ASSESSING MOTIVATIONAL AND ASSOCIATIVE LEARNING MECHANISMS UNDERLYING COMPULSIVE DRINKING
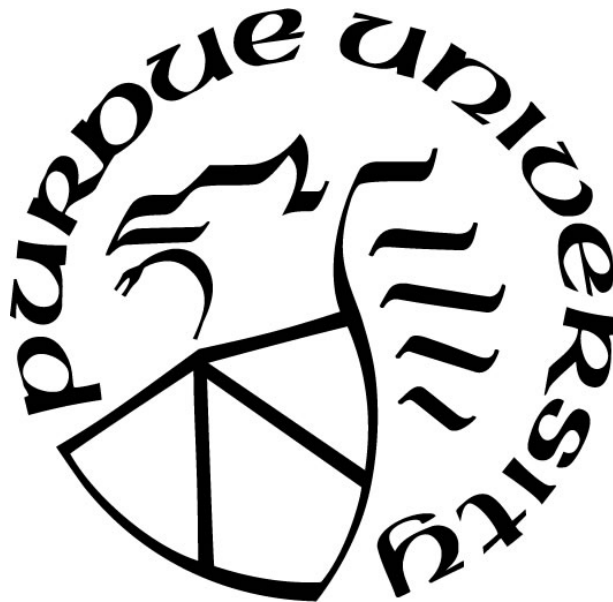
by

Claire R. Carron

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STATEMENT OF COMMITTEE APPROVAL

Dr. Nicholas Grahame, Chair

Department of Psychology

Dr. Cristine Czachowski

Department of Psychology

Dr. Christopher Lapish

Department of Psychology

Dr. Fredric Hopf

IU School of Medicine, Department of Psychiatry

Approved by:

Dr. Nicholas Grahame

To my parents. Your love, support, and encouragement made me who I am. I love you.

Also, to a lesser extent, to dogs. See above.

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I would like to acknowledge my mentor Dr. Nicholas Grahame for his willingness to let me follow my interests and work on new behavioral projects, for the significant or the null. Thank you to my dissertation committee, for providing such positive encouragement and thoughtful feedback throughout my entire graduate career. To the Addiction Neuroscience faculty, thank you for always answering my questions, and encouraging me to continue asking them. To my fellow graduate students, thank you for cheering with me when I was right, and telling me when I was wrong. Thank you for the constant encouragement, support, and joy you brought to my graduate experience.

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ABSTRACT

Continued consumption of alcohol despite the knowledge of negative consequences is a hallmark of alcohol use disorder (AUD), yet much remains unknown about what motivates these behaviors. Compulsive drinking may require motivational resources that are not necessary when drinking in unchallenged conditions in order to counteract the addition of these negative consequences. Increased sensitivity to drug-paired stimuli via associative learning processes may provide this additional motivation. To evaluate if alcohol-paired stimuli enhance alcohol seeking, selectively bred crossed High Alcohol Preferring mice experienced Pavlovian conditioning procedures with an alcohol unconditioned stimulus. We hypothesized that after repeated pairings, alcohol cues would elicit seeking conditioned responses. Then, to determine if the motivation provided by these cues influenced responding, mice were trained to respond for alcohol and tested in the presence of alcohol cues. Finally, to test if alcohol-paired cues influence compulsive drinking, this same test was repeated with the addition of response-contingent footshock. We hypothesized the cue paired with alcohol would increase responding for alcohol in unchallenged conditions, but especially in challenged conditions, contributing to compulsivity. An auditory stimulus paired with alcohol did elicit enhanced seeking responses, but contrary to hypothesis, we observed no effect of these same cues on instrumental responding. To validate these findings, training and testing procedures must be optimized to ensure conditioning has properly occurred and compulsivity is being appropriately measured.

INTRODUCTION

Compulsive Alcohol Use and Craving

Drinking alcohol despite aversive consequences, or compulsive drinking, is a core feature of alcohol use disorder (AUD; American Psychiatric Association, 2013), and contributes significantly to the personal and societal problems experienced by those with AUD (Ahmed, 2012; Epstein & Kowalczyk, 2018; Koob & Volkow, 2010; NIDA, 2017). Symptoms of this compulsive use are outlined in the AUD criteria found in the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013), such as continued use despite recurrent interpersonal problems, failure to fulfill major role obligations, and withdrawal from important activities. Although compulsion is generally accepted as an important facet of addictive disorders (e.g Ahmed, 2012; Koob & Volkow, 2010) it remains poorly understood. Robinson and Berridge (1993) propose that to understand addiction, we first need to understand how drug taking behaviors become compulsive; they suggest compulsivity involves the development of craving, which leads to greater drug seeking and taking.

Although drug seeking and taking are essential components of addiction, their presence alone is not sufficient to encapsulate the disorder. Compulsion, like craving, is a somewhat abstract concept that has proven difficult to consistently operationalize and study in humans and animals alike. This difficulty may arise from inconsistences in parameters and in defining compulsion. In order to effectively investigate what contributes to compulsive use, it is necessary to first define compulsion. The hallmark feature of compulsive drug use is its occurrence despite both the presence and knowledge of costs associated with use. Hopf and Lesscher (2014) suggest that compulsion involves both aversion-resistant seeking and inflexibility towards a reinforcer, defined as inability to resist the urge to use drugs even if an individual desires to do so. Inflexibility is further characterized by patterns of responding remaining unchanged regardless of shifts in the value of the reinforcer.

This definition of compulsive drinking requires knowledge of the negative consequences associated with alcohol use, with a user continuing to drink despite this conflict. This behavior likely requires motivational processes distinct from those used in unchallenged drinking conditions. This additional motivation may be provided by craving. For example, when

compared to light drinkers, heavy drinkers engage in more alcohol seeking behaviors under threat of punishment and also report higher levels of craving (Grodin et al., 2018). Significant correlations have been uncovered between self-reported craving and the compulsive drive to consume alcohol (Anton et al., 1995; Modell et al., 1992). Moreover, greater craving and compulsion scores contribute to relapse and negative alcohol use outcomes (Bottlender, 2004). A study of individuals admitted to the emergency department for alcohol-related problems demonstrated that the compulsive dimension of craving significantly contributes to AUD severity (Flaudias et al., 2019). The additional motivation provided by craving may underlie the ability to drink despite punishment, and craving may develop as a result of a history of drinking alcohol.

The effect of drug history on compulsive use has been observed in animals repeatedly with alcohol (Fachin-Scheit et al., 2006; Hopf et al., 2010; Houck et al., 2019; Lesscher et al., 2010; Randall et al., 2017; Seif et al., 2013; Wolffgramm, 1991) as well as with aversion resistance and other drugs (Jonkman, Pelloux, & Everitt, 2012; Kenny, 2007; Pelloux, Everitt, & Dickinson, 2007; Torres et al., 2017; Vanderschuren, 2004). It is important to note that in these experiments, as is the case with human addiction, a history of extended drug use is not easily dissociable from an extended conditioning history of repeated parings between a drug and stimuli associated with that drug. Some methods of drug self-administration explicitly present a cue to signal drug delivery (e.g. Randall et al., 2017; Torres et al., 2017). Other procedures, specifically alcohol self-administration conducted in the animal's home cage, may not present experimenter-controlled cues with the drug delivery. However, the animals in these studies still undergo associative learning in which the taste and smell of the alcohol, the context in which the drug is delivered, and the sensory aspects of the sipper tube are all paired with the reward and intoxication produced by alcohol's ingestion. Moreover, these implicitly conditioned stimuli still gain incentive value, exerting motivational control over drinking behaviors (Cofresí et al., 2018).

