THE INTERACTION OF ETHANOL AND NICOTINE IN MALE AND FEMALE ADOLESCENT RATS: EXAMINATION OF THEIR REWARDING EFFECTS AS A FUNCTION DOSE

A THESIS

Presented to the Department of Psychology

California State University, Long Beach

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Psychology

Option in Psychological Research

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August 2021

ABSTRACT

Ethanol and nicotine are two of the most commonly abused recreational substances, and adolescence is the most common stage of life to initiate their use. Currently, there is little preclinical research investigating the effects of coadministration of ethanol and nicotine in adolescence. While the rewarding effects of nicotine are well-established in adolescent rats, the data on ethanol reward is not as well-established. The most common and validated animal model to examine drug reward is the conditioned place preference (CPP) paradigm. Thus, the purpose of this study is twofold. First, we conducted an experiment to establish ethanol preferences in adolescent male and female rats using the CPP paradigm. Second, we then investigated the rewarding effects of ethanol and/or nicotine in male and female rats using the CPP paradigm. The CPP procedure took place over 10 days and consisted of three phases: preconditioning, conditioning, and postconditioning. During preconditioning, the rats had free access to both sides of the CPP box so that an initial side-preference could be established. After preconditioning, the rats underwent an 8-day conditioning period, consisting of four 2-day cycles. On day one, rats are administered ethanol and/or nicotine and then confined to their initially non-preferred side. The next day rats are administered saline and confined to their initially preferred side.
A biased CPP design was used, meaning the drug was paired with the rats' initially non-preferred side throughout conditioning to make shifts in preference easier to detect. During postconditioning, rats were again allowed free access to both sides of the CPP box to reevaluate their preference. In Experiment 1, ethanol-induced CPP depended on the pattern and quantity of ethanol dosing; however, unlike our hypothesis, the fixed pattern of ethanol produced more robust CPP preferences than the ascending pattern. Using the fixed pattern of ethanol dosing from Experiment 1, the coadministration of the two drugs did not produce strong preferences in

Experiment 2, although there was some indication of an interaction. These experiments have added to the literature concerning ascending versus fixed dosing in CPP and the understanding of how ethanol and nicotine interact in adolescent male and female rats. Future experiments are needed to uncover potential brain mechanisms involved.

ACKNOWLEDGEMENTS

I would first like to express my deepest appreciation to my mentor, my advisor, and my committee chair, Dr. Arturo R. Zavala. I am grateful for his continued support through the years and that he never gave up on my ability to finish this thesis. He was always willing to meet, first in-person and later over Zoom, to help me reach my goals and push me to be my best. I could never have persevered without his mentorship.

I am also extremely grateful to my other committee members, Drs. Guido G. Urizar and Yada Treesukosol. Thank you for dedicating your time and expertise to help me progress my academic career to the next level, and for your patience in being members of my committee. I would also like to extend my sincere thanks to Diane Roe who was always very kind and willing to help, quickly responding to any questions I asked.

Finally, I would like to thank my parents whose love and support have never wavered, even when I wanted to move to California. You both have shown me what it means to be a strong, smart, self-sufficient adult, and I appreciate the important life lessons you taught me that have led me to a great life. I don't know what I would do if I didn't have you two to look to as role models: thank you for everything.

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LIST OF ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CSULB	California State University, Long Beach
CPA	Conditioned Place Aversion
CPP	Conditioned Place Preference
GABA	gamma-Aminobutyric acid
IACUC	Institutional Animal Care and Use Committee
IP	Intraperitoneal
NA	Nucleus Accumbens
nAChRs	Nicotinic Acetylcholine Receptors
NIDA	National Institute of Drug Abuse
PD	Postnatal Day
PFC	Prefrontal Cortex
SAMHSA	Substance Abuse and Mental Health Services
SC	Subcutaneous
US	United States
USDHHS	U.S. Department of Health and Human Services
VTA	Ventral Tegmental Area

CHAPTER 1

INTRODUCTION

Overview

Alcohol and tobacco are two of the most commonly abused recreational drugs, with both producing a heavy burden on society. Each year, excessive drinking costs the United States (US) approximately $249 billion in economic costs and causes 8,800 deaths (Centers for Disease Control and Prevention [CDC], 2016a). Smoking costs the U.S. approximately $300 billion and causes 480,000 deaths annually, making it the leading cause of preventable death (U.S. Department of Health and Human Services [USDHHS], 2014; Xu et al., 2014). When considered together, the use and abuse of these drugs cost the US economy over $500 billion annually (Substance Abuse and Mental Health Services Administration [SAMHSA], 2015).

In addition to economic costs, several health risks are associated with the co-use of alcohol and nicotine. For example, people who drink and smoke are at a significantly higher risk of certain cancers, particularly mouth and throat, than people who only drink or smoke (Negri et al., 1993). Adolescents who reported high rates of both drinking and smoking exhibited higher violence and deviant behavior rates, even compared to adolescents who only reported high rates of drinking (Tucker et al., 2005). Although we know that the co-use of alcohol and nicotine affects health, our understanding of the mechanisms of co-use is poorly understood.

Preclinical research has primarily focused on examining the singular use of alcohol, nicotine, or tobacco products and how exposure to one may impact later use of the other, but there is a lack of preclinical research examining how alcohol and nicotine interact when taken simultaneously, particularly during adolescence. This study investigated the rewarding properties of ethanol and nicotine coadministration in male and female adolescent rats to examine the

potential interactive effects between the drugs. Delineating the interaction between ethanol and nicotine during this developmentally vulnerable age is the first step towards understanding the neurobiological consequences of simultaneously using the drugs during adolescence (Spear, 2015; Yuan et al., 2016). This review begins by examining the epidemiology of ethanol and nicotine, then discusses the mechanisms of action of each drug. It concludes by examining adolescent animal models and preclinical studies examining the effects of alcohol, nicotine, and their interaction.

Epidemiology of Alcohol Use

Alcohol is the most commonly used and abused drug among adolescents in the United States (USDHHS, 2007). The World Health Organization reported that 91.6% of people use alcohol at one point in their life, with initiation often beginning in adolescence and prevalence continuing well into adulthood. Though alcohol consumption is not legal until 21 years old in the US, the median age of first alcohol use occurs during 16-19 years (Degenhardt et al., 2008). National surveys have found that 20% to 33% of 12-20-year-olds reported using alcohol within the past 30 days (Center for Behavioral Health Statistics and Quality, 2016; CDC, 2016c). The likelihood of adolescents drinking also increases as they age, as drinking within the past 30 days escalates from 10% to 35% in 8th and 12th graders, respectively (Johnston et al., 2016). Early alcohol use has proven to be especially dangerous for adolescents. Compared to those who wait to drink until 21 years old, adolescents who start drinking before 15 years old are 6 times more likely to develop alcohol dependence or abuse as adults (Miller et al., 2007).

The non-health related consequences of underage drinking range from social to safety issues. Adolescents who drink alcohol experience more school problems (i.e., more absences from school, worse grades) compared to adolescents who do not drink (USDHHS, 2007).

Adolescents who drink alcohol are also at a higher risk for physical assault, sexual assault, suicide, and homicide compared to those who do not drink (Miller et al., 2007). In summary, alcohol use is initiated and is widespread throughout adolescence, and the health and behavioral consequences for early users are increasingly adverse. The need to understand alcohol effects during adolescence is important in reducing alcohol abuse during this early developmental period. Tobacco use, one of the main ways to ingest nicotine, is similarly initiated and abused in adolescence, especially with new smoking technologies.

Epidemiology of Tobacco Use

Smoking is the leading cause of preventable death in the US (SAMHSA, 2015; USDHHS, 2014). Cigarettes contain up to 482 ingredients that contribute to cigarette smoke chemistry and toxicity, with the main addictive substance being nicotine (Baker et al., 2004). Although nicotine products are not legal until age 18 in the US, tobacco use is often initiated and continued during early adolescence (Bakar et al., 2013; Degenhardt et al., 2008; SAMHSA, 2015). National epidemiological studies have found that seven million adolescents ages 12 to 17 reported using tobacco within the past month (SAMHSA, 2015), with 43.6% smoking by age 15 and 71.6% smoking by age 21 (Degenhardt et al., 2008). In a large-scale study of university students, the prevalence of tobacco use was as high as 38.6% (Bakar et al., 2013).

While rates of tobacco use have been historically high, especially during adolescence, the rates of traditional tobacco products (e.g., cigarettes) have been decreasing, while other forms of nicotine use have been on the rise (National Institute of Drug Abuse [NIDA], 2015). Overall rates of smoking have declined in the past 30 years, from 42.4% of the overall population in 1965 to only 17.8% in 2013 (CDC, 2016c). However, use of multiple tobacco products (e.g., hookah, electronic cigarette, cigar, cigarette, pipe tobacco, all types of smokeless tobacco) have

been on the rise among adolescents, with 3.3% and 13% of middle and high schoolers, respectively, using two or more tobacco products in the past 30 days (CDC, 2016b). Adolescents who use multiple tobacco products have a higher risk for developing dependence to nicotine and are more likely to continue using tobacco products in the future (CDC, 2016b).

The highest increase in nicotine use among adolescents has been seen in the use of electronic cigarettes, which vaporize liquid nicotine so that it can be inhaled (CDC, 2015, 2016b). The rates of electronic cigarette use dramatically increased from 1.5% of high schoolers in 2011 to 16% in 2015. The NIDA (2015) reported that 12th graders used electronic cigarettes most (16.2%), followed by cigarettes (11.4%), and then smokeless tobacco (6.1%). Taking these statistics together, adolescents are using electronic cigarettes more frequently than normal cigarettes. Compared to a regular tobacco cigarette, electronic cigarettes allow the user to increase his or her exposure to nicotine and the concentration of nicotine consumed (Cameron et al., 2014; Goniewicz et al., 2014; USDHHS, 2014). These changes can lead to more nicotine being consumed overall, allowing more potential for addiction. Electronic cigarette use in adolescence is a significant health concern that needs to be addressed and investigated (USDHHS, 2014).

Even more concerning is that tobacco companies target youth and young adult populations with advertisements, specifically for electronic cigarettes. Between 2011 and 2013, there was a 256% increase in youth exposure to television electronic cigarette advertisements. Tobacco companies are also appealing to a younger market by using and promoting flavored tobacco products (Duke et al., 2014). The Population Assessment of Tobacco and Health Study, a national longitudinal cohort study, found that the tobacco product most commonly tried first among 12–17-year old's was a flavored tobacco product, a majority being electronic cigarettes or

hookah (Ambrose et al., 2015). The rising popularity of electronic cigarettes has the potential to worsen nicotine use and abuse among the adolescent population.

Epidemiology of Alcohol and Tobacco Use

The prevalence of co-use of alcohol and tobacco during adolescence has varied between 19-22% (Hoffman et al., 2001) to 35-45% (Anthony & Echeagaray-Wagner, 2000), with the highest prevalence occurring between 18-24 years old (Falk et al., 2006). Overall, data suggest that the co-use of alcohol and nicotine was high among adolescents (12-17 years old), peaked around young adulthood (25-34 years old), and declined steadily into adulthood (35 years or older; Anthony & Echeagaray-Wagner, 2000). These epidemiological findings demonstrate a strong link between ethanol and nicotine use; however, experimental studies offer more insight into the complex interaction.

Interaction of Alcohol and Tobacco in Adolescence

Literature examining the co-use of alcohol and tobacco reveals a complicated interaction in which the effects of one drug influence the other, which changes depending on one's experience with each drug (Bobo & Husten, 2000). Studies show that initially, an increase in alcohol consumption is associated with starting to smoke. As users begin to smoke more, less alcohol consumption is necessary to continue smoking (Harrison & McKee, 2008; Harrison et al., 2009). As smoking and drinking continue to increase, it becomes more likely for a person to binge drink (i.e., consume five or more drinks in one sitting; Harrison & McKee, 2008). Alcohol-use disorder is significantly higher among heavy smokers, defined as smoking three or more cigarettes daily (Dierker et al. & Tobacco Etiology Research Network, 2007). Altogether, these studies demonstrate how one drug interacts with the other, with the relationship getting stronger the more each is used. There are several possibilities why the drugs interact in this manner.

