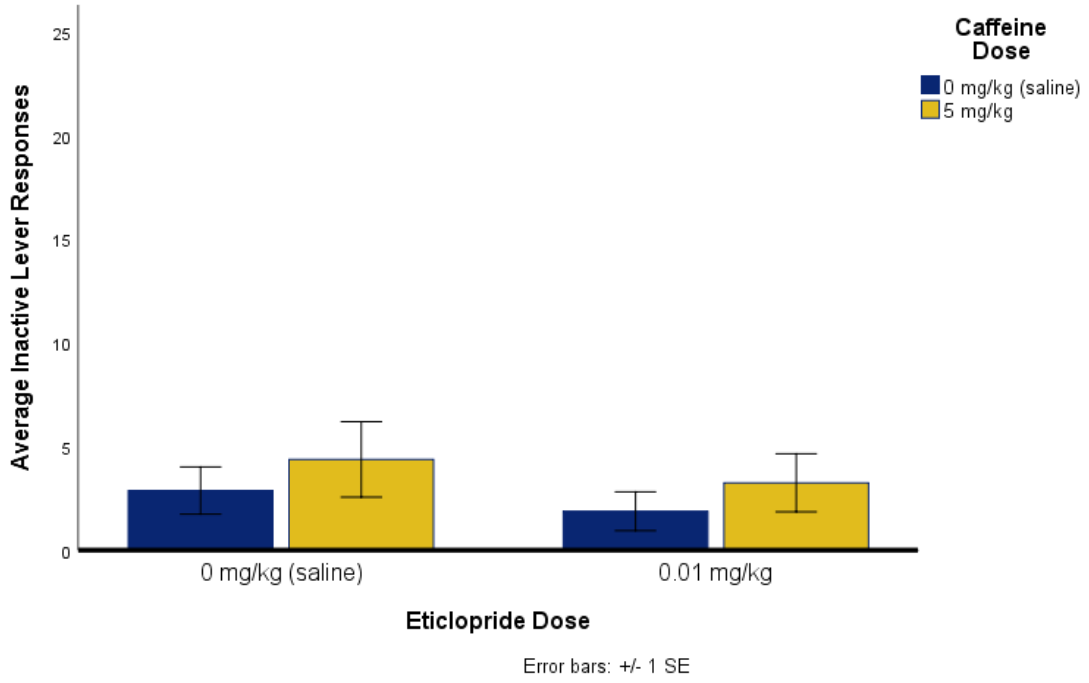

Figure 16. There was no significant effect of 5 mg/kg caffeine or 0.01 mg/kg eticlopride on inactive lever responses on an FR2 schedule of reinforcement. There was also no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.