The emergence of craving and compulsive alcohol use may involve disturbances in motivation resulting from reactivity to alcohol cues (Clemens & Holmes, 2018a). This pathological shift in motivation is proposed to occur via increased drug-wanting following repeated exposure to drug-paired stimuli (Robinson & Berridge, 1993, 2008). After alcohol-paired cues have been repeatedly presented, the cues themselves may become behavioral motivators, or "motivational magnets" promoting further consumption (Berridge & Robinson,

2003). Appetitive associative learning may therefore be intrinsic to the development of craving and compulsive drinking. A longitudinal study following young drinkers' response to alcohol supports this claim – greater alcohol-induced stimulation and wanting increases across 10 years were observed in those who exhibited the highest AUD symptomology, whereas liking, though high to begin with, did not exhibit this same increase (King et al., 2021).

Many experiments that aim to investigate compulsive alcohol drinking include classically conditioned cues, some experimenter-controlled visual or auditory stimuli (Giuliano et al., 2019; Halladay et al., 2017; Hopf et al., 2010; Marchant et al., 2018; Radke et al., 2017; Randall et al., 2017; Seif et al., 2013; Timme et al., 2019), whereas other cues are more implicit. These implicit cues include the taste and smell of alcohol, or the context and apparatus in which the alcohol is delivered (Darevsky et al., 2019; Hopf et al., 2010; Lei et al., 2016; Seif et al., 2013; Spoelder et al., 2015). These implicitly conditioned stimuli still gain incentive motivational control over drinking behaviors (Cofresí et al., 2018). While alcohol cues are present in compulsive drinking experiments, associative learning's contribution to compulsive drinking has yet to be evaluated.

Associative Learning Contributes to Compulsive Alcohol Use

Associative learning generally refers to Pavlovian (classical) and instrumental conditioning processes. During instrumental conditioning, a subject learns to make a behavioral response that results in delivery of a reinforcer. Instrumental conditioning involves pairing a response with the outcome contingent on this response. In Pavlovian conditioning, a neutral stimulus which initially elicits no response is paired with an unconditioned stimulus (US) that does not require training to prompt an unconditioned response (UR). Over repeated parings, the previously neutral stimulus becomes a conditioned stimulus (CS), eliciting some response (conditioned response, CR) even in absence of the US. For example, when an individual has no experience with alcohol, a neutral stimulus such as a pint glass would elicit no reaction. However, after repeated pairings of the pint glass with beer, a US that causes intoxication and elicits motivation to drink, the glass becomes the CS. The glass, having been paired with alcohol, comes to evoke craving and motivation to drink, even in absence of the beer itself. While this is a relatively simple example of Pavlovian conditioning, often the learning processes involved in alcohol seeking are more complex, as conditioned stimuli differ in the type of associative relationships with unconditioned stimuli (Clemens & Holmes, 2018b).

Different stimuli may elicit distinctive reactions depending on their modalities, yet in appetitive conditioning, all seem to obtain a motivational influence, thus driving behavior. At least three experimental indications may be used to confirm a CS has itself become a behavioral incentive: (1) The CS elicits conditioned approach and contact, (2) the CS is able to reinforce a novel instrumental response, or (3) presence of the CS enhances instrumental responding (Everitt et al., 2001; Valyear et al., 2017).

The first indication, conditioned approach (also known as sign tracking or autoshaping) occurs when a subject approaches and interacts with a discrete, localized CS that signals a reward. Importantly, the reward is not contingent on this interaction, yet the subject is still drawn to the CS. This suggests the CS has become imbued with incentive properties similar to the initial reward. Animals display some variability in conditioned approach behaviors with CSs signaling alcohol availability, and this variability is thought to relate to addiction severity (Davis et al., 2008; Flagel et al., 2010; Meyer et al., 2012). A second method to experimentally validate if a CS has obtained incentive salience during conditioning is conditioned reinforcement – the ability of a CS to reinforce new instrumental behaviors in absence of the original US. For example, animals that exhibit more conditioned approach behaviors for a food reward also exhibit greater conditioned reinforcement (Hughson et al., 2019). These data suggest conditioned reinforcement and autoshaping may be distinct, yet related, psychological constructs that both can be used to measure incentive salience (Cardinal et al., 2002). This relatedness is also observed in animals responding for drug reinforcers, such as ethanol (Srey et al., 2015). Experiments analyzing sign tracking and conditioned reinforcement behaviors support the assertion that drug-associated CSs acquire incentive salience, which may pathologically drive seeking (Villaruel & Chaudhri, 2016). This claim is further supported by the third experimental indication of incentive sensitization, Pavlovian to Instrumental Transfer (PIT).

Pavlovian to Instrumental Transfer and Alcohol

PIT paradigms are particularly useful for investigating the possible motivational influence of conditioned stimuli. In these experiments, increases in instrumental responding when in the presence of an appetitively-conditioned stimulus are thought to signify an incentive motivational state. Generally, these procedures are divided into three phases: Pavlovian conditioning, instrumental conditioning, and a test phase. In the Pavlovian conditioning phase, a

stimulus is presented in conjunction with delivery of a reinforcer, forming an association between the CS and the reinforcer. Importantly, reinforcer delivery is not contingent on any response in this phase. In the instrumental conditioning phase, the subject learns the response required for reinforcer delivery. The CS is not present during this phase, therefore preventing formation of any explicit association between stimulus and response. The instrumental stage may occur before or after the Pavlovian stage, however the length and order of these stages may affect PIT magnitude (Holmes et al., 2010). Following separate Pavlovian and instrumental conditioning stages, the effect of an appetitive CS on instrumental responding is tested by measuring responding with and without the CS present. In most PIT studies, the test is conducted in extinction, without presentation of the reward. An appetitive PIT effect is found when the presence of the CS increases responding in the experimental group versus the control group.

PIT effects vary depending on the reinforcer used during conditioning. While there is limited preclinical research using ethanol as a reinforcer in PIT procedures, findings have been consistent that cues associated with ethanol rewards facilitate instrumental responding (Alarcón & Delamater, 2019; Corbit et al., 2016; Glasner et al., 2005; Krank, 2003; Krank et al., 2008; Lamb et al., 2016; Milton et al., 2012), and extended ethanol self-administration increases this effect (Corbit et al., 2016). However, there is a lack of research in this field involving animals that will voluntarily self-administer alcohol to the high levels of intoxication seen in humans with AUD (e.g. Mello & Mendelson, 1970), especially without the addition of a natural reinforcer like saccharin (Alarcón & Delamater, 2019; Glasner et al., 2005; Krank et al., 2008) or exposure to ethanol prior to training (Corbit et al., 2016; Corbit & Janak, 2016; Milton et al., 2012) which may interfere with conditioning. Furthermore, while these experiments highlight the incentive effects of cues paired with alcohol, it has yet to be examined if these cues have a larger effect on behavior in situations involving punishment to measure compulsivity.