Increased alcohol use among heavy smokers may be due to increased alcohol tolerance, as smokers reported feeling less intoxicated than nonsmokers after identical doses of alcohol (Madden et al., 1995). Interestingly, a similar pattern is seen with users using e-cigarettes: people who used e-cigarettes experienced more problematic alcohol use than people who did not smoke (Hershberger et al., 2016). Sex differences also influence the interaction between alcohol and smoking, as males are more likely than females to drink and smoke (Falk et al., 2006; Hoffman et al., 2001; Lorenzo Di Bona & Erausquin, 2014). Moreover, while alcohol caused both men and women to experience increased smoking urges, it also caused men to increase smoking behavior (King et al., 2009). Early alcohol use also appears to influence later tobacco use, as prior alcohol use was a strong predictor of later tobacco use (Jackson et al., 2002). For example, adolescents who experimented with alcohol when they were in seventh grade were significantly more likely to smoke a pack of cigarettes a day or more by 12th grade (Griffin et al., 1999). Collectively, these findings suggest that the interaction between alcohol and smoking changes as these drugs are consumed over time and may be different among male and female users. These interactions could explain why smoking leads to additional consumption of alcohol. Although the biological mechanisms underlying the interaction between alcohol and smoking are not known, they have been examined individually.

Ethanol and Nicotine: Mechanisms of Action

Ethanol

Ethanol, the type of alcohol that is drinkable (unlike methanol and isopropyl), is produced by the fermentation of yeast, sugars, and starches and is typically absorbed from the gastrointestinal tract and spread via passive diffusion throughout the entire body (Meyer & Quenzer, 2013). Once absorbed, ethanol affects neurons in several ways, as it does not bind

specifically to one receptor within the brain. Rather, neurochemical effects associated with ethanol consist of changes in several different neurotransmitter systems in the brain. Ethanol acts as an antagonist or blocker for glutamate, the brain's main excitatory site, and an agonist or activator for GABA, the brain's main inhibitory site (Tsai et al., 1998). The combination of these effects leads to an overall decrease in brain activity. In addition to the excitatory and inhibitory effects, both dopamine and opioids are involved in the rewarding effects of ethanol. Ethanol indirectly activates dopamine receptors in the mesolimbic dopamine pathway, one of the main reward pathways in the brain (Brodie et al., 2006; Gonzales et al., 2004). The mesolimbic dopamine pathway includes three main brain regions: the ventral tegmental area (VTA), the nucleus accumbens (NA), and the prefrontal cortex (PFC; Alcaro et al., 2007). Specifically, the mesolimbic dopamine pathway consists of projections from the VTA to the NA, PFC, and other limbic regions. The NA has been extensively researched for its involvement in the rewarding effects of drugs (Funk et al., 2006). The rewarding effects of ethanol are believed to result from the activation of dopaminergic VTA neurons, which project to the NA and trigger the release of dopamine (Brodie et al., 2006; Gonzales et al., 2004). For instance, directly injecting dopamine-releasing agents into the NA increased ethanol intake, while injecting dopamine antagonists into the dopaminergic VTA neurons decreased ethanol intake (Gonzales et al., 2004).

The effects of ethanol can vary depending on the dose administered, a phenomenon known as a dose-response curve (Chan & Anderson, 2014). In humans, low and medium doses of ethanol caused relaxation, reduced anxiety, intoxication, impaired judgment and memory, and sleep; however, at high doses, ethanol caused respiratory depression, and even coma and death (Chan & Anderson, 2014). Sustained use of ethanol can damage the liver and significantly impair the metabolism of ethanol and other drugs.

The rate of absorption of ethanol is proportional to the concentration of alcohol consumed, and its proportionality is moderated by gender differences (Cederbaum, 2012; Frezza et al., 1990). The same dose of ethanol per unit of body weight can produce widely different effects due to variations in fat and water content between genders (Cole-Harding & Wilson, 1987; Frezza et al., 1990). Compared to females, males contain 60% more alcohol dehydrogenase, the enzyme responsible for the breakdown of ethanol in the stomach. Males also have a larger body size on average compared to females, meaning that when equal amounts of ethanol are consumed, blood alcohol levels are more concentrated in females. Females also have a higher percentage of body fat compared to men, causing females to have a higher peak blood alcohol level compared to males. Due to these facts, males are more efficient at absorbing ethanol and show less sensitivity to the effects of ethanol (Frezza et al., 1990). While ethanol has more widespread effects on the mesolimbic dopamine pathway, nicotine has more specific biological effects.

Nicotine

Nicotine is a compound found in tobacco leaves, a plant native to North and South America. Nicotine can be consumed in several ways, and the rate at which nicotine reaches the brain depends on how it is consumed (Digard et al., 2013; Rose et al., 2010). When smoked in cigarettes, nicotine takes approximately 7 seconds to reach the brain. When administered intravenously, nicotine takes approximately 14 seconds to reach the brain. This rapid metabolism of nicotine, especially when smoked, is thought to strongly reinforce nicotine use and lead to high addiction rates. Frequent nicotine use leads to progressively higher nicotine levels in the body because the dose builds off the previous levels, even building off the previous day's peak (Rose et al., 2010). Throughout the day, at least some tolerance to nicotine’s effects develops;

however, this tolerance dissipates while the user sleeps, so he or she wakes up in a state of mild withdrawal. Like ethanol, the effects of nicotine are dose-dependent: low and medium doses of nicotine are rewarding, while high doses can lead to nicotine intoxication and may be fatal (Tuesta et al., 2011).

Nicotine activates both the sympathetic and parasympathetic nervous systems. In the sympathetic nervous system, nicotine signals the adrenal glands to release epinephrine and norepinephrine, causing an increase in heart rate, blood pressure, and arousal (Yildiz, 2004). Though these indications of arousal contribute to the rewarding effects of nicotine, the long-term increase in heart rate raises the chance for cardiovascular disease and stroke. In the parasympathetic nervous system, nicotine signals an increase in stomach acid secretion and muscle contractions in the bowel (Yildiz, 2004). These effects are considered responsible for the negative consequences of prolonged use of nicotine, such as the formation of stomach ulcers and diarrhea. Other side effects include an increase in metabolic rate and appetite suppression, both of which contribute to weight loss. While nicotine causes a wide variety of behavioral responses, it exhibits specific neurochemical effects.

Nicotine mimics the neurotransmitter acetylcholine by directly binding to nicotinic cholinergic receptors (nAChRs), 1 of the 2 basic subsets of acetylcholine receptors (Funk et al., 2006). These high affinity nAChRs can be found in many areas of the brain and can modulate the release of several other neurotransmitters (i.e., norepinephrine, dopamine, serotonin, and glutamate; Yildiz, 2004). The main reinforcing effects of nicotine are thought to be mediated through the nAChRs located within the dopaminergic neurons of the VTA that extend to the NA, known as the mesolimbic dopamine system (Levin & Rose, 1995; Pontieri et al., 1996). When nAChRs in the VTA were blocked pharmacologically, rats reduced their nicotine self-

administration, suggesting nicotine's reinforcing effects are based in the VTA (Corrigall et al., 1994). Furthermore, stimulation of nAChRs in the VTA led to the firing of dopaminergic neurons in the NA. When these dopaminergic neurons were lesioned, self-administration of nicotine in rats was significantly attenuated, demonstrating that a large portion of nicotine's reinforcing effects results from dopamine release in the NA (Corrigall et al., 1992). Though ethanol and nicotine have different mechanisms of influence, they both act on the mesolimbic dopamine pathway.

Interaction

The mesolimbic dopamine pathway is thought to mediate the rewarding and reinforcing properties of ethanol and nicotine, as well as other drugs of abuse (DiChiara & Imperato, 1988). There is a clear interaction between alcohol and nicotine in how they activate the mesolimbic pathway. For example, pharmacologically blocking nAChRs in the VTA led to a decrease in ethanol intake, suggesting that nAChRs mediate the reinforcing effects of ethanol (Söderpalm et al., 2000). When cultured neurons from the VTA and NA were exposed to ethanol and nicotine simultaneously, they exhibited enhanced gene expression resulting in more nAChR receptors (Inoue et al., 2007). Studies using in vivo microdialysis (allowing researchers to measure levels of neurotransmitters in specific brain areas) have found that coadministration of ethanol and nicotine produced an additive release of dopamine in the NA (Tizabi et al., 2007; Tizabi et al., 2002).

Moreover, ethanol modulates how nicotine binds to nAChR receptors by enhancing these receptors and activating dopamine release (Gotti et al., 2006; Salminen et al., 2006). It is possible that enhancing receptor function is the mechanism by which dopamine functioning in the VTA increases during coadministration. Supporting evidence shows that when nAChR receptors are

blocked using a non-specific antagonist, ethanol-induced CPP is not expressed (Bhutada et al., 2012). Altogether, these findings demonstrate that the mesolimbic dopamine pathway is associated with the reinforcing properties of ethanol and nicotine. When used together, these drugs collectively impact the activity of the mesolimbic dopamine pathway differently than when used alone. In order to study the behavioral interaction of ethanol and nicotine's rewarding effects, this study used CPP, an animal model of drug reward.

Animal Models of Addiction

Animal models are an important tool for studying processes, behaviors, and relationships related to drug use. Furthermore, animal models are indispensable to the understanding and study of addiction because they allow researchers to bypass the ethical and methodological concerns associated with human studies (Jain, 2003). There are three main parallels between animal and human addiction research. First, drugs function as a reward for both animals and humans, influencing behavior accordingly (Willner, 1997). Both animals and humans will also self-administer drugs, meaning they will voluntarily engage in behaviors that result in drug use (Panlilio & Goldberg, 2007). Finally, animals and humans will also seek out the reinforcing and rewarding effects of drugs, as demonstrated in paradigms such as self-administration and CPP.

While there are several parallels, there are also a few limitations when using animal models to model human behaviors (Panlilio & Goldberg, 2007). Research involving animals necessarily uses different methodologies than human studies. Some methods, like self-administration, translate well between human and animal studies. Other methods, like testing fear response or cognitive ability, are necessarily different (Delgado et al., 2006; Wallace et al., 2015). Although the study methods may differ dramatically, animal addiction research is crucial in understanding the neurobiological influences on human drug use.

There are two main paradigms used to study the effects of drugs: self-administration and CPP. Self-administration is when an animal performs a response, like pressing a lever, to access a drug. CPP is when an animal establishes a preference for an environment that has been associated with the effect of a drug (Carr et al., 1989). For this study, a CPP paradigm was used as a behavioral measure of the rewarding effects of drugs.

The CPP paradigm is one of the most frequently used methods to measure the rewarding effects of drugs (Bardo & Bevins, 2000; Carr et al., 1989). It relies on the concept of secondary conditioning, which is when a neutral stimulus is paired with a reward sufficiently enough, so the neutral stimulus begins to act as a reward itself (Skinner, 1953). In CPP, the neutral stimulus is the CPP environment, and the drug is the reward. The CPP environment comprises two separate rooms with distinct internal environments, with the possibility of movement in-between. Although procedures may differ between various labs, there are three primary phases in the CPP paradigm: preconditioning, conditioning, and postconditioning. During preconditioning, the animal can move freely in-between boxes to gain an initial assessment of preference. During conditioning, the drug is repeatedly paired with the initially non-preferred box side by administering the drug and restricting the animal's movement to that side; saline is paired with the preferred side. Because the animal is administered the drug before being placed in the CPP box, the location is associated with the effects of the drug. Finally, during postconditioning, the animal is again given the freedom to move between boxes in a drug-free state, and preference for the drug-paired compartment is assessed. If the animal increases the time spent approaching and maintaining contact with the drug-paired environment, it is inferred that the drug was rewarding to the animal and that the animal spent time in the chamber because it made an association with the drug (Carr et al., 1989).

One of the first studies demonstrating that the rewarding effects of drugs could be measured was done using CPP. Beach (1957) found that non-addicted rats spent more time in the compartment paired with morphine than the compartment paired with saline. Since this study, CPP has been validated using other drugs (i.e., cocaine, amphetamine, ethanol, and nicotine) during various age periods for decades (Ahsan et al., 2014). This study focuses exclusively on ethanol- and nicotine-induced CPP during adolescence, an age period often neglected in animal research.