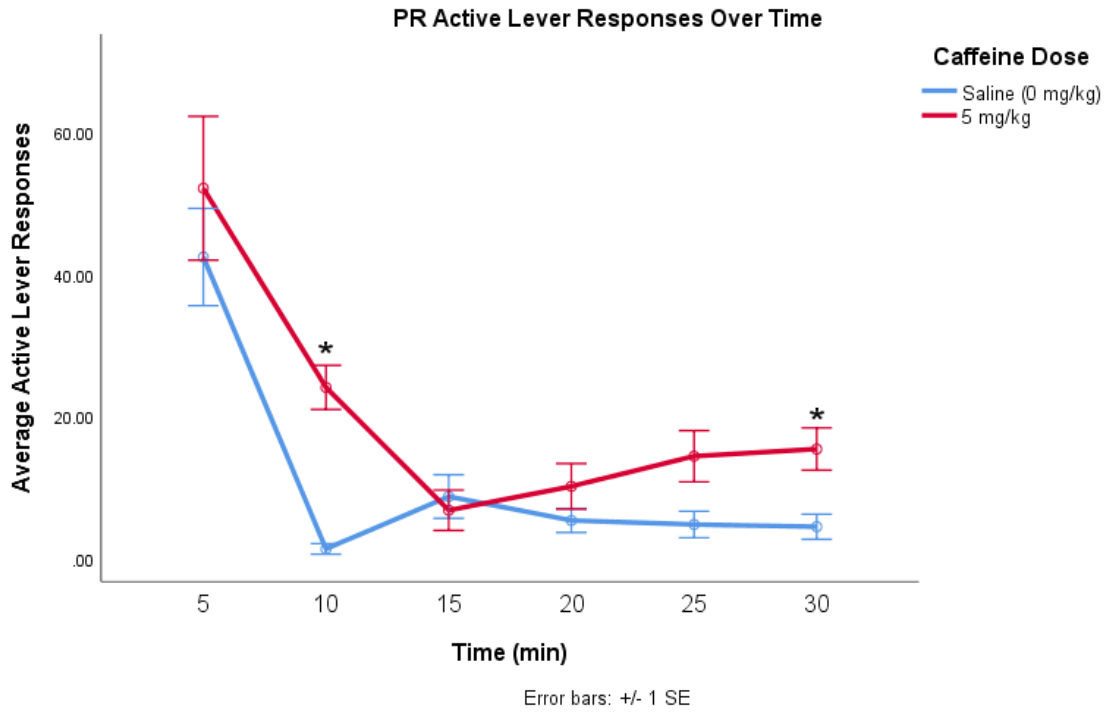

Figure 17. A 5 mg/kg dose of caffeine significantly increased active lever responses on a PR schedule at 10 and 30 minute time points relative to saline. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

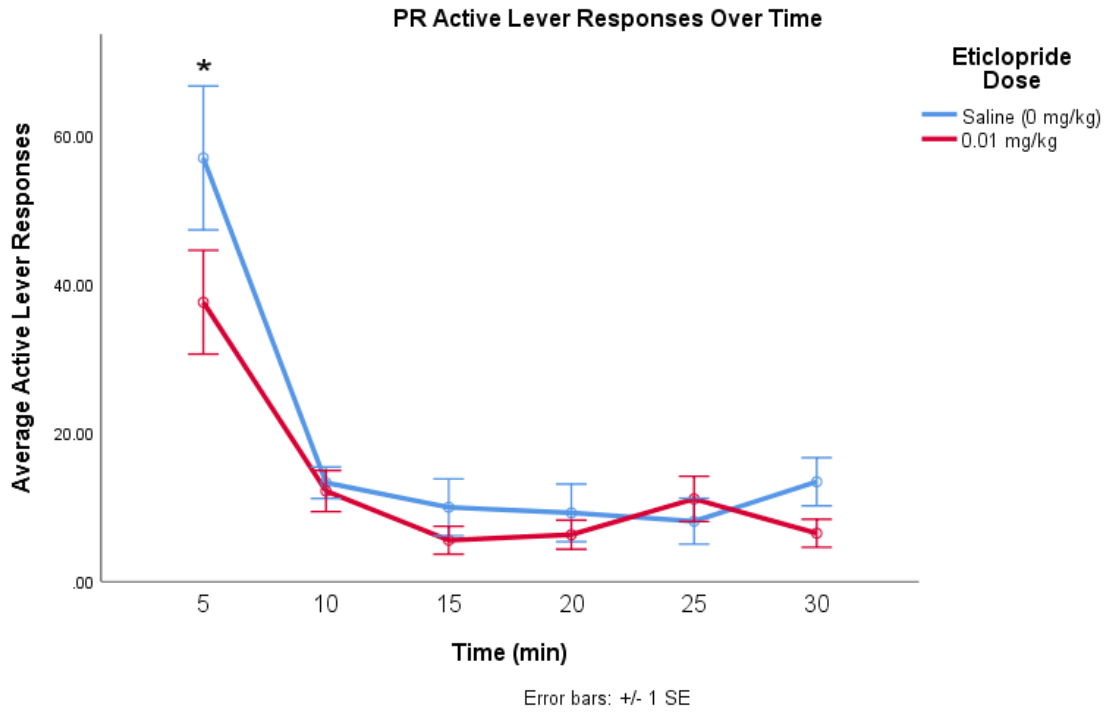

Figure 18. The 0.01 mg/kg dose of eticlopride significantly decreased active lever responses at the 5 minute time bin as compared to the saline control. Error bars represent standard error. * indicates a significant difference ($p < .05$) from the saline control condition.