Recently, PIT paradigms have been translated for use in human subjects, investigating the relationship between elevation of responding and alcohol use with both alcohol and non-alcohol stimuli (for review see Doñamayor et al., 2021). These studies have supported alcohol-paired stimuli's ability to invigorate instrumental responding (Hardy et al., 2017; Mahlberg et al., 2019; Rose et al., 2018). Furthermore, a consistent relationship has been revealed in individuals with problematic drinking behaviors between more intense PIT responses (for non-alcohol rewards) and subsequent alcohol use, compared to abstainers and healthy controls (Chen et al., 2020;

Garbusow et al., 2014, 2016, 2019; Schad et al., 2019; Sommer et al., 2017, 2018; but see van Timmeren et al., 2020). PIT effect size is also greater in highly impulsive individuals (Sommer et al., 2017), and subjects with AUD who later relapsed had more difficulty inhibiting stimulus approach behaviors even when punishment was expected (Sommer et al., 2018). These studies provide additional support for the incentive value of conditioned stimuli's relationship to compulsive drinking, yet animal research may be useful for explicitly investigating this association and developing a model to research possible pharmacotherapeutic targets.

Using Selectively Bred Mice as a Model of Alcohol Use Disorder

While EtOH has been used as a reinforcer in previous experiments investigating PIT, it is important to consider the amount of EtOH consumed during each stage, as well as how EtOH drinking was first induced. In all previous experiments, EtOH pre-exposure methods (Alarcón & Delamater, 2019; Corbit & Janak, 2007, 2016; Glasner et al., 2005; Krank et al., 2008; Milton et al., 2012) and adding other natural reinforcers like sucrose (Alarcón & Delamater, 2019; Corbit & Janak, 2007; Glasner et al., 2005; Krank, 2003; Milton et al., 2012) were utilized to induce EtOH drinking during training stages. To accurately investigate conditioned responding for EtOH, and the possible motivation of EtOH-paired cues, the reinforcer should be experienced initially during training and should be unadulterated to ensure any effects are due to EtOH itself rather than any additives. Furthermore, to more closely model the drinking patterns of individuals with AUD, the animals should self-administer to levels of intoxication similar to that seen in compulsive drinkers. Only one previous experiment investigating EtOH PIT reported intakes during Pavlovian conditioning, these intakes were relatively low and confounded by the addition of polycose to the EtOH (Alarcón & Delamater, 2019). Therefore, in order to more accurately model the disorder, animals that self-administer high amounts of EtOH without any pre-exposure should be utilized.

The High Alcohol Preferring (HAP) lines of mice were developed by selecting mice from the heterogeneous Hs/Ibg progenitor strain that drink high amounts of EtOH during a two-bottle choice procedure and repeatedly mating these high drinkers across generations (Grahame et al., 1999; Oberlin et al., 2011). Crossed HAP (cHAP) mice were subsequently bred by crossing the first and second replicate HAP lines, theoretically resulting in the fixation of any genes related to alcohol drinking. This high-drinking line consumes 23.85 g/kg/day on average, resulting in blood

ethanol concentrations (BECs) of over 250 mg/dL (Matson et al., 2013). Importantly, these mice do not require any preexposure to the drug or additional reinforcers to drink to these high levels. Furthermore, cHAP mice exhibit a strong genetic predisposition to compulsive-like drinking behaviors, as they continue to drink high levels of EtOH despite addition of the bitter tastant quinine (Houck et al., 2019). Utilizing cHAP mice will thus allow the investigation of EtOH CRs, CS-induced motivation, and their possible contributions to drinking in spite of aversive consequences.

Summary

The inability to cease drinking despite knowledge of the possible resulting negative consequences is a core component of AUD. There is substantial evidence connecting craving to these compulsive drinking behaviors. Furthermore, excessive craving may be the result of reactivity to alcohol-paired cues. Associative learning processes may contribute to craving when stimuli associated with alcohol become incentive motivators, increasing alcohol-wanting following repeated pairings. It is therefore possible that these associative learning processes contribute to compulsive drinking, with alcohol-cues providing additional motivation that is necessary to continue administering alcohol despite punishment. Using cHAP mice, I first sought to determine if repeatedly pairing alcohol delivery with presentation of initially neutral cues would result in CRs indicative of alcohol seeking. Then, I evaluated if these cues were able to increase responding for alcohol in a PIT procedure, and if this motivated behavior facilitated responding when alcohol seeking was punished via footshock, as a model of compulsive drinking.

SPECIFIC AIMS

Aim 1: Do cHAP mice exhibit appetitive conditioned responses to alcohol-paired cues?

If alcohol-paired cues play a role in motivating compulsive drinking, it is first necessary to demonstrate that an alcohol reinforcer is sufficient to produce appetitive CRs. Male and female cHAPs underwent classical conditioning in which an auditory cue (CS+) predicts delivery of 10% EtOH, and another predicts no reward (CS-). I hypothesized that cHAPs would develop CRs to the alcohol-paired cue, such that magazine entries increase, and time spent near the alcohol sipper is greater when the CS+ is presented compared to the CS-.

Aim 2: Are alcohol-paired cues involved in aversion-resistant responding for alcohol?

If classically conditioned alcohol cues provide the incentive motivation necessary to respond for alcohol under punished conditions, these cues should increase responding in a punished PIT test. Following classical conditioning in Aim 1, male and female cHAPs underwent instrumental conditioning. During this phase, mice learned an instrumental lever press response to gain access to alcohol. Importantly, the CSs present in the classical conditioning phase were not used here. Following training, mice first underrwent traditional PIT testing, during which the levers were present, and CS+/CS- were presented. Following additional classical and instrumental retraining sessions, was the shock PIT (sPIT) test. This test was identical to the first PIT test, with the addition of footshock occurring for a proportion of EtOH responses. I hypothesized that when punished by footshock, cHAPs would exhibit greater response rates in the presence of an alcohol-paired cue than responding in the presence of an unpaired cue, and this difference between CS+ responding and CS- responding would be greater than the difference under unpunished conditions

MATERIALS AND METHODS

General Design

cHAP mice were initially trained on a Pavlovian Conditioning procedure, and conditioned approach behaviors were evaluated after 14 days of training. The same mice subsequently underwent instrumental conditioning. The effect of Pavlovian conditioned stimuli on instrumental responding was then assessed via a PIT test. Then mice experienced another PIT test, with the addition of footshock (sPIT). After confirming instrumental responding had not changed following footshock, mice completed a final instrumental session. Retro-orbital blood samples were collected immediately following this instrumental session to evaluate BECs. Figure 1A depicts this experimental timeline.

Subjects

50 cHAP mice (25 male) were counterbalanced by sex, family, CS assignment, and EtOH side for operant testing. Mice were single housed in standard Plexiglas cages with pine bedding and acclimated to a standard 12-hour reverse light cycle (lights off at 0700) for 10 days prior to the start of behavioral testing. Mice were between 107-112 days old on the first day of Pavlovian Conditioning. All experiments were performed following the Institutional Animal Care and Use Committee (IACUC) of IUPUI and NIH Guide for the Care and Use of Laboratory Animals (NRC, 2011).