Adolescent Animal Models

Characterizing human adolescence in animal models can be difficult. However, animals undergo unique developmental changes that are comparable to adolescent humans. The primary difficulty in using adolescent animal models is defining the age period, which is complicated in animals and humans. For example, sometimes adolescence is defined as puberty, the attainment of sexual maturation, while other times it is described as the entire period between childhood and adulthood (Spear, 2000). Often in animal research, rats are tested early in life before postnatal day (PD) 21, when they are weaned (i.e., when they are separated from the dam), and then again in adulthood (PD 70+)—completely neglecting the period of adolescence (Spear, 2000). The best estimate for early adolescence in rats is PD 28-42 because they go through several adolescent-related changes that make it comparable to humans: large growth spurts, sexual maturation, and, most importantly, several neurological developments (Spear & Brake, 1983).

The neurological developments associated with adolescence in rats include modifications in the brain, alterations in the mesolimbic dopamine pathway, and synaptic pruning (Spear, 2000, 2004). Brain circuitry in the frontal, temporal, and parietal cortices are remodeled and reorganized during this period. For example, the mesolimbic dopaminergic system is more active

during early adolescence, with activity declining during late adolescence, leading to increased dopamine levels in early adolescence (Spear, 2004). The amygdala and the NA also show similar activity across adolescence. In addition, synaptic pruning occurs in the prefrontal cortex, hippocampus, and amygdala during adolescence (Spear, 2000). Similar alterations in the dopamine system and synaptic pruning have also been observed in humans.

The brain transformations also correspond to behavioral changes, such as an increase in reckless behavior, sensation-seeking, risk-taking, and drug use (Andrucci et al., 1989; Baumrind, 1987; Trimpop et al., 1999; Wills et al., 1992). Not only do adolescents use drugs more, but there is also evidence that they become dependent on drugs at a faster rate than adults (Clark et al., 1998; Estroff et al., 1989). While there are limitations to using animal models, the fundamental underpinnings are sufficiently similar to human addiction and adolescence. Several studies examining ethanol, nicotine, and their coadministration have been conducted using animal models of drug reward.

Effects of Ethanol: Preclinical Studies

Adult Preclinical Studies

Although alcohol is one of the most widely abused drugs by humans, preclinical studies examining the rewarding effects of ethanol in an animal model have been difficult because studies seeking to establish ethanol-induced CPP in adult animals have produced varied results. In many studies, either no significant preference was established or conditioned place aversion (CPA) was established (Bienkowski et al., 1996; Cunningham, 1979; Fidler et al., 2004; Gauvin & Holloway, 1992; Philpot et al., 2003; Torres et al., 2014). CPA is procedurally similar to CPP, but instead of the animal spending more time in the drug-paired side, the animal spends less time in the drug-paired side, reflecting the aversive nature of the drug (Cunningham, 1979; Fidler et

al., 2004). Collectively, these findings suggest that ethanol produces a weak rewarding effect in animals. CPP has been established in adult animals under a few conditions.

One study using adult animals established ethanol-induced CPP using 2.0 g/kg doses through four drug conditioning sessions (Dickinson et al., 2009). Ethanol-induced CPP was also more robust when adult animals were exposed to ethanol before CPP conditioning through a self-administration paradigm (Gauvin & Holloway, 1992; Reid et al., 1985) or by administering 20 injections of ethanol (Bienkowski et al., 1996). Ethanol-induced CPP was also established using genetically selected alcohol-preferring rats, but again, the rats were pretreated with ethanol for 15 days before CPP conditioning (Ciccocioppo et al., 1999). Overall, establishing ethanol-induced CPP in adult preclinical studies was difficult and often unsuccessful, unless the relationship to ethanol was strengthened by using higher doses, being exposed to ethanol for more extended periods, or pre-exposing the animal.

Adolescent Preclinical Studies

Adolescents' response to ethanol differs significantly from that of adults. Because the rewarding effects of ethanol have been challenging to study in preclinical settings, there is limited research with strong conclusive results using CPP as a model for ethanol reward due to differences in age, ethanol doses, or the number of drug pairings between studies. However, some preclinical studies examined the differences between adolescent and adult animals' responses to ethanol. For example, adolescent mice (PD 31) established CPP at 4.0 g/kg ethanol, while adult mice (PD 70) established CPP at 2.0 g/kg ethanol, suggesting that it required more ethanol to establish a preference in adolescent rats, meaning they were less sensitive to the rewarding effects of ethanol (Dickinson et al., 2009).

Other studies demonstrate that adolescence was a special period in which animals were more sensitive to the effects of ethanol (Little et al., 1996; Philpot et al., 2003). Late adolescent (PD 30) and adult (PD 60) rats were twice as active as early adolescent (PD 20) rats in an ethanol locomotor activity assessment, demonstrating that adolescent animals were more sensitive to the sedative effects of ethanol (Little et al., 1996). Philpot et al. (2003) compared several age periods throughout adolescence and adulthood (PD 25, 35, 45, and 60) in rats using an unbiased CPP, wherein the rats were trained over four days with two conditioning sessions per day. In the conditioning sessions, rats were injected with ethanol (0.2, 0.5, 1.0, or 2.0 g/kg) and then placed in the CPP apparatus after waiting five minutes. Among all the ages and doses tested, CPP was only established using a small (0.2 g/kg ethanol) dose at PD 25 and medium (0.5 and 1.0 g/kg ethanol) doses at PD 45. While this data suggests that low and medium doses of ethanol vary in their ability to establish a preference as a function of age, the results were inconsistent across the many ages and doses used.

When examining sex differences, both adolescent female and adult intact female rats (PD 45) established CPP at 1.0 g/kg ethanol (Torres et al., 2014). Meanwhile, adolescent males, adult males, and ovariectomized adult females failed to establish CPP, and instead demonstrated CPA at higher doses of ethanol. These results provide strong evidence that the rewarding effects of ethanol are hormone-dependent in adolescent and adult female rats.

Though it has been difficult to establish a consistent ethanol-induced CPP in animals, studies examining how rats metabolize and process ethanol have consistently shown developmental differences between adolescent and adult animals (Pyapali et al., 1999; Swartzwelder et al., 1995a, 1995b). For example, ethanol suppressed long term potentiation induction in the hippocampus of adolescent rats but did not in adult rats, indicating that memory-

related synaptic plasticity in the hippocampus is inhibited by ethanol in adolescent rats but not adult rats (Pyapali et al., 1999; Swartzwelder et al., 1995a). In addition, similar patterns were observed when studying NMDA synaptic activity in the hippocampus: adolescent rats demonstrated significantly reduced NMDA activity after small amounts of ethanol concentration were used, while adult rats required the highest concentration of ethanol to reduce activity (Swartzwelder et al., 1995b). Because developmental differences have been observed in many other aspects of ethanol's effects, we plan to establish a consistent ethanol-induced CPP using a different methodology. While ethanol-induced CPP has not been conclusive, nicotine-induced CPP has more consistent findings.

Effects of Nicotine: Preclinical Studies

Adult Preclinical Studies

Although there is some inconsistency in the findings, nicotine-induced CPP has successfully been established in several studies (Ahsan et al., 2014; Lenoir et al., 2015; Torres et al., 2008). Specifically, nicotine-induced CPP has been established using 0.6 mg/kg nicotine in adult (PD 56) rats using a biased design with six 30 minute conditioning sessions, half of which were drug-paired (Ahsan et al., 2014). Using a similar procedure, Torres et al. (2008) successfully established nicotine-induced CPP in adult (PD 60) rats using a low dose (0.2 mg/kg nicotine), but established CPA using higher doses (1.2 mg/kg nicotine). When examining sex differences, adult (PD 71) male rats established CPP at lower doses (0.1 and 0.2 mg/kg nicotine) compared to adult female rats who established CPP at 0.4 mg/kg (Lenoir et al., 2015). While several studies have successfully established nicotine-induced CPP, others either (a) fail to establish CPP (Belluzzi et al., 2004; Dannenhoffer & Spear, 2016; Shram et al., 2006; Vastola et

al., 2002) or (b) establish CPA (Jorenby et al., 1990). Preclinical adult nicotine studies have successfully used biased CPP designs and several drug pairings in conditioning sessions.

Adolescent Preclinical Studies

The effects of nicotine have been reported to be age-dependent, with adolescent rats being more sensitive to the rewarding effects and less sensitive to the aversive effects when compared to adults (Ahsan et al., 2014; Belluzzi et al., 2004; Dannenhoffer & Spear, 2015; Lenoir et al., 2015; Natividad et al., 2013; Shram et al., 2006; Torres et al., 2008; Vastola et al., 2002). While adolescent (PD 28-38) rats established significant CPP using doses within the range of 0.03 to 0.6 mg/kg nicotine, adult rats either required a larger dose of nicotine or did not establish significant CPP (Ahsan et al., 2014; Dannenhoffer & Spear, 2015; Lenoir et al., 2015; Shram et al., 2006; Torres et al., 2008; Vastola et al., 2002). When studying withdrawal effects from nicotine exposure, adolescent rats produced fewer aversive measures of withdrawal than adult rats, providing evidence of the increased vulnerability to nicotine during adolescence (O'Dell, Bruijnzeel et al., O'Dell, Torres et al., 2006). Specifically, rats displayed fewer somatic withdrawal symptoms, no decreases in reward, and a lack of avoidance to the nicotine-paired side during withdrawal.

These age-related patterns of younger rats being more sensitive to rewarding and less sensitive to aversive effects apply when comparing different windows within adolescence. Early adolescent (PD 28) rats showed significant nicotine-induced CPP after one 20 min conditioning session using 0.5 mg/kg nicotine injected subcutaneously. Late adolescent (PD 38) and adult (PD 90) rats showed no preference (Belluzzi et al., 2004). When comparing nicotine use in a self-administration paradigm, adolescent rats had higher nicotine intake than adults; furthermore, adult rats displayed robust appetite and weight suppressant effects, while adolescent rats did not

(Natividad et al., 2013). Taken together, these studies demonstrate that adolescence is a special time in development marked by an increased vulnerability to nicotine use, either through increased rewarding effects or decreased withdrawal effects.

Coadministration of Ethanol and Nicotine: Preclinical Studies

The majority of preclinical studies have examined how use of ethanol or nicotine early in life influences use of the other drug later in life (i.e., early use of ethanol on later use of nicotine or vice versa), but few studies have examined the effects of ethanol and nicotine coadministration (Bianchi et al., 2017; Mantella & Youngentob, 2014; Philpot et al., 2014). Exposure to nicotine during adolescence led to a more robust ethanol-induced CPP in adult rats willing to explore new environments, demonstrating that one drug influences the use of the other (Philpot et al., 2014). Conversely, using an ethanol pretreatment in a nicotine-induced CPP led to a weaker preference, most likely because ethanol diminished the discriminative stimulus effect in each environment (Boutros et al., 2015). Though these studies have investigated parts of the relationships between ethanol and nicotine, they do not directly study the coadministration of both during adolescence.

A few studies have examined the coadministration of ethanol and nicotine during adolescence using a variety of paradigms. Adolescent mice exposed to cigarette smoke for six hours a day for three weeks and then given access to 10% (w/v) ethanol solution for two hours, consumed three to five times more ethanol and had a blood alcohol content about four times higher compared to animals not exposed to cigarette smoke (Burns & Proctor, 2012). Another study using self-administration of both ethanol and nicotine found that, though the availability of ethanol decreased the amount of nicotine rats self-administered, they would still self-administer significant amounts of both ethanol and nicotine when both were available (Lê et al., 2010).

Moreover, when the self-administration of ethanol was extinguished while nicotine was still available, the extinction of ethanol was significantly slower. After co-administering ethanol and nicotine in a radial arm maze, rats experienced significant choice accuracy impairment at doses that, when given alone, had no effect (Rezvani & Levin, 2002). These results provide further evidence of an interaction between ethanol and nicotine and how one influences the amount consumed and the extinction process of the other.