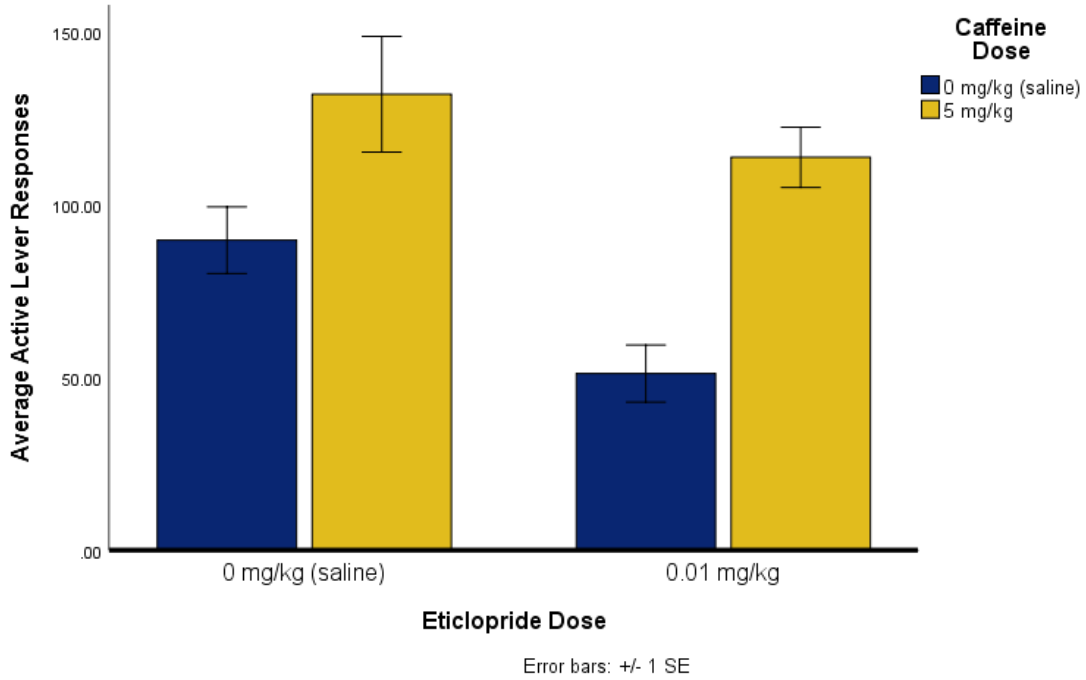

Figure 19. On a PR schedule of reinforcement, the 5 mg/kg dose of caffeine significantly increased active lever responses as compared to the saline control. The 0.01 mg/kg eticlopride dose significantly decreased active lever responses as compared to the saline control. There was no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.