Apparatus

Ten operant boxes were used for all behavioral testing (Med-Associates, St. Albans, VT). Each chamber measured 21.6 x 19.7 x 12.7 cm and was located inside a light- and sound-attenuating box. Two retractable sippers located on the same wall of the operant box were used; only one of these was equipped with a 10-mL graduated sipper tube to provide the 10% (v/v) ethanol (EtOH) reinforcer, the other remained empty. The sipper that contained the EtOH was counterbalanced across subjects. EtOH intakes were measured by recording the fluid levels before and after each session. Two retractable levers were located on the side of the boxes opposite the sippers, and the lever paired with EtOH delivery was located directly opposite the

sipper. Levers were retracted during Pavlovian Conditioning and extended for all other sessions. A 2900 Hz tone generator (model ENV-323AW) was located above the lever towards the back of the box, and a mechanical clicker (model ENV-335W) was located above the other lever towards the front. Footshock for the sPIT test was produced via a metal grid floor connected to a shock generator.

Pavlovian Conditioning

Mice received 14 sessions of Pavlovian conditioning. All experimental procedures were conducted 7 days/week. Two auditory stimuli (the clicker and tone described above) served as the CSs. One stimulus was paired with EtOH delivery, while the other was paired with the sound of the descending sipper, but no reinforcer. The sound of sipper descent is a salient cue that would be predictive of EtOH delivery; therefore the purpose of descending the empty sipper during the CS- was to control for this cue. CS assignment was counterbalanced across subjects, with 25 mice (13 male) in the click CS+/tone CS- (C+/T-) group and the other 25 (12 male) in the tone CS+/click CS- group (T+/C-). Conditioning sessions consisted of 20 presentations of each CS (40 presentations total), in a pseudorandomized order. Each CS was presented for 25 seconds total; 18 seconds after CS onset, the sipper descended (EtOH for CS+ or empty for CS-). The sipper remained descended for 10 seconds, resulting in a CS/US overlap of 7 seconds (Figure 1B). A variable intertrial interval averaging 60 seconds (VI60) separated each CS presentation. Conditioning terminated following the last CS presentation, resulting in sessions approximately 58 minutes in length.
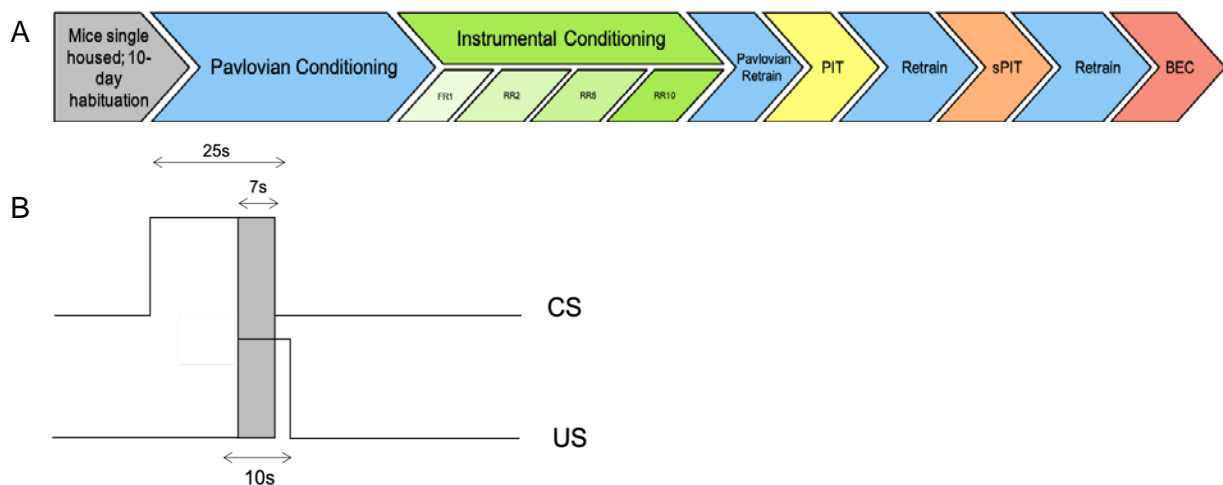
Figure 1. Experimental timeline (A) and timing of Pavlovian stimulus presentation (B)

Sessions 1 and 14 were recorded for behavioral scoring. Time spent in front of the EtOH sipper and EtOH magazine entries during the CS+ vs CS-, only prior to the sipper descent, were used as measures of conditioned approach. A 58 x 44 mm area, landmarked by vertical bars on the walls surrounding the sipper and the 5th bar of the grid floor behind the sipper, was used as the sipper zone.

Mice were water restricted for 22 hours only prior to the first Pavlovian conditioning session. However, after four conditioning sessions, a subset of 13 animals failed to drink 0.2 mL or greater during one session. These mice were subsequently water deprived prior to the fifth conditioning session. Following this additional water deprivation session intakes increased, and sessions 6-14 were not water deprived.

Instrumental Conditioning

Following Pavlovian conditioning, the mice were then trained to lever press to self-administer EtOH. Importantly, all instrumental conditioning procedures occurred in absence of the auditory CSs. For mice that were assigned the EtOH sipper towards the back of the box, the lever towards the back was reinforced with 8 seconds of EtOH sipper access. For the other half of the mice with EtOH in the front sipper, the front lever was reinforced with 8 seconds of EtOH access. Pressing the non-EtOH lever resulted in the empty sipper descending for 8 seconds, but no reinforcer, again to control for the auditory stimulus of sipper descent. For all instrumental conditioning, the levers operated on independent but equivalent schedules. All instrumental conditioning sessions were 60 minutes in duration. Mice received 2 days of FR1 training, followed by 5 days of a random ratio (RR) 2 schedule, in which 2 responses on average resulted in EtOH reinforcement. Following the 5 days of RR2, mice were trained on 5 days of RR5 and finally shifted to 5 days of RR10, for a total of 17 days of instrumental training.

Pavlovian to Instrumental Transfer Test

Mice received one additional day of Pavlovian conditioning prior to the PIT test. The possible excitatory influence of the conditioned stimuli on responding for EtOH was assessed via the PIT test. This test began with a 2-minute habituation period, during which levers were retracted and no CSs were played. The rest of the test consisted of 10 presentations of each CS in

a pseudorandomized order. All mice received the same order of CS presentation. Due to CS assignment being counterbalanced, this resulted in the first CS presentation being the CS+ for half of the mice and the CS- for the other half. CS presentations were 25 seconds long, separated by 60-second interstimulus intervals. Following the 2-minute habituation period, both levers were available for the duration of the test and reinforced on independent RR10 schedules.

Shock Pavlovian to Instrumental Transfer Test

Following the PIT test, mice received two retraining days, one Pavlovian and one instrumental, using the RR10 schedule. Next, to determine if conditioned stimuli provide additional motivation to respond for alcohol despite punishment, the sPIT test was performed. The timing of habituation and CS presentation in the sPIT test was identical to the PIT test. Both levers were also available during sPIT, reinforced on independent RR10 schedules. However, during this test, every third RR10 sequence for the EtOH lever, the mouse completed a shock sequence instead of the normal RR10 lever pressing sequence. During the shock sequence, a 0.75 second, 0.2 mA footshock was delivered two-thirds of the way through the RR value (For example, if the RR value was 9, a shock would be administered on the sixth lever press, and EtOH delivery would still occur after the ninth lever press). This structure of shock delivery was chosen to prevent predictability of the shock delivery.