Some studies used inbred strains of mice that were either ethanol-avoiding, DBA/2J, or ethanol-preferring, C57BL/6J (Gubner et al., 2015; Gulick & Gould, 2008; Korkosz et al., 2006). Using fear conditioning, Gulick and Gould (2008) examined the interactive effects of acute ethanol on acute, chronic, or withdrawal from chronic nicotine use. This study found that low doses of nicotine (0.09 mg/kg) reversed ethanol-induced deficits (1.0 and 5.0 g/kg) in contextual and cued fear conditioning. On the other hand, low doses of ethanol (0.25g/kg) reversed nicotine withdrawal-induced deficits in contextual conditioning. Essentially, nicotine may reduce the aversive effects of ethanol at the cost of developing a tolerance to nicotine. Similarly, ethanol may decrease nicotine withdrawal symptoms. These polar effects suggest that the high co-use may result from each drug ameliorating the aversive effects of the other. When examining the effect of nicotine on ethanol-induced CPP in adult mice, 1.0 mg/kg nicotine did not enhance ethanol-induced CPP; however, 2.0 mg/kg of nicotine did suppress ethanol-induced CPP (Gubner et al., 2015). Another study found that ethanol enhanced nicotine-induced CPP, though not to a significant level (p =.07), and suppressed seizures elicited by high doses (6.0 mg/kg) of nicotine (Korkosz et al., 2006). Together, these studies suggest an interaction between ethanol and nicotine using the CPP paradigm; however, results were inconsistent. The goal of the current

study was to determine if the coadministration of nicotine and ethanol produced a greater preference compared to administration of either drug alone in an adolescent rat population.

Purpose

The purpose of this study was to investigate the interaction of the rewarding effects of ethanol and nicotine coadministration in adolescent rats. Experiment 1 established a dose-response curve for an ethanol-induced CPP in adolescent (PD 23-32) Sprague-Dawley rats to address the lack of adolescent ethanol-induced CPP research. Although CPP studies traditionally use a fixed daily dose of the drug during conditioning (Belluzzi et al., 2004; Dickinson et al., 2009; Lenoir et al., 2015; Philpot et al., 2003), this pattern of drug administration does not mimic the progressive pattern of drug intake generally found in adolescent populations (Townshend & Duka, 2002). Therefore, a more fitting examination of ethanol-induced CPP may be using ascending doses of ethanol, which has the potential to more accurately model the human experience with ethanol and achieve more robust CPP results compared to traditional dosing patterns. Indeed, recent studies have demonstrated that using ascending dosing patterns of cocaine during conditioning produce more robust CPP than using high or low fixed dosing patterns during conditioning (Conrad et al., 2013; Itzhak & Anderson, 2011). Thus, this study first compared ascending dosing patterns of ethanol to fixed dosing patterns using CPP in Experiment 1. Then, using the results from Experiment 1 and previously established nicotine dose-response curves, Experiment 2 investigated the primary purpose of this study – the effects of the coadministration of ethanol and nicotine during that same period in adolescence. The first hypothesis was that rats would establish CPP for the ethanol-paired side, explicitly using the ascending dose pattern due to its more accurate representation of alcohol use. The second

hypothesis was that the coadministration of ethanol and nicotine would lead to a more robust CPP than the administration of either drug alone.

CHAPTER 2

METHODOLOGY

Subjects

Subjects were 241 male and female Sprague-Dawley rats (Charles River Farms, Hollister, CA). All rats were born at the California State University, Long Beach (CSULB). Litters were culled to 10 pups (5 male, 5 female when possible) on postnatal day (PD) 3. Culling liters to 10 pups ensured pups are cared for and raised in similar environments. Rats were kept in the home cage with the dam until PD 21, at which time they were weaned with two or three same-sex littermates. To control for litter effects, no more than one rat from each litter was assigned to a particular group (Zorrilla, 1997). Rats were housed in a polycarbonate cage in a temperature-controlled colony (21-24 °C) and kept on a 12:12 light/dark cycle with lights turning on at 7:30 am. Food and water were provided ad libitum. On PD 21 and 22, rats were handled 2 minutes per day to reduce any anxiety from experimenter handling. Animals were treated according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at CSULB and in compliance with the Guide for the Care and Use of Laboratory of Animals (National Research Council of the National Academies, 2011).

Apparatus

Five CPP apparatuses were used in both experiments. As shown in Figure 1, each CPP apparatus was composed of two compartments (20 × 25 × 45 cm) separated by a removable partition. The two main compartments were distinguishable using visual, tactile, and olfactory cues. One compartment had horizontal black and white stripes, a metal rod floor painted black (20 × 17.5 cm) with rods spaced 6.5 mm apart, and Aspen chip bedding beneath the rod floor. The other compartment had vertical black and white striped walls, a sheet metal floor painted

black (20 × 17.5 cm) with 0.5 cm perforated holes, and Beta chip bedding beneath the perforated metal floor.

Two removable partitions were used. One was a solid partition, which prevents access between compartments, and one had an 8 × 8 cm opening, allowing rats to move freely between the two compartments. During the assessment of compartment preferences, the latter partition was used. The center portion (20 × 7.5 cm) of the box between the two floors of each compartment floor was made up of solid wood painted black. Approximately 1.12 m above the center of each CPP box, there was a 60Hz 7W LED strip light and a PanaVise Fujinon digital video camera to record sessions. The strip light ensured equal lighting throughout both compartments.

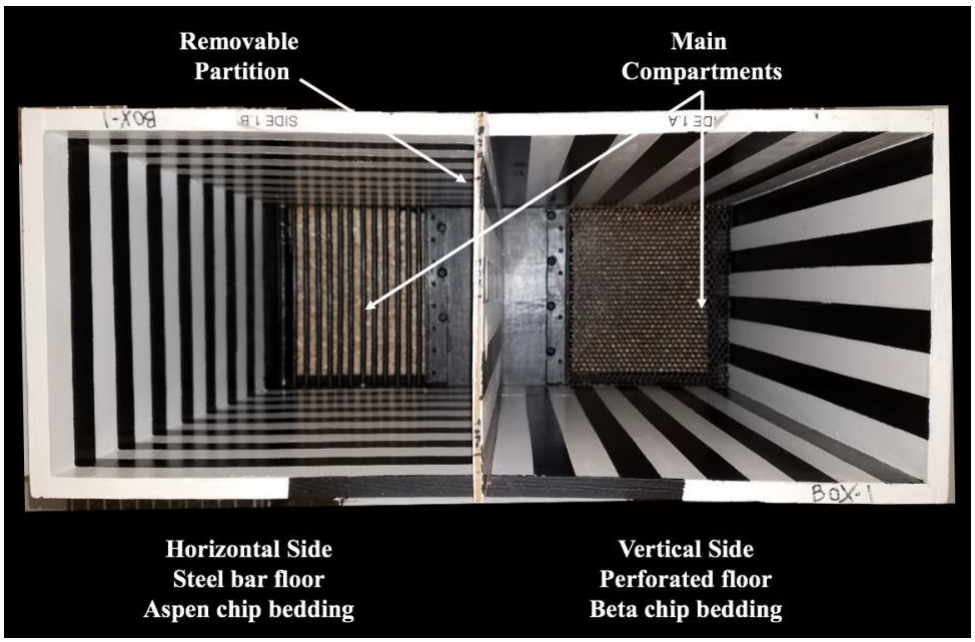

FIGURE 1. Conditioned place preference (CPP) apparatus. The removable partition can either have a small opening to allow free access to both compartments or is closed to confine subjects to one side of the compartment.

Drugs

Ethanol (95%) was obtained from Pharmaco-AAPER (Catalog 111000190, ACS/USP grade). (-)Nicotine hydrogen tartrate salt (Catalog Number N5260) was obtained from Sigma-Aldrich. Ethanol was administered intraperitoneally (IP) at a 20% v/v ethanol solution in 0.9% saline. Nicotine was adjusted to a pH of 7.4 using NaOH and injected subcutaneously (SC) at a volume of 1 ml/kg (doses refer to the free base weight). Saline (0.9% NaCl) control injections were administered either IP or SC for ethanol or nicotine injections. All drugs were administered at room temperature.

Procedures

General CPP Procedure

The CPP procedure took place over 10 days and consisted of three phases: preconditioning, conditioning, and postconditioning. Preconditioning and postconditioning were conducted on days 1 and 10 of the CPP procedure, respectively, with conditioning occurring on days 2-9 (see Table 1). Each phase consisted of 15 min sessions. After each session, the inside walls and floors of the compartments were sanitized with double distilled water, and soiled bedding was replaced. In addition, floors were cleaned with 50% ethanol daily. Throughout the three phases, a continuous white noise machine (Brookstone Model #46709, Merrimack, NH) set at 10 dB above background noise was used to minimize ambient noise disturbances and enhance the salience of the CPP environment from the animal holding room.

TABLE 1. CPP Phases and Associated Ages

Handling	Preconditioning	Conditioning	Postconditioning
Day 1-2	Day 3	Day 4-11	Day 12
PD 21-22	PD 23	PD 24-31	PD 32

Note. Ages are expressed as postnatal days (PD) and are used in both Experiment 1 and 2. On PD 21-22, subjects were handled for two days before the start of the experiment. On PD 23, subjects were allowed into each side of the apparatus to determine initial preference for each side. Through PD 24-31, subjects alternated between ethanol and saline conditioning. On PD 32, the final day of the experiment, subjects were again allowed access to both containers to establish a final preference for each side.

Preconditioning. During preconditioning, rats shuttled freely for 15 min between the two different compartments. Each session began when the rat was placed in one of the two compartments facing the wall of the chamber opposite the removable partition. Half the rats started in the vertical-striped compartment and half in the horizontal-striped compartment. The time spent (sec) in each compartment was recorded and analyzed using ANY-maze (Version 5.0 Stoelting Co., Wood Dale, IL), which used the rats' head to determine the location of the rat (Figure 2).

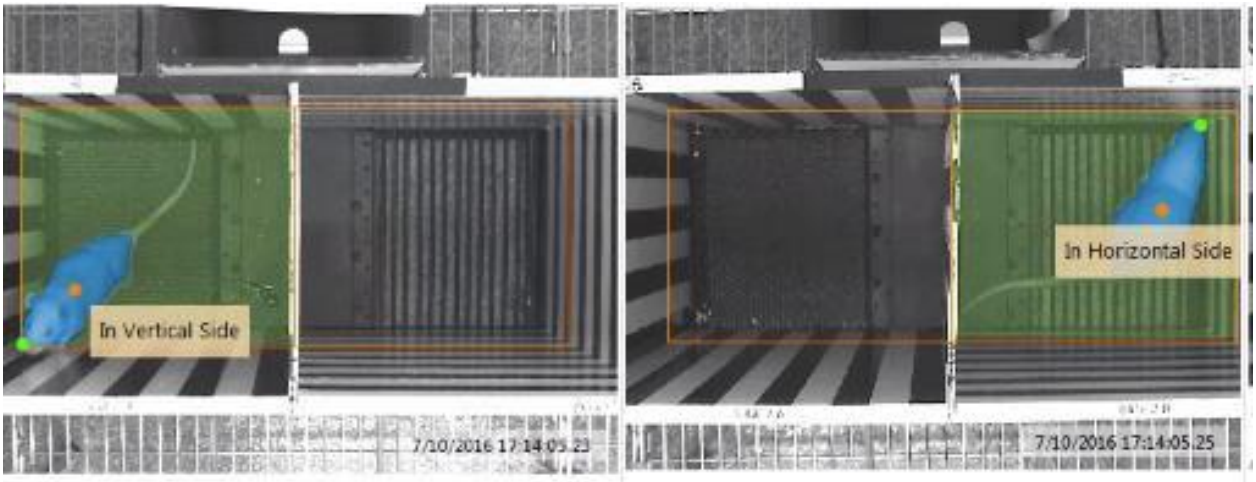

FIGURE 2. Screenshot of live ANY-Maze tracking. Rats were tracked using ANY-Maze technology to track the amount of time spent in each side of the apparatus during preconditioning and postconditioning.