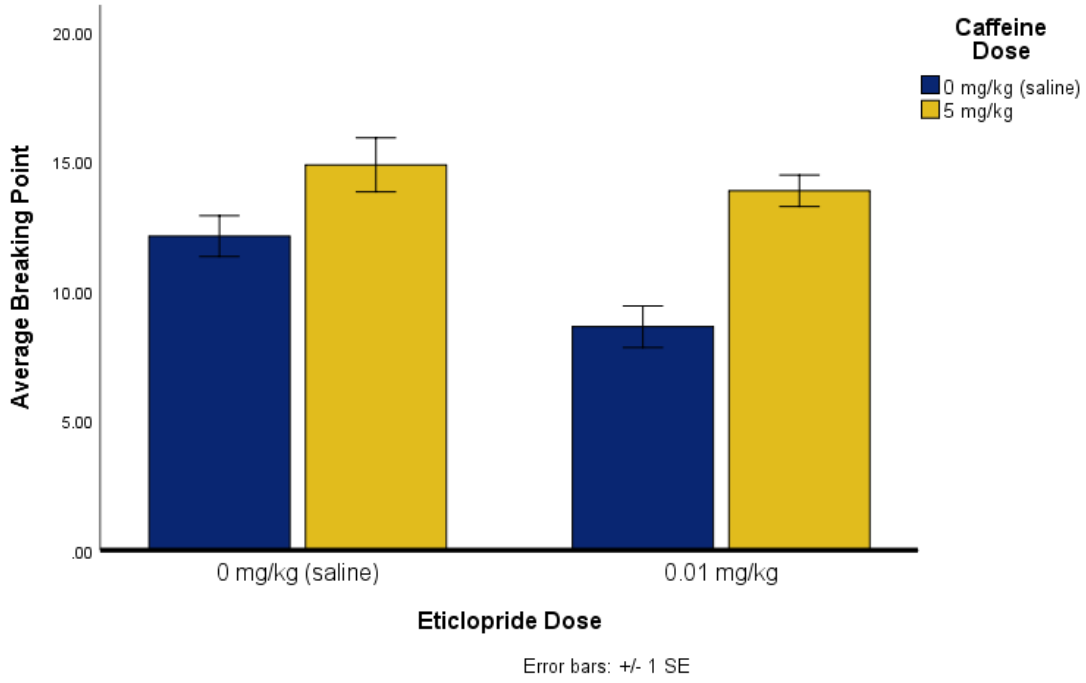

Figure 20. On a PR schedule of reinforcement, a 5 mg/kg dose of caffeine significantly increased breaking point relative to saline. The 0.01 mg/kg dose of eticlopride significantly decreased breaking point relative to saline. There was no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.

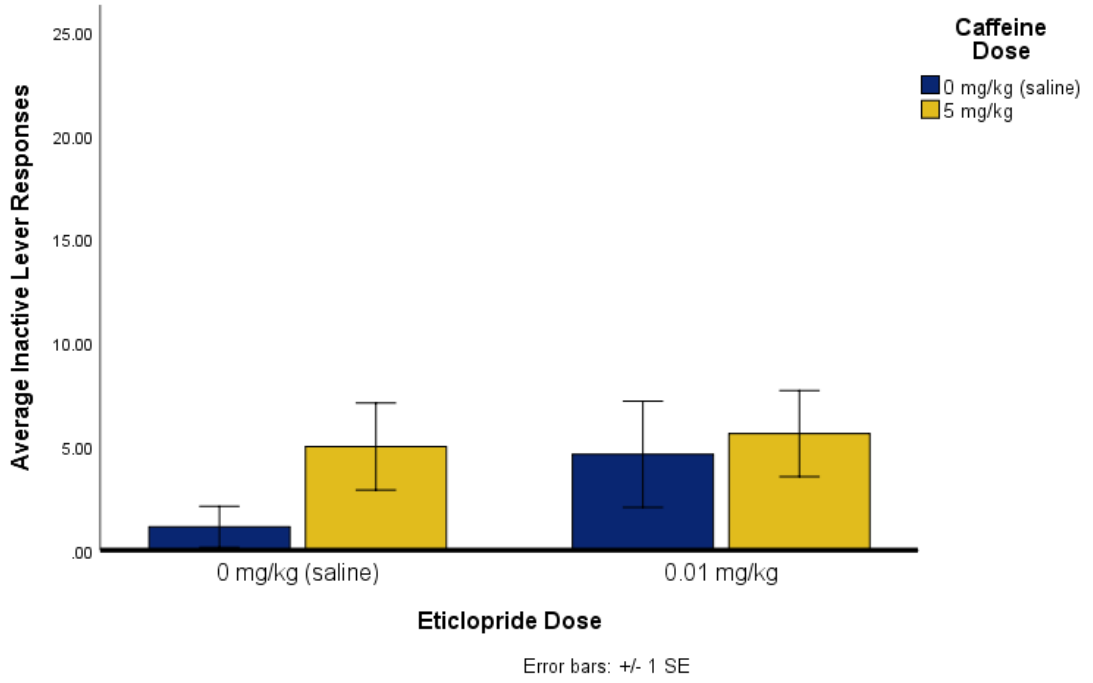

Figure 21. On a PR schedule of reinforcement, there was no significant effect of 5 mg/kg caffeine or 0.01 mg/kg eticlopride on inactive lever responses. There was also no significant interaction of caffeine and eticlopride dose. Error bars represent standard error.