Traditionally, PIT tests are conducted in extinction (e.g. (Alarcón & Delamater, 2019; Corbit & Balleine, 2011; Corbit & Janak, 2007). However, in these experiments, the ethanol reinforcer was still delivered during testing in order to incentivize continued responding despite shock. The reinforcer was delivered during the PIT test to allow for comparison between the two tests.

Blood Ethanol Concentrations

The day following the sPIT test, two additional 60-minute, RR10 instrumental days were completed. The first confirmed that rates of lever pressing had not changed following footshock. Immediately upon completion of the second instrumental session, retro-orbital blood samples were collected using 25-µL heparinized capillary tubes. Samples were centrifuged to separate plasma and stored at -20ºC until analysis. BECs were determined using an Analox EtOH Analyzer (Analox Instruments, Lunenburg, MA).

Additional sPIT Test

Following initial analyses of the PIT and sPIT tests and completion of all other experimental procedures, an additional sPIT test was completed to ensure the mice were being appropriately challenged. This test was double the length of the initial tests, with 20 presentations of each CS, in the same order as the PIT and sPIT tests. After the 10th presentation of both CSs, the order repeated itself. Instead of shock occurring every third reinforcer sequence as in the previous sPIT test, mice were shocked two-thirds of the way through each RR10 string.

Statistical Analysis

Data were analyzed using SPSS software (SPSS, Version 27, Chicago, IL) and graphed and analyzed with GraphPad Prism software (GraphPad Prism, v. 9.0, La Jolla, CA). Significance values were set at $p < 0.05$. All data were assessed for normality using the Kolmogorov-Smirnov Test, and if normality was violated, non-parametric tests were used.

Pavlovian Conditioning

A repeated measures ANOVA (RMANOVA) was used to compare the 14 sessions of Pavlovian Conditioning EtOH intake by Sex and CS Assignment. A paired samples t-test compared day 2 intake to day 14. All raw conditioned approach data violated normality and were evaluated by Wilcoxon Signed Ranks Tests. 15 subjects were randomly selected and scored by an additional researcher. A Spearman correlation on ranked data was utilized to compare and verify conditioning scores. A Spearman correlation on ranked data was completed for time near the sipper for T+/C- mice to support trending results. CS+ preference ratios were calculated for time spent near the sipper, CS+ time/ (CS+ time + CS- time), and magazine entries, CS+ magazine entries/(CS+ magazine entries + CS- magazine entries). RMANOVAs compared these ratios by sex and CS assignment.

Instrumental Conditioning

A RMANOVA compared daily instrumental EtOH intake (17 days total) by sex and CS assignment. Intake was also evaluated in a schedule X session X sex X CS assignment RMANOVA to assess the effect of schedule on intake. Mean lever presses for both the EtOH

and non-reinforced levers, and reinforcers earned were calculated for each stage of training (FR1, RR2, RR5, RR10) and compared altogether via a Friedman Test followed by individual Wilcoxon Tests. Mean lever presses for each stage for each sex were compared via a non-parametric Mann-Whitney U test. Preference for the EtOH lever was calculated for each session (EtOH lever presses/total lever presses) and schedule X sex X CS assignment RMANOVA compared these values. FR1 data were excluded from this RMANOVA because there were only 2 days of training on this schedule versus the 5 days used with the RR schedules.

Pavlovian to Instrumental Transfer Test

Intake for the PIT test was assessed via a sex X CS Univariate ANOVA. The excitatory influence of the CS+ was assessed by comparing lever presses during the CS+ to lever presses during the CS- via Wilcoxon Signed Ranks Tests for each sex and CS assignment. A Spearman regression on ranks was completed for non-reinforced lever presses to support trending results. An elevation ratio was calculated (CS+ lever presses/CS+ lever presses + CS- lever presses) as in Wiltgen et al. (2007) and compared via a Sex X CS assignment ANOVA. A simple linear regression predicted elevation ratio from PIT intake, and Pearson correlations between elevation ratio and total Pavlovian intake, total instrumental intake, and CS preference ratios were calculated.

Shocked Pavlovian to Instrumental Transfer Test

A univariate ANOVA analyzed sPIT intake by sex and CS assignment. A RMANOVA compared PIT and sPIT intake by sex and CS assignment. Wilcoxon signed ranks tests compared CS+ and CS- lever presses for each sex and CS assignment. Elevation ratios were calculated as in the PIT test and compared via a Univariate sex X CS assignment ANOVA. A test X sex X CS assignment RMANOVA compared PIT and sPIT elevation ratios. sPIT elevation ratio was correlated with sPIT intake via Pearson correlation.

Additional sPIT Test

Intake in the additional sPIT test was not normally distributed and was compared to PIT and sPIT intake via Wilcoxon signed ranks tests. Wilcoxon tests also were utilized to compare CS+

and CS- EtOH lever pressing for each sex and CS group. Elevation ratios for this test were calculated and compared to PIT and sPIT tests via a test X sex X CS group RMANOVA.

Blood Ethanol Concentrations

A Univariate ANOVA compared BECs by sex and CS group. Additionally, EtOH lever presses were compared to mean RR10 lever pressing by a Wilcoxon signed ranks test. Finally, a simple linear regression was utilized to predict BEC from session intake, and the formula obtained from this regression was used to estimate BECs for Pavlovian and instrumental conditioning.

RESULTS

Attrition

One mouse was excluded from all instrumental, PIT, and sPIT analyses because it failed to learn to drink during instrumental conditioning. On day 1 of Pavlovian conditioning, two mice were excluded from video scoring – one escaped the operant box during the conditioning session, and another was not recorded due to experimenter error. One mouse was excluded from day 14 of Pavlovian conditioning because of a camera malfunction. Three mice were excluded from all analyses involving PIT testing due to experimental error. EtOH intakes were not recorded for 10 mice on day 2 of RR5 training. Median intakes were calculated using the previous 2 and following 2 training days so that RMANOVAs could still be calculated with these animals.
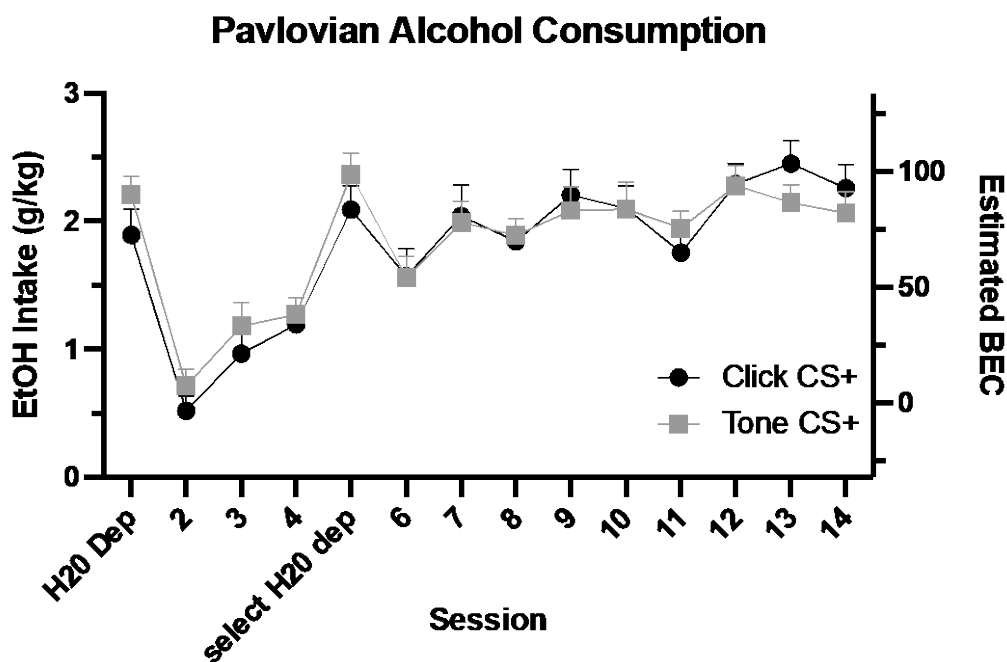
Figure 2 Alcohol intake during Pavlovian Conditioning phase. Mice were water deprived on the first day of conditioning, and select mice received an additional day of water deprivation on day 5. There was no difference in intakes between mice assigned the the click CS+ and mice assigned the tone CS+. Estimated blood ethanol concentrations (BECs) are plotted on the right Y-axis. BEC values were generated from the simple linear regression calculated from the BEC collection day. Mean estimated BEC on the last day of conditioning was 86.7 mg/dL (SEM = 6.68).