The amount of time spent on each side was analyzed to establish the rats' initially-preferred (> 50% of the time) and nonpreferred (< 50% of the time) side. This experiment used a biased experimental design in that the drug was paired with the initially non-preferred side, and saline was paired with the preferred side. This design was chosen because it is more sensitive to the potential changes in drug reward (Tzschentke, 1998). The preferences for each side of the compartment in Experiment 1 and 2 for both males and females are presented in Table 2. The time spent in each of the two compartments revealed an unbiased box, as there was an equal amount of time spent in each of the two compartments.

TABLE 2. Preconditioning Preferences in Male and Female Rats Across Experiments

Experiment	Sex	Non-Preferred Side	#	Percent	Vertical Time ±SEM	Horizontal Time ± SEM
One	Male					
(900 sec)		Horizontal	23	38%	399 ± 8.51	***501 ± 8.51***
		Vertical	38	*62%*	***510 ± 6.51***	390 ± 6.51
		Total	61		467 ± 8.60	432 ± 8.60
	Female					
		Horizontal	25	36%	399 ± 7.09	**501 ± 7.09**
		Vertical	45	*64%*	**521 ± 5.98**	379 ± 5.98
		Total	70		477 ± 8.40	423 ± 8.40
Two	Male					
(900 sec)		Horizontal	27	35%	402 ± 6.74	**498 ± 6.74**
		Vertical	50	*65%*	**514 ± 5.03**	386 ± 5.03
		Total	77		475 ± 7.30	425 ± 7.30
	Female					
		Horizontal	29	31%	395 ± 6.96	**505 ± 6.96**
		Vertical	64	*69%*	**513 ± 4.09**	387 ± 4.09
		Total	93		477 ± 6.82	423 ± 6.82

Note. **Bold** numbers indicate the non-preferred side, which signifies the side in which ethanol was paired with during the conditioning phase of the CPP procedure. *Italics* indicate the overall non-preferred side.

Rats that spent less than 33% of the time (< 300 sec) in the non-preferred side were excluded from the study because such strong preferences make it difficult to establish CPP (O'Dell et al., 2004). Out of 241 animals total, 37 (20.9%) of the animals were lost due to issues with preconditioning in Experiment 1, and 82 were lost from Experiment 2 (27.8%).

Conditioning. The day after preconditioning, rats started the conditioning phase. Using the established initial preference, rats underwent 8 consecutive days of 15 min conditioning sessions, alternating between drug- and saline-conditioning sessions. During drug days, rats received an injection of drug prior to being confined to their initially nonpreferred compartment. During saline days, rats received an injection of saline prior to being confined to their initially preferred compartment. This two-day conditioning cycle was repeated three more times over the next six consecutive days. The order of drug and saline days was counterbalanced such that half of the rats received drug on the first day of conditioning and the other half of the rats received drug on the second day of conditioning. A biased CPP procedure was used, meaning drug injections were paired with the non-preferred side, because ethanol and nicotine have weak reinforcing properties and pairing the drug to the non-preferred side made it easier to detect a change in preference (Bienkowski et al., 1996; Vastola et al., 2002). Group assignments were counterbalanced across the five CPP boxes to ensure all groups were equally represented across all boxes.

Postconditioning. The postconditioning session procedure was identical to the preconditioning session procedure, except that rats were first placed in the same chamber in which they were initially placed in during baseline. No drug injections were given during postconditioning sessions. The day after postconditioning was completed, rats were euthanized via CO_2 and decapitated as a secondary measure.

Experiment 1: Ethanol Dose-Response Curve

The purpose of Experiment 1 was to examine ethanol-induced CPP using fixed or ascending doses of ethanol during conditioning in male and female periadolescent (PD 23-32) rats. Experiment 1 used an experimental, longitudinal, between-subjects Pre-test / Post-test control group design.

Rats went through preconditioning on PD 23 and were assigned to receive either fixed or ascending doses of ethanol during conditioning (PD 24-31), counterbalancing for time spent in the nonpreferred side to ensure equal preference during drug preconditioning session. There was a total of seven groups to account for the administration of fixed doses, ascending doses, and the saline control group, see Table 3. Ethanol doses were based on previous published data on ethanol-induced CPP (Philpot et al., 2003). Rats in the fixed dosing group were given 0.5, 1.0, or 2.0 g/kg of ethanol across the four drug days of the conditioning phase. Rats in the ascending dosing groups were given one of three dosing patterns during the four drug days of the conditioning phase: 1) 0.0625, 0.125, 0.25, and 0.5 g/kg of ethanol; 2) 0.125, 0.25, 0.5, and 1.0 g/kg of ethanol; or 3) 0.25, 0.5, 1.0, and 2.0 g/kg of ethanol). Immediately after injections, rats were returned to their home cage to reduce the initial aversive effects of ethanol (Cunningham et al., 1997; Song et al., 2007). After waiting 5 minutes, rats were placed into the CPP apparatus for the 15 minute conditioning session. During the four saline days, rats were placed in the initially preferred side and received saline injections. After conditioning, rats underwent one day of postconditioning (PD 32) to reevaluate their preferences without any administration of ethanol.

TABLE 3. Groups and Dosing Patterns of Ethanol (g/kg) for Experiment 1

	Conditioning Days							
Group	1	2	3	4	5	6	7	8
Saline Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fixed Low	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0
Ascending Low	0.0625	0.0	0.125	0.0	0.25	0.0	0.5	0.0
Fixed Medium	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
Ascending Medium	0.125	0.0	0.25	0.0	0.5	0.0	1.0	0.0
Fixed High	2.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0
Ascending High	0.25	0.0	0.5	0.0	1.0	0.0	2.0	0.0

Note. There were seven total groups used to evaluate the rewarding effects of ethanol: one saline control group, three ascending dosing patterns, and three fixed dosing patterns. Rats were randomly assigned to each group; both male and female rats were used.

Experiment 2: Ethanol and Nicotine Coadministration

Experiment 2 compared the effects of the coadministration of ethanol and nicotine to the administration of either drug alone on CPP in male and female periadolescent (PD 23-31) rats. After preconditioning on PD 23, rats were randomly assigned to receive ethanol, nicotine, or ethanol and nicotine during conditioning (PD 24-30), counterbalancing for time spent in the non-preferred side to ensure equal preference during preconditioning. There were eight drug groups to account for the administration of low and medium doses of ethanol, administration of low and medium doses of nicotine, and coadministration of ethanol and nicotine in various doses (see Table 4). Nicotine doses (0.0067 and 0.02 mg/kg nicotine) were derived from previous lab experiments and other studies that were able to induce nicotine CPP during adolescence (Ahsan et al., 2014; Dannenhoffer & Spear, 2015; Natarajan et al., 2011; Shram et al., 2006). The fixed doses of ethanol (1.0 g/kg or 2.0 g/kg) used were determined by the results from Experiment 1. Similar to Experiment 1, rats were administered saline or ethanol and then immediately returned to the home cage. Once 5 minutes elapsed, the rats were injected with nicotine or saline and then immediately placed into the CPP box for the 15 minute conditioning session. Other than these changes, the CPP procedure for Experiment 2 was identical to the procedure described for

Experiment 1. After conditioning, rats underwent one day of postconditioning (PD 32) to reevaluate their preferences without any administration of ethanol and/or nicotine.

Statistics

Body Weight

Body weight (g) was collected for each rat at the start of each of the 10-day CPP procedure to determine if any treatment impacted the body weight of the animal. Body weight is a good overall indicator of health and can signal when the animal is ill or under distress (exhibited as a decrease in body weight). For Experiment 1 and 2, body weight data were

TABLE 4. Groups and Dosing of Ethanol (g/kg) and Nicotine (mg/kg) for Experiment 2

	Conditioning Days							
Group	1	2	3	4	5	6	7	8
Saline Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ETOH Low	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
NIC Low	0.067	0.0	0.067	0.0	0.067	0.0	0.067	0.0
ETOH Medium	2.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0
NIC Medium	0.02	0.0	0.02	0.0	0.02	0.0	0.02	0.0
ETOH Low / NIC Low	1.0 / 0.067	0.0	1.0 / 0.067	0.0	1.0 / 0.067	0.0	1.0 / 0.067	0.0
ETOH Low / NIC Med	1.0 / 0.022	0.0	1.0 / 0.022	0.0	1.0 / 0.022	0.0	1.0 / 0.022	0.0
ETOH Med / NIC Low	2.0 / 0.067	0.0	2.0 / 0.067	0.0	2.0 / 0.067	0.0	2.0 / 0.067	0.0
ETOH Med / NIC Med	2.0 / 0.022	0.0	2.0 / 0.022	0.0	2.0 / 0.022	0.0	2.0 / 0.022	0.0

Note. Nine experimental groups were used to evaluate the rewarding effects of ethanol and nicotine separately, as well as when coadministered. There was one saline control group, two ethanol-only groups, two nicotine-only groups, and four ethanol and nicotine coadministration groups. Rats were randomly assigned to each group; both male and female rats were used.

examined separately for males and females using a two-way repeated-measures ANOVA with conditioning day (PD 23-31) and group (1-7) or (1-9), respectively, as the independent factors. Mauchly's test analyzed the assumption of sphericity for all analyses involving repeated

measures (i.e., body weight). Given that assumption of sphericity was violated in all the analyses, the Greenhouse-Geisser correction was used to correct the degrees of freedom.

CPP

Based on previous experiments examining ethanol-induced CPP, Experiment 1 required a sample of approximately 10 subjects per group to obtain statistical significance (Bienkowski et al., 1996; Ciccocioppo et al., 1999; Dickinson et al., 2009; Fidler et al., 2004; Philpot et al., 2003). Likewise, based on previous experiments examining ethanol- and nicotine-induced CPP, Experiment 2 required a sample of approximately 10 subjects per group to obtain statistical significance (Ahsan et al., 2014; Dannenhoffer & Spear, 2015; Natarajan et al., 2011; Philpot et al., 2014; Shram et al., 2006).

A preference score was calculated and served as the dependent variable for both experiments to evaluate shifts in preference to the drug-paired compartment between preconditioning and postconditioning. The preference score was calculated by subtracting seconds spent in the non-preferred side (i.e., drug paired side) during postconditioning from seconds spent in the non-preferred side during the preconditioning session. Positive preference scores indicated a preference to the drug-paired side, while negative preference scores indicated an aversion to the drug-paired side. Rats were removed from the analysis if they were more than two standard deviations away from the group mean. Overall, two outliers (1.2%) were excluded from Experiment 1 and three outliers (1.0%) were excluded from Experiment 2.

The CPP results were analyzed in two ways: Analysis of Variance (ANOVA) and planned comparisons. The ANOVA allowed for a between-group analysis, which involved comparisons between the experimental and saline control groups. In contrast, the planned comparisons allowed for within-group analysis, which involved comparing the time spent on the

drug-paired side during preconditioning and postconditioning. Male and female data were analyzed using separate ANOVAs, given sex differences were not the primary goals of this study. Moreover, sex was not considered as a factor for several reasons. First, we are studying the rats during a periadolescent stage before major sex differences begin to emerge. Second, the possible sex differences would be negligible due to the rats' young ages (Schramm-Sapyta et al., 2014). Lastly a wide range of doses would be necessary to reveal any possible differences (Torres et al., 2008).

Experiment 1 was not a completely factorial design (i.e., there are no ascending versus fixed saline groups), and thus a group variable was used as the independent variable for the analysis. In Experiment 1, a one-way ANOVA with group as the independent variable was used to examine the preference score. In Experiment 2, a 3×3 ANOVA (ethanol × nicotine) was used to examine the preference score. All significant main effects and interactions were analyzed further with simple main effects and Tukey's test ($p < .05$).

Planned comparisons examined if there was a significant increase in time spent in the drug-paired side from preconditioning to postconditioning. To test for significant shifts in preference between the two, a paired-samples *t*-test was analyzed for each individual group. Planned comparisons allow for an examination of CPP using weak reinforcers like ethanol and nicotine (Lenoir et al., 2015).