Pavlovian Conditioning

Across all 14 sessions, mean daily EtOH intake was 1.80 g/kg *(SEM* = 0.07). Intake differed across days, F (6.784, 312.07) = 13.02, $p < 0.001$. However, there was no effect of Sex, $F(1,46) = 1.96$, $p = 0.17$, or CS assignment on intake, $F(1,46) = 0.005$, $p = 0.94$ (Figure 2). Calculation for escalation of alcohol intake began at Day 2, as the mice were water deprived on Day 1. Intake on day 2 (the first day of conditioning without water deprivation) was less than intake on day 14, suggesting escalation did occur, $t(49) = -10.47$, $p < 0.001$. Intake on Day 6 (following the water deprivation of select mice) was also less than day 14, $t(49) = -4.596$, $p < 0.001$, but greater than day 2, $t(49) = -6.085$, $p < 0.001$. Time spent near the sipper and magazine entries were significantly correlated between scorers, $ps < 0.05$. Sipper site approach was assessed by measuring the time in a zone near the EtOH sipper during the CS+ and CS-, prior to sipper descent. On day 1, time spent in the zone was the same for both CSs, $Z = -0.359$, $p = 0.72$. After 14 days of Pavlovian conditioning, mice spent more time near the sipper during the CS+ than the CS-, $Z = -3.85$, $p < 0.001$ (Figure 3A). Previous experiments that used these auditory stimuli indicated that the click may be a stronger stimulus than the tone. To confirm conditioning effects, variables were split by CS assignment and analyzed separately. On day 14, mice assigned the C+/T- spent more time in the zone near the sipper, $Z = -3.514$, $p < 0.001$ during the CS+ compared to the CS-. Mice assigned the opposite CS grouping, T+/C-, trended towards spending more time by the sipper during the CS+, $Z = -1.87$, $p = 0.061$ ($r_s = 0.715$, $p < 0.001$) (Figure 3B).

Overall, mice spent more time near the sipper on day 1 compared to day 14, $Z = -4.74$, $p < 0.001$. To account for this time difference while comparing the days, a CS+ preference ratio was calculated. Indicative of appetitive conditioning, more of the total time near the EtOH sipper occurred during the CS+ on day 14 than day 1, $F(1,43) = 7.950$, $p = 0.007$. There was no effect of Sex on this preference ratio, $F(1,43) = 1.161$, $p = 0.287$, but mice assigned the C+/T condition had a higher preference ratio than mice in the T+/C- group, $F(1,43)= 6.298$, $p = 0.016$. There was no interaction between Sex and CS+, $F(1,43) = 0.701$, $p = 0.407$.

Appetitive conditioning was also assessed by comparing EtOH magazine entries between CS+ and CS-. As with the time variable, there was no difference in magazine entries between CS+ and CS- on day 1, $Z = 0.175$, $p = 0.861$. After conditioning, however, mice committed more magazine entries during the CS+ compared to the CS-, $Z = -3.99$, $p < 0.001$ (Figure 3C). Mice

Pavlovian Conditioning
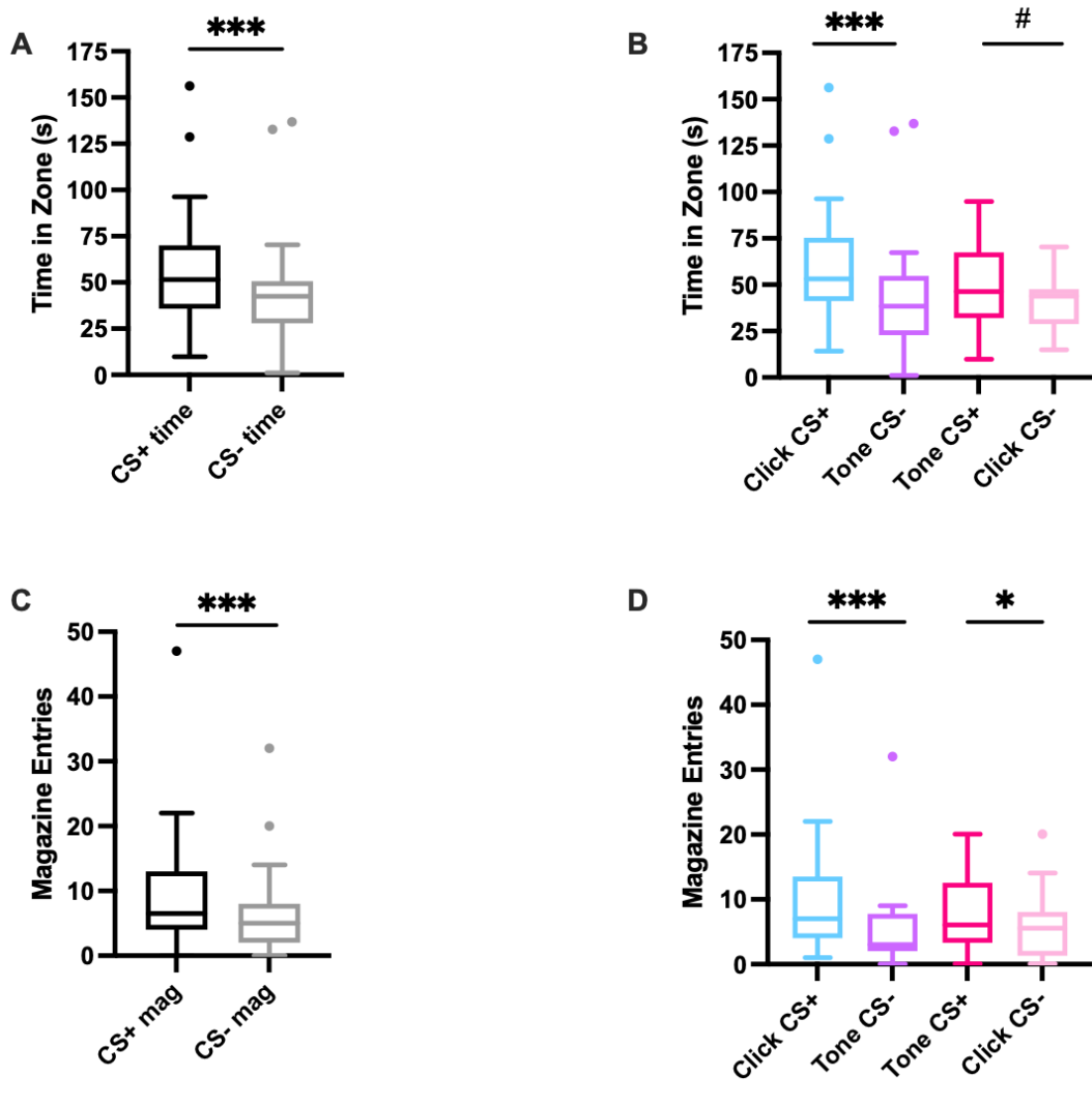
Figure 3. Pavlovian Conditioning Effects. Following 14 days of Pavlovian conditioning, mice spent more time in the zone near EtOH delivery (A) and exhibited more magazine entries, indicative of EtOH seeking (C). When analyzed by CS assignment (n = 25 C+/T- and 25 T+/C-), mice assigned the click CS+ showed a stronger response to conditioning than mice assigned the tone CS+. ***p<0.001, *p<0.05, #p= 0.061

entered the magazine more on day 1 than day 14, $Z = -2.325$, $p = 0.020$, and out of those total magazine entries, more were CS+ on day 14 than day 1, $Z = -3.019$, $p = 0.003$. When split by CS assignment, both C+/T- mice, $Z = -3.162$, $p = 0.002$, and T+/C- mice, $Z = -2.481$, $p = 0.013$, entered the magazine more during the CS+ compared to the CS-. (Figure 3D). A preference ratio

was also calculated for magazine entries. A greater percentage of total magazine entries occurred during the CS+ on day 14 compared to day 1, $F_{(1,41)} = 11.111$, p = 0.002. Neither Sex, $F_{(1,41)} = 2.622$, $p = 0.113$, nor CS assignment, $F_{(1,41)} = .938$, $p = 0.339$, affected this ratio.

Instrumental Conditioning

EtOH intake averaged 2.36 g/kg ($SEM = 0.099$) across all instrumental training sessions. The was a main effect of session on intake, $F_{(6.612, 297.536)} = 3.842$, $p = 0.001$, but no clear pattern of escalation. There was also a main effect of Sex on intake, $F_{(1,45)} = 51.282$, $p < 0.001$, with female mice ($M = 2.868$, $SEM = 0.99$) drinking more than male mice($M = 1.873$,
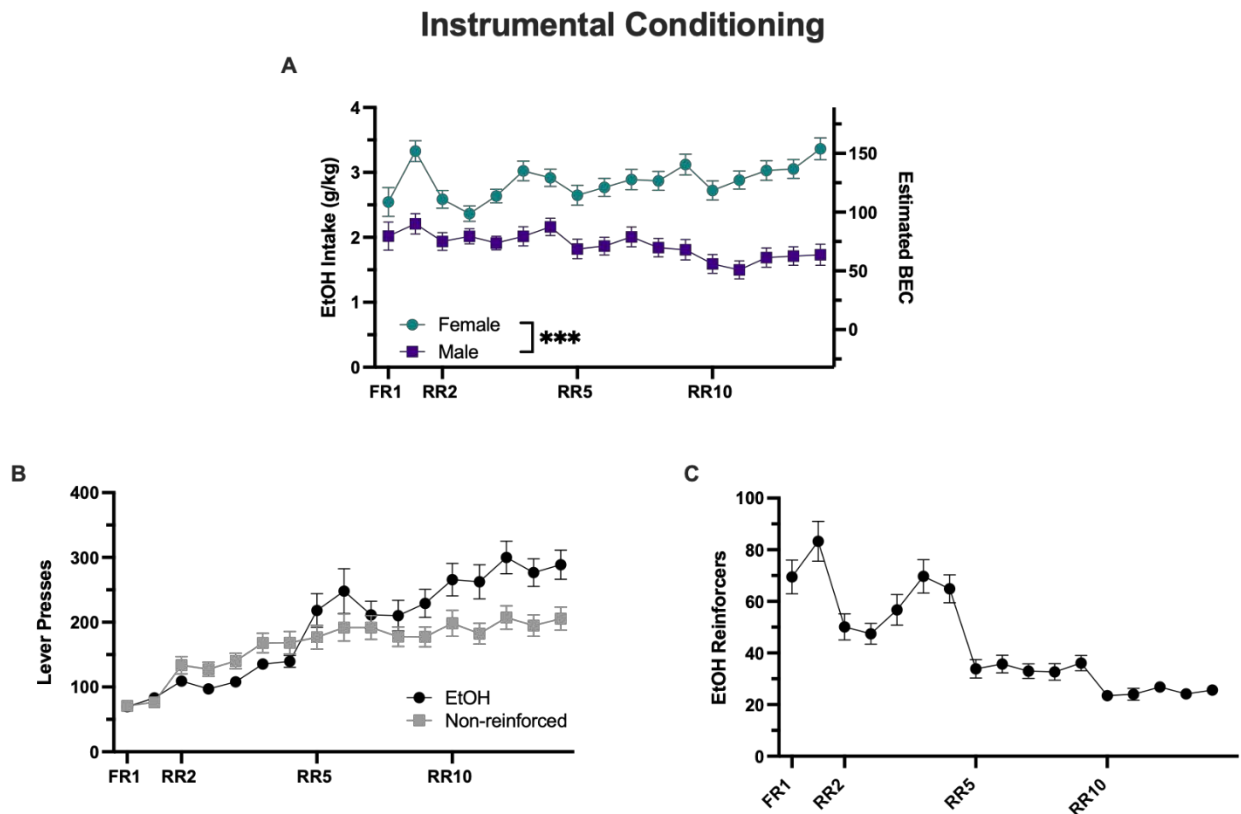
Figure 4. Instrumental Conditioning. Throughout the instrumental conditioning stage, female cHAPs drank more than males (A). As response demands increased, so did lever presses on the EtOH and non-reinforced lever. BEC values were generated from the simple regression calculated from the BEC collection day. However, EtOH lever preference increased with schedule demand (B) while EtOH reinforcers decreased, suggesting the mice were able to learn the relationship between instrumental response and outcome (C). ***p<0.001 (Note: lever press and reinforcer data are presented as mean ± SEM, but all statistical comparisons were non-parametric).

SEM = 0.97). An interaction between Sex and session was present, $F(6.61, 297.54) = 4.302$, $p < 0.001$, so male and female intakes were plotted separately (Figure 4A).