To summarize data was analyzed using planned comparisons and ANOVA. To establish CPP, the following criteria had to be met: (a) rats spent more time in the drug-paired compartment than the saline control (between-group comparison) and/or (b) rats spent more time in the drug-paired side during postconditioning than during preconditioning (within-group comparison). Rats that met both criteria were determined to have exhibited robust CPP.

CHAPTER 3

RESULTS

Experiment 1

Body Weight

Body weight (data not shown) did not differ across the seven groups for males or females across the 10-day CPP procedure (i.e., PD 23-31). Mauchly's test indicated that the assumption of sphericity had been violated for both males and females, $\chi^2(44) = 444.51$, $p = .000$ and $\chi^2(44) = 620.74$, $p = .000$ respectively, meaning the Greenhouse Geiser correction was used. However, both male and female rats exhibited predictable weight gain across the 10-day procedure, main effect of day, $F(2.84, 153.41) = 1260.29$, $p = .000$, $\eta_p^2 = .96$ and $F(2.05, 129.36) = 1799.34$, $p = .000$, $\eta_p^2 = .97$, respectively.

CPP

Males. The individual and mean preference scores for male rats are presented in Figure 3. The one-way between-subjects ANOVA revealed no significant main effect of group, $F(6, 54) = 1.81$, $p = .114$, $\eta_p^2 = .17$. Results from the paired-samples *t*-test revealed that male rats in the fixed high dose (2.0 g/kg) ethanol group spent significantly more time in the drug-paired side during postconditioning than preconditioning, $t(8) = 3.72$, $p = .006$, as seen in Table 5.

TABLE 5. Significant Results of Planned Comparisons for Experimenr 1 Male Rats

Group	*t*-test statistic	DF	*p*-value	Cohen's D
Saline	1.02	8	.340	.338
Fixed 0.5 g/kg Ethanol	1.20	8	.266	.398
Fixed 1.0 g/kg Ethanol	1.93	8	.101	.730
Fixed 2.0 g/kg Ethanol	3.72	8	.006*	1.24
Ascending 0.5 g/kg Ethanol	.57	8	.587	.189
Ascending 1.0 g/kg Ethanol	1.96	8	.085	.654
Ascending 2.0 g/kg Ethanol	.10	8	.921	.033

Note. Group indicates which drug group demonstrated a significant increase in time spent in the drug-paired compartment from preconditioning to postconditioning. * indicates a significant finding at $p < .05$.

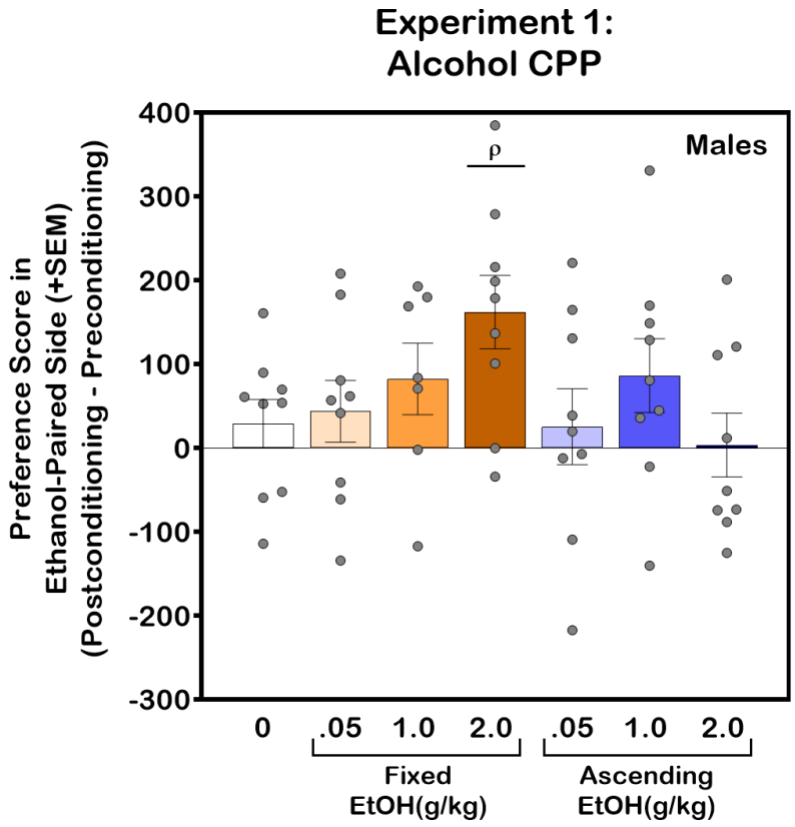

FIGURE 3. Experiment 1 ethanol-induced CPP for male rats. Individual preference scores are shown for males conditioned with fixed or ascending ethanol doses (0.5, 1.0, and 2.0 g/kg). Error bars represent standard error for each respective group. ρ represents a significant planned comparison, meaning the preference scores show a significant difference between the amount of time spent in the ethanol-paired side between preconditioning and postconditioning.

Females. The individual and mean preference scores for female rats are presented in Figure 4. The one-way between-subjects ANOVA revealed significant effect of group, $F(6, 63) = 3.21$, $p = .008$, $\eta_p^2 = .23$. Tukey HSD post hoc analyses revealed that rats in the fixed 2.0 g/kg ethanol group had higher preference scores than the saline control group ($p = .004$). As shown in Table 6, planned comparisons using the paired-samples *t*-test also revealed that female rats in the ascending 1.0 g/kg ethanol group and the fixed 2.0 g/kg ethanol group spent significantly more

time in the drug-paired side during postconditioning compared to preconditioning, $t(8) = 3.46$, $p = .009$ and $t(8) = 5.22$, $p = .001$, respectively.

TABLE 6. Significant Results of Planned Comparisons for Experimenr 1 Female Rats

Group	*t*-test statistic	DF	*p*-value	Cohen's D
Saline	1.08	10	.306	.325
Fixed 0.5 g/kg Ethanol	1.338	9	.214	.432
Fixed 1.0 g/kg Ethanol	1.85	10	.095	.57
Fixed 2.0 g/kg Ethanol	5.22	8	.001*	2.78
Ascending 0.5 g/kg Ethanol	1.79	9	.107	.57
Ascending 1.0 g/kg Ethanol	3.46	8	.009*	1.15
Ascending 2.0 g/kg Ethanol	1.51	9	.166	.48

Note. Group indicates which drug group demonstrated a significant increase in time spent in the drug-paired compartment from preconditioning to postconditioning. * indicates a significant finding at $p < .05$.

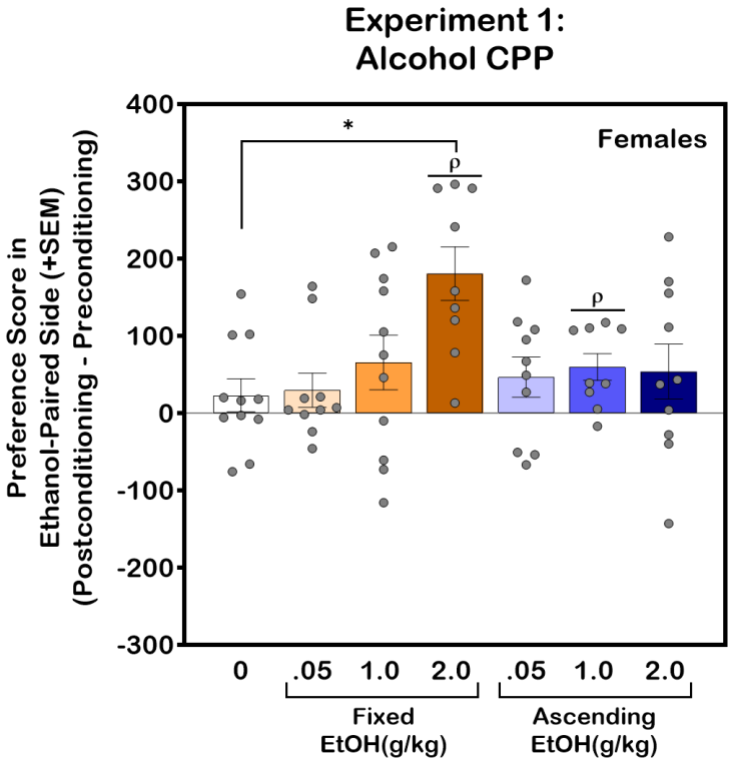

FIGURE 4. Experiment 1 ethanol-induced CPP for female rats. Individual preference scores are shown for females conditioned with fixed or ascending ethanol doses (0.5, 1.0, and 2.0 g/kg). Error bars represent standard error for each respective group. *Represents a significant difference between the saline group and the 2.0 g/kg fixed dosing pattern ethanol group. ρ Represents a significant planned comparison, meaning the preference scores show a significant difference between the amount of time spent in the ethanol-paired side between preconditioning and postconditioning.

Experiment 2

Body Weight

Body weight for males or females (data not shown) did not differ between groups across the 10-day CPP procedure (i.e., PD 23-31). Similar to Experiment 1, Mauchly's test showed that the assumption of sphericity was violated and the Greenhouse Geiser correction was used, $\chi^2(44) = 973.08$, $p = .000$ and $\chi^2(44) = 881.96$, $p = .000$ respectively. The only significant difference was the predictable weight gain as an effect of day for both males and females, $F(1.48, 97.48) = 801.32$, $p = .000$, $\eta_p^2 = .92$ and $F(2.28, 186.79) = 565$, $p = .000$, $\eta_p^2 = .87$ respectively.

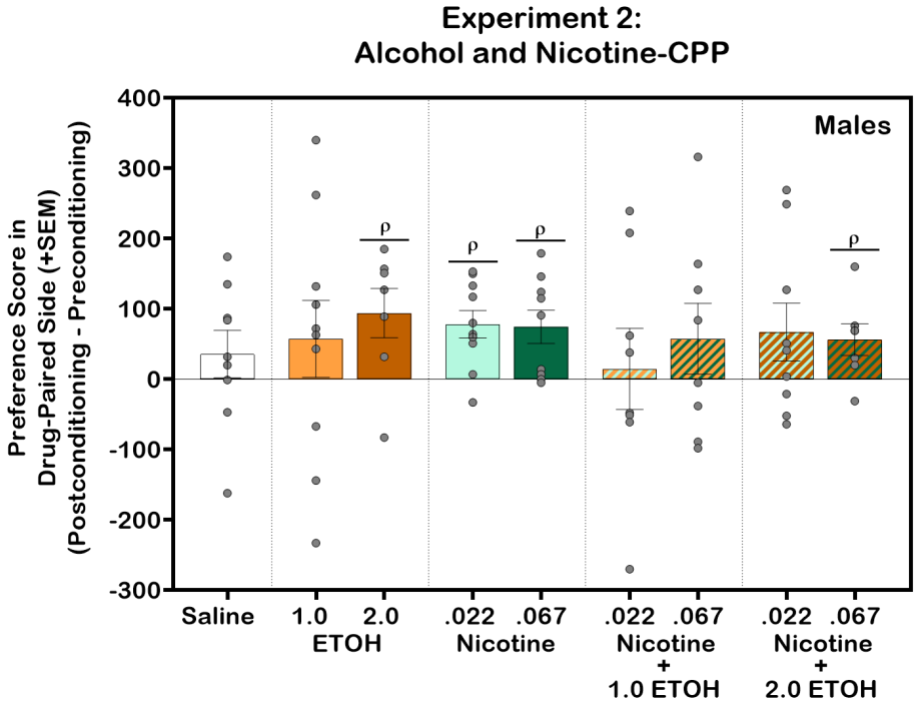

FIGURE 5. Experiment 2 ethanol and nicotine coadministration CPP for male rats. There were nine groups to examine the rewarding effects of ethanol (g/kg), nicotine (mg/kg), and ethanol and nicotine coadministration in male rats. Error bars represent standard error for each respective group. ρ Represents a significant planned comparison, meaning the preference scores show a significant difference between the amount of time spent in the drug-paired side between preconditioning and postconditioning.

CPP

Males. The individual and mean preference scores for male rats are presented in Figure 5. The 3×3 ANOVA did not reveal any significant main effect of ethanol [$F(2, 68) = .389$, $p = .680$, $\eta p^2 = .01$], nicotine [$F(2, 68) = .052$, $p = .949$, $\eta p^2 = .00$], or interaction [$F(4, 68) = .441$, $p = .779$, $\eta p^2 = .03$]. Planned comparisons using the paired-samples *t*-test found significant differences for a subset of groups, as indicated in the Table 7. Specifically, male rats in the 2.0 g/kg ethanol group or 0.022 mg/kg and 0.067 mg/kg nicotine groups spent significantly more time in the drug-paired side from preconditioning to postconditioning, $t(6) = 2.67$, $p = .037$, d = 1.009, $t(9) = 4.01$, $p = .003$, d = 1.269, and $t(8) = 3.14$, $p = .014$, d = 1.045, respectively. Finally, the only coadministration group to spent significantly more time in the drug paired side during postconditioning compared to preconditioning was the 2.0 g/kg ethanol plus 0.067 mg/kg nicotine group, $t(6) = 2.49$, $p = .047$, d = .942. There were no other statistically significant results between any of the other groups.

TABLE 7. Significant Results of Planned Comparisons for Experiment 2 Male Rats

Group	*t*-test statistic	Degree of Freedom	*p*-value	Cohen's D
Saline	1.44	11	.177	.417
1.0 g/kg Ethanol	.99	8	.351	.330
2.0 g/kg Ethanol	2.82	10	.018*	.850
0.022 mg/kg Nicotine	1.66	11	.125	.480
0.067 mg/kg Nicotine	9.79	8	.000*	3.262
1.0 g/kg Ethanol + 0.022 mg/kg Nicotine	3.15	7	.016*	1.114
1.0 g/kg Ethanol + 0.067 mg/kg Nicotine	2.22	8	.032*	.741
2.0 g/kg Ethanol + 0.022 mg/kg Nicotine	2.48	10	.057	.748
2.0 g/kg Ethanol + 0.067 mg/kg Nicotine	1.6	10	.152	.467

Note. Group indicates which drug group demonstrated significant differences between time spent in the drug-paired compartment from postconditioning and preconditioning. * indicates a significant finding at $p < .05$.

Females. The individual and mean preference scores for female rats are presented in Figure 6. The 3×3 ANOVA did not reveal any significant main effect of ethanol [$F(2, 83) = .478$, $p = .622$, $\eta p^2 = .01$], nicotine [$F(2, 83) = .894$, $p = .413$, $\eta p^2 = .02$], or interaction [$F(4, 83) = .2.06$, $p = .093$, $\eta p^2 = .09$]. Planned comparisons using the paired-samples *t*-test found significant differences for a subset of groups, as indicated in the Table 8. Specifically, female rats in the 2.0 g/kg ethanol group or the 0.022 mg/kg nicotine group spent significantly more time in the drug-paired side from preconditioning to postconditioning, $t(10) = 2.82$, $p = .018$, d = .85 and $t(8) = 9.79$, $p = .000$, d = 3.0, respectively. Finally, the two groups that received 1.0 g/kg ethanol coadministered with either 0.022 mg/kg or 0.067 mg/kg nicotine groups spent significantly more time in the drug paired side during postconditioning compared to preconditioning, $t(7) = 3.15$, $p = .016$, d = 1.114 and $t(10) = 2.48$, $p = .032$, d = .741, respectively. There were no other statistically significant results between any of the other groups.

TABLE 8. Significant Results of Planned Comparisons for Experiment 2 Female Rats

Group	*t*-test statistic	Degree of Freedom	*p*-value	Cohen's D
Saline	1.44	11	.177	.417
1.0 g/kg Ethanol	.99	8	.351	.330
2.0 g/kg Ethanol	2.82	10	.018*	.850
0.022 mg/kg Nicotine	1.66	11	.125	.480
0.067 mg/kg Nicotine	9.79	8	.000*	3.262
1.0 g/kg Ethanol + 0.022 mg/kg Nicotine	3.15	7	.016*	1.114
1.0 g/kg Ethanol + 0.067 mg/kg Nicotine	2.22	8	.032*	.741
2.0 g/kg Ethanol + 0.022 mg/kg Nicotine	2.48	10	.057	.748
2.0 g/kg Ethanol + 0.067 mg/kg Nicotine	1.6	10	.152	.467

Note. Group indicates which drug group demonstrated significant differences between time spent in the drug-paired compartment from postconditioning and preconditioning. * indicates a significant finding at $p < .05$.

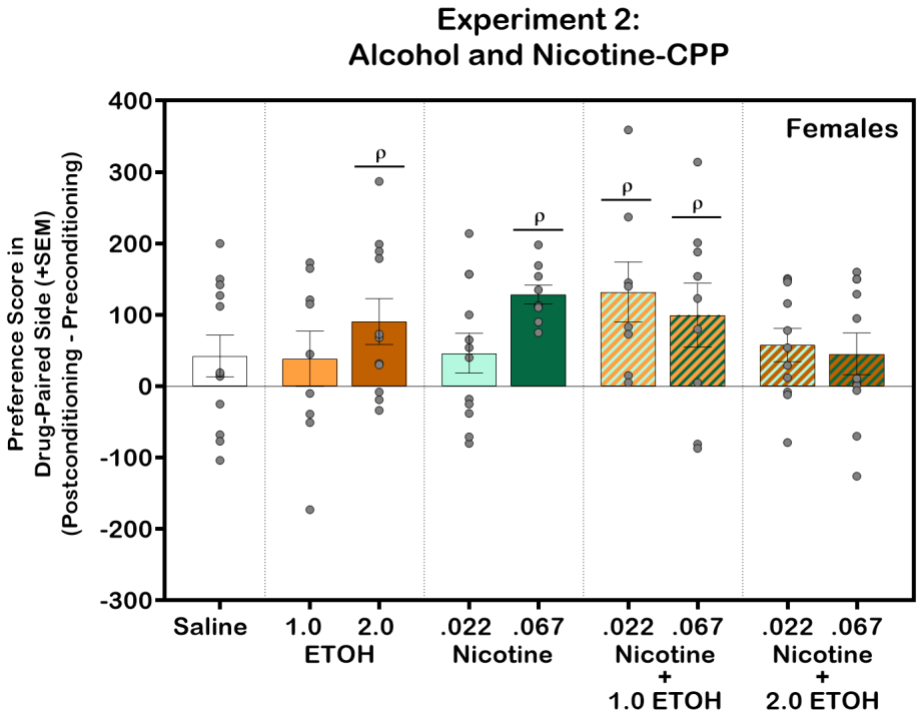

FIGURE 6. Experiment 2 ethanol and nicotine coadministration CPP for female rats. There were nine groups to examine the rewarding effects of ethanol (g/kg), nicotine (mg/kg), and ethanol and nicotine coadministration in female rats. Error bars represent standard error for each respective group. ρ Represents a significant planned comparison, meaning the preference scores show a significant difference between the amount of time spent in the drug-paired side between preconditioning and postconditioning.

CHAPTER 4

DISCUSSION

Although several studies have examined the effects of nicotine in an adolescent population, very few studies have examined the rewarding effects of ethanol in an adolescent model, and few studies examine the coadministration of ethanol and nicotine in an adolescent model. The study of the coadministration of these drugs is critical due to the high rates of their simultaneous use, often beginning in early adolescence. The CPP paradigm was used to analyze the rewarding effects of both drugs. The overall focus of this study was to examine the rewarding effects of ethanol and nicotine coadministration in an adolescent animal model. Because a dose-response curve for ethanol reward is not well established in the literature, our first experiment established an ethanol-induced CPP dose-response curve. Previous research has demonstrated that different dosing patterns of drug administration (e.g., fixed dosing vs. ascending dosing) result in a greater CPP magnitude. Therefore, the first experiment used fixed and ascending dosing patterns for ethanol during conditioning in male and female periadolescent (PD 23-31) rats. It was hypothesized that ascending doses of ethanol would more closely mimic the human experience of using ethanol and therefore demonstrate a more robust ethanol preference using the CPP paradigm. Once a solid ethanol-induced CPP dose-response curve was established, those doses and dosing patterns were used in Experiment 2 to study the coadministration of ethanol and nicotine – the primary purpose of the study. The hypothesis of Experiment 2 was that rats who were administered both ethanol and nicotine would demonstrate a higher CPP magnitude compared to rats who were only administered one of the drugs.

In summary, there were two purposes to this study: (a) to establish a dose-response curve using ethanol-induced CPP (Experiment 1), and (b) using those established ethanol doses from

Experiment 1, to examine the coadministration of ethanol and nicotine during adolescence (Experiment 2). The overall results of Experiment 1 demonstrated that both male and female rats were able to establish ethanol-induced CPP. Specifically, both male and female rats demonstrated ethanol-induced CPP in a fixed dosing pattern, while only female rats demonstrated CPP in both the fixed and ascending dosing patterns. Thus, the results contrasted with the hypothesis that the ascending dosing pattern would lead to stronger CPP. Based on the results from Experiment 1, Experiment 2 used fixed doses of ethanol and again demonstrated that both male and female rats were able to establish CPP to ethanol and demonstrate nicotine-induced CPP in a dose-dependent manner (see Figures 5 and 6). However, the preferences were not as robust, given that none of the groups differed from the saline control. Furthermore, the coadministration of ethanol and nicotine also did not produce robust CPP. However, there was an indication that the coadministration may have facilitated the preference for nicotine when the 1.0 g/kg of ethanol dose was administered to female adolescent rats (see Figure 6). Thus, overall there was no strong support for the hypothesis that coadministration of ethanol and nicotine would produce strong place preferences.

Ethanol Conditioned Place Preference

The main results from the first experiment found that both males and females successfully demonstrated ethanol-induced CPP. When examining the results for Experiment 1 in male rats, the ANOVA analysis revealed no significant differences between groups, demonstrating that no group significantly differed from the saline group. Conversely, the planned comparisons revealed that male rats in the fixed 2.0 g/kg alcohol group spent significantly more time in the drug-paired side during postconditioning compared to preconditioning. This is considered to be a weaker CPP because only one criterion for CPP was established. Robust CPP

is evident when the experimental group is significantly different from the control group (between-group criterion), and the experimental group shows a significant increase in the time spent in the drug-paired side between preconditioning and postconditioning (within-group criterion). Experiment 1 female rats showed a significant difference in both ANOVA (between groups) and planned comparisons (within groups) for the fixed 2.0 g/kg ethanol group, which indicated that this group demonstrated a robust ethanol-induced CPP. Experiment 2 results confirmed the results for Experiment 1 because both male and female rats again established CPP using the fixed 2.0 g/kg ethanol doses. However, the preference in females was less robust in Experiment 2. Nevertheless, these results for fixed 2.0 g/kg ethanol doses are consistent across males and females in both experiments.

The ethanol preference in adolescent male and female rats is noteworthy, since it has been challenging to establish a consistent ethanol-induced CPP in adult populations, and even more so in adolescent populations. Prior studies in adult animals reveal a lack of ethanol-induced CPP or establish ethanol-induced CPA (Bienkowski et al., 1996; Cunningham, 1979; Fidler et al., 2004; Gauvin & Holloway, 1992; Philpot et al., 2003; Torres et al., 2014). Nonetheless, this study's results are consistent with an adult ethanol-induced CPP study in which CPP was successfully established using the fixed 2.0 g/kg ethanol doses (Dickinson et al., 2009). When comparing this study to past adolescent ethanol research, this study adds to the already inconsistent results. In Dickinson et al. (2009), adolescent mice (PD 31) required higher (4.0 g/kg) doses of ethanol to establish CPP, while adults were able to establish CPP using lower (2.0 g/kg) doses. This contrasts with Philpot et al. (2003), wherein CPP was established using small (0.2 g/kg) doses of ethanol at PD 25, and using larger (0.5 and 1.0 g/kg) doses of ethanol at PD 45. Similar to the successful CPP at 1.0 g/kg in Philpot et al. (2003), Torres et al. (2014)

demonstrated that both adolescent female rats (PD 45) established CPP at 1.0 g/kg ethanol, while adolescent males failed to establish CPP, even showing CPA at higher (2.5 g/kg) doses. This study fits somewhere in-between these three studies in that rats at PD 32 rats established CPP using 2.0 g/kg doses of ethanol, which is a lower dose than Dickenson et al. (2003), but a higher dose than Philpot et al. (2003) and Torres et al. (2014).