Increasing the average lever presses required for reinforcement (i.e. changing the response requirement from 1 to an average of 2, 5, then 10) resulted in EtOH lever presses differing between schedules, $X^2(3) = 120.546$, $p < 0.001$. Wilcoxon tests confirmed that lever presses increased with each new response requirement, $ps < 0.001$ (Figure 4B). Although females drank more than males during instrumental conditioning, there was no effect of Sex on mean EtOH lever presses for FR1, RR2, or RR5 instrumental training, $ps > 0.114$. There was a trend for females pressing the EtOH lever more than males for the RR10 schedule, $U = 206.0$, $p = 0.060$. Interestingly, lever pressing on the non-reinforced lever also changed with schedule demands overall, $X^2(3) = 86.914$, $p < 0.001$, increasing with each new response requirement $ps < 0.05$ (Figure 4B). While the mice did interact with the non-reinforced lever, their preference for the EtOH lever increased with response requirement demands, $F(1.704, 76.672) = 15.377$, $p < 0.001$. As a result of response requirements increasing within a set session time, reinforcers earned decreased when demand increased, $X^2(3) = 116.192$, $p < 0.001$ (Figure 4C). While the mice earned fewer EtOH deliveries, their intake was not affected by change in instrumental schedule, $F(2,90) = 0.144$, $p = 0.866$, suggesting they learned to drink more efficiently within the session.

Pavlovian to Instrumental Transfer Test

The excitatory influence of appetitive conditioned stimuli was assessed via the PIT test. Overall intake during this approximately 30-minute test averaged 1.823 g/kg (*SEM* = 0.101). Intake did not differ between CS groups, $F(1,42) = 0.508$, $p = 0.480$. As in instrumental conditioning, females drank more than males during the test, $F(1,42) = 37.090$, $p < 0.001$ (Figure 5A). Due to these Sex effects in drinking, PIT data were split by Sex and analyzed separately.

During the CS-on periods, an excitatory effect of Pavlovian conditioning was observed in female mice that pressed the lever more during the CS+ than the CS-, $Z = -2.360$, $p = 0.018$; the. This same effect was not seen in the male mice, $F = -1.248$, $p = 0.212$ (Figure 5B). As CS type affected conditioned approach differently, PIT data were also split and analyzed by this variable,

across Sex. Mice assigned the C+/T- pressed the EtOH lever more during the CS+ than the CS-tone, $Z = -2.372$, $p = 0.018$. However, mice assigned to the T+/C- did not show this same PIT response, $Z = -1.170$, $p = 0.242$ (Figure 5C). Responses were also split by Sex and CS assignment together; females in the C+/T- condition pressed the lever more during their respective CS+ on periods, $Z = -2.322$, $p = 0.020$. Males in the T+/C- condition pressed the lever more during the CS- periods, $Z = -2.383$, $p = 0.017$. Females in the T+/C- group and males in the C+/T- group did not respond differently between CS+ and CS-, $Z = -0.849$, $p = 0.396$ and $Z = -0.756$, $p = 0.449$, respectively. There was a trend for lever pressing on the non-reinforced lever occurring more during the CS- than the CS+, $Z = -1.817$, $p = 0.069$ ($r_s = 0.669$, $p < 0.001$).

An elevation ratio for PIT responding was also calculated, with values greater than 0.5 indicating an excitatory effect of the CS+. Similar to the raw values, females ($M = 0.557$, $SEM = 0.029$) had a higher elevation ratio than males ($M = 0.453$, $SEM = 0.032$), $F(1,42) = 7.589$, $p = 0.009$ (Figure 5D). Mice assigned the C+/T-showed greater excitation by their CS+ ($M = 0.563$, $SEM = 0.030$) than mice in the T+/C- group ($M = 0.446$, $SEM = 0.029$), $F(1,42) = 9.325$, $p = 0.004$ (Figure 5E). There was no interaction between Sex and CS assignment, $F(1,42) < 0.001$, $p = 0.894$. A simple linear regression revealed PIT intake significantly predicted 30.4% of the variance in PIT elevation ratio, $F(1,44) = 19.185$, $p < 0.001$ (Figure 5F). There was, however, no relationship between the elevation ratio and total intake throughout Pavlovian, $r(44) = 0.169$, $p = 0.261$, or instrumental conditioning, $r(44) = 0.225$, $p = 0.132$. Elevation ratio was also not correlated with the Pavlovian CS+ preference ratio for time spent near the sipper, $r(43) = 0.224$, $p = 0.139$, or magazine entries, $r(41) = -0.022$, $p = 0.889$.
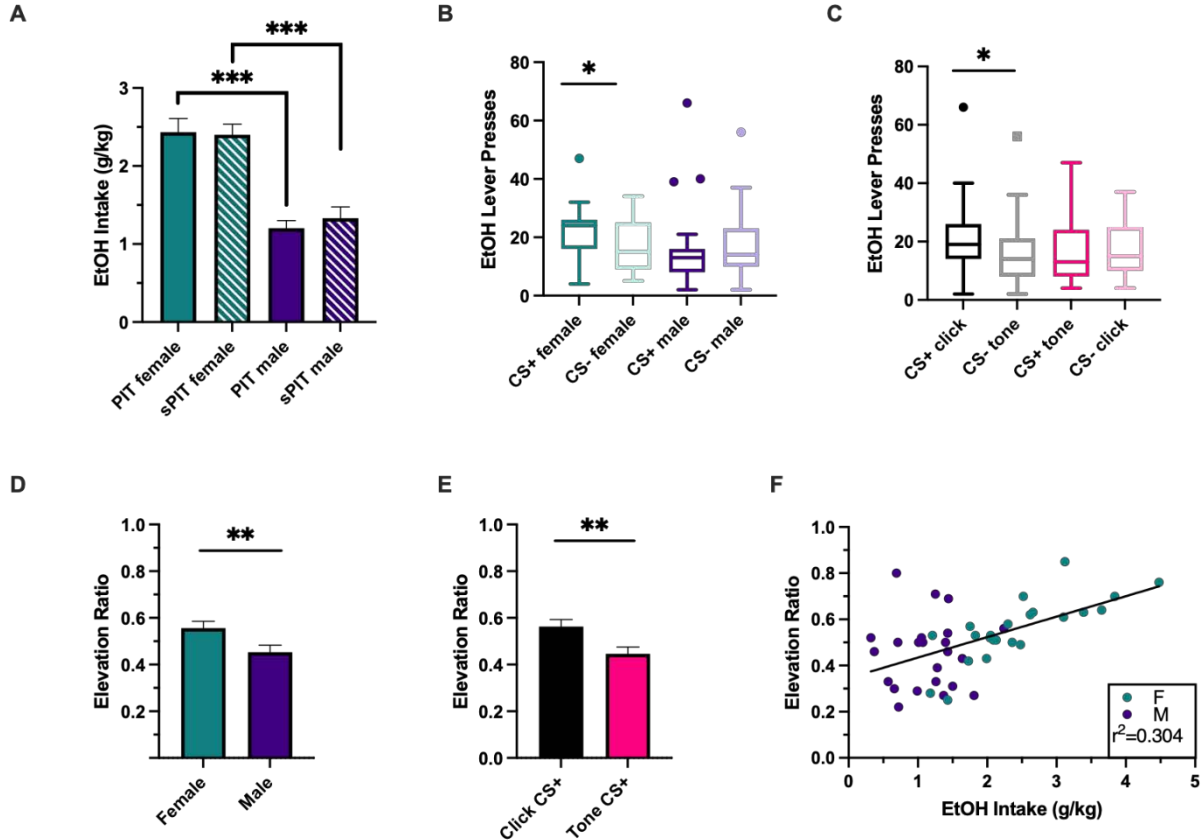
Figure 5. Pavlovian to Instrumental Transfer. Females drank more than males in both the PIT and sPIT tests, with 30-minute durations. Intakes between PIT and sPIT