The use of ascending doses of ethanol did not produce consistent results. Planned comparisons for female rats showed that the ascending 1.0 g/kg dose of ethanol successfully established CPP. However, these results are inconsistent in that females could establish CPP at only one ascending dose, and the preference for this dose was lower than that of a fixed 2.0 ethanol dose and not significantly different from saline control (see Figure 4). Males also did not demonstrate a greater preference from the use of ascending ethanol doses to establish CPP. Indeed, CPP was not established with any ascending dose. It was hypothesized that using an ascending dosing pattern would lead to a stronger ethanol-induced CPP because the ascending pattern more closely mimicked the human experience of drinking alcohol and because prior work had been successful using this approach with a different drug of abuse (Conrad et al., 2013; Itzhak & Anderson, 2011). Because we successfully establish CPP in both males and females using a fixed dosing pattern, but not with the ascending dosing with males and not reliably with females, these results did not support that hypothesis. It is possible that the ascending doses used were not high enough to produce ethanol preference, given that the rats only received 2.0 g/kg once on the last day of conditioning. It is also possible that the methodology would need to be altered to successfully establish ethanol-induced CPP using the ascending dosing pattern, such as more conditioning days, longer conditioning sessions, or more gradual increases in ethanol administration. There are currently no published studies using ethanol in an ascending dosing

pattern, so further research is needed.

Ethanol and Nicotine Coadministration

The fixed 2.0 g/kg ethanol dosing pattern from the first experiment and the already established doses of nicotine were used for the second experiment, which examined the coadministration of ethanol and nicotine during adolescence. The ANOVA revealed no significant differences for both males and females. However, using planned comparisons, both male and female rats established CPP after receiving 2.0 g/kg ethanol, replicating Experiment 1 and demonstrating the reliability of the ethanol data. Male rats also exhibited CPP after administering both nicotine doses (.022 and .067 mg/kg) alone, confirming the established doses used in the nicotine CPP literature (Ahsan et al., 2014; Dannenhoffer & Spear, 2015; Natarajan et al., 2011; Shram et al., 2006). Notably, the preference exhibited for both ethanol and nicotine was minor, given that these groups did not differ from saline control rats. In males, the only coadministration group that demonstrated a modest change in preference was the group given the high dose of ethanol (2.0 g/kg) and nicotine (.067 mg/kg; see Table 5 and Figure 5). However, it is difficult to ascertain whether this dose combination was effective because the preferences for the coadministration were not significantly greater than that of nicotine or ethanol by itself. Thus, the preferences for the coadministration may only be reflecting the nicotine or ethanol dose. Alternatively, it is possible that 1.0 g/kg of ethanol could be shifting the nicotine dose response curve to the right – administration of the 1.0 ethanol and either the 0.22 or 0.067 mg/kg dose of nicotine resulted in no preference for the drug-paired side, which is typically seen when higher doses of nicotine are used (Torres et al., 2008). In other words, the ethanol could have enhanced the effect of nicotine, such that there is a decrease in preference for the drug-paired side. However, this seems unlikely, as the same pattern was not evident in the group that received the

2.0 g/kg dose of ethanol. Further research with higher doses of nicotine and/or ethanol is needed to parse out any coadministration effects.

In females, the coadministration of ethanol and nicotine also produced mixed results. Females demonstrated CPP with the high dose of nicotine (.067 mg/kg), but not the low dose (.022 mg/kg), which is consistent with prior work (Torres et al., 2008). Small doses of nicotine were purposefully used, so it would be possible to see an enhancement of preferences when both ethanol and nicotine were coadministered. Higher doses of nicotine are less variable, but may not have allowed increases in preference to be detected with coadministration. Female rats also demonstrated CPP using the low ethanol dose (1.0 g/kg) coadministered with both nicotine doses (.022 and .067 mg/kg), perhaps suggesting an enhancement of the effects of the 0.22 mg/kg nicotine dose, given that the 0.22 mg/kg nicotine alone failed to shift the time spent in the drug-paired side during postconditioning. Similarly, the 2.0 g/kg dose of ethanol may have enhanced the effects of the two doses of nicotine (see Figure 6). As mentioned before, higher doses of nicotine result in a decrease in preference. Nonetheless, all rats showed a modest preference to the drug-paired side, given that none of the groups differed from the saline control group. As with the males, further research with different doses of nicotine are needed to confirm a shift to the right of the dose response curved to nicotine.

Overall, the present study failed to convincingly show a strong preference for the coadministration of ethanol and nicotine in male and female adolescent rats. These results are consistent with prior work that has examined this question in adult rats. Specifically, past CPP studies have been inconsistent in their ability to find evidence of interaction when nicotine and ethanol are coadministered, with some studies finding no evidence of interaction using CPP (Korkosz et al., 2006). The present student extended this past study by examining adolescent

rats, which typically are more sensitive to the rewarding effects of nicotine and ethanol. Moreover, there is evidence that the two drugs affect each other, although not within the CPP paradigm. For example, other studies were able to find evidence of increased locomotor activity between the two drugs (Gubner et al., 2015) or a decrease in ethanol-induced seizures (Korkosz et al., 2006) when the drugs were coadministered. Thus, this study is unique because it provides partial evidence of a behavioral interaction when ethanol and nicotine are used together in the CPP paradigm during adolescence. Additional research is needed to replicate the present findings, perhaps using additional intermediary doses to investigate the coadministration of ethanol and nicotine in male and female adolescent rats more deeply. Because this experiment analyzed rats in early adolescence, future work could examine mid- and late-adolescent male and female rats. In addition, future work should examine the mesolimbic dopamine pathway in rats after coadministration of ethanol and nicotine to begin to uncover potential brain mechanisms.

The mesolimbic dopamine pathway is known to mediate the rewarding and reinforcing properties of ethanol and nicotine. There is evidence of interaction in how these drugs interrelate with the pathway (DiChiara & Imperato, 1988). Specifically, nAChRs in the VTA and NA show evidence of this interaction, and there are several examples of this interaction: pharmacologically blocking nAChRs in the VTA led to a decrease in ethanol intake (Söderpalm et al., 2000), exposing cultured neurons from the VTA and NA to ethanol and nicotine simultaneously led to an increase in nAChR receptors (Inoue et al., 2007), and in vivo microdialysis has found that coadministration of both drugs produced an additive release of dopamine in the NA (Tizabi et al., 2002, 2007). Altogether, these studies contribute to the finding that ethanol and nicotine interact in the mesolimbic dopamine pathway, specifically at the nAChR receptors. This study, by using CPP to examine the rewarding properties of these drugs, provides some behavioral

evidence that ethanol and nicotine interact along this pathway. Future studies could examine the impact of coadministration of ethanol and nicotine on these receptors more directly.

Strengths and Limitations

The current study had several limitations and strengths that need to be addressed in future studies. The most significant limitation was the struggle to get enough animals for each group to be appropriately powered, statistically. Specifically, rats were not included in the study if they spent more than 67% of the preconditioning phase in the preferred side of the apparatus because such an initial strong preference makes it difficult to establish CPP (O'Dell et al., 2004). Typically, about 5% of animals are expected to be lost due to strong preconditioning preferences (Franco et al., 2020); however, in this study, 20.9% were lost from Experiment 1 and 27.8% were lost from Experiment 2. An essential difference between the present and prior studies, however, is that we used younger rats in the present study. Thus, the strong initial preferences may be due to age, with younger animals showing stronger preferences. Regardless, this high attrition made it difficult to properly power the groups, leading to more variable and weaker results. When examining the statistical power, several of the groups only demonstrate low or medium statistical power according to Cohen's D. The ANOVA analyses for the coadministration group also demonstrated low power as interpreted using ηp^2. A future iteration of this experiment would need to have strong power for each group. It is possible that our results were weak because the animals were too young and formed extremely strong initial preferences; however, it is also possible that the groups were too underpowered to produce significant results.

One limitation of CPP is that it does not allow for a direct measure of drug abuse. Instead, it measures the degree to which the animal can be classically conditioned to associate the effects of the drug with a particular environment. On the other hand, self-administration

allows for animals to choose when to administer the drug (typically via an intravenous route), which more closely mimics the human experience (Panlilio & Goldberg, 2007). Though self-administration has some advantages, CPP also has several unique strengths. First, CPP requires as little as one dose of the drug to show the rewarding effects, while self-administration requires multiple infusions to establish a behavioral response (Bardo & Bevins, 2000). Second, CPP can show responses to low doses of a drug, which allows for a greater assessment of the rewarding value of drugs (Carr et al., 1989). Finally, unlike self-administration, where surgery is required so the animal can receive infusions of the drug during the procedure, CPP does not require a surgical procedure. Surgery is invasive, can lead to health complications, and generally produces more pain for the animal compared to CPP studies (Panlilio & Goldberg, 2007). Moreover, the CPP paradigm allows for tight control over the amount of drug dose administered (Bardo & Bevins, 2000), which allows for more specific findings, especially when examining interaction effects from the coadministration of two drugs.

Animal models, in general, are also limited in that they do not take into account the social environments (i.e., peer pressure) associated with drinking and smoking in clinical populations, which can have significant effects on drug use during adolescence (Fujimoto & Valente, 2012). Animal models can address the effect of social contact on an animal's experience by manipulating exposure to other rats; however, it is not currently possible to study an animal equivalent of social drug use, with the associated peer pressures to engage in drug use (Peartree et al., 2017). While the social aspect of drug use cannot be accounted for, using CPP (and animal models in general) to study drug abuse allows for high experimental control. When using CPP, all rats receive a standard level of care from their mother, the same level of socialization with other rats, the same exposure to the CPP box, and the same number of exposures to the drugs

(Bardo & Bevins, 2000). Altogether, CPP is a strong preliminary paradigm for measuring the rewarding effects of drugs, with some unavoidable limitations.

Implications

Ethanol and nicotine are the two most commonly abused drugs in the United States, and adolescence is the most common time period in life to initiate their use (Falk et al., 2006). This study explored how specific doses of ethanol and nicotine interacted in heavily controlled circumstances using an adolescent rat model. Previous research focused on the long-term effects of early ethanol and nicotine use but not on the effects of coadministration during adolescence. While this study was able to establish a dose-response curve for ethanol-induced CPP, there was minimal data to support the hypothesis that the potential sensitivity of these drugs would be heightened when co-administered.

The most significant success achieved in this study was establishing an ethanol dose (2.0 g/kg) that successfully established CPP across both experiments in an adolescent model. Future studies could examine the extinction and reinstatement of an ethanol-induced CPP and expand the knowledge base of ethanol's effects in adolescence. The ethanol dose-response curve can also extend to examine how ethanol interacts with other drugs. Examining these drug interactions is crucial, especially the high level of co-use of drugs high-school students reported and the increased risk for potential drug-abuse problems into young adulthood (Moss et al., 2014).

Through these experiments, we were able to determine which ethanol dose pattern established CPP preference and some of the interaction effects of coadministration of ethanol and nicotine. Most importantly, these questions were applied to an adolescent population, which is often overlooked in animal research despite their unique vulnerability to drug use. When considering biological implications, future studies are needed to expand upon Experiment 2 to

examine the underlying mechanisms of ethanol and nicotine coadministration. Specifically, research should be focused on the mesolimbic dopamine pathway, given the critical role dopamine plays in drug reward.

Conclusion

The primary purpose of this study was to examine the coadministration of ethanol and nicotine during adolescence. Though we successfully established CPP using both nicotine or ethanol, the results were mixed between males and females. Additionally, we found some evidence that the coadministration of ethanol and nicotine produces preferences for the drug-paired side, although the magnitude of the preference was modest, given that none of the groups differed from the saline control group. Finally, one unanticipated result was that ethanol-induced CPP was successfully established using a fixed dosing method instead of the hypothesized ascending dosing method. The coadministration of ethanol and nicotine is difficult to examine, but this study provides some evidence that further research should be conducted in this area.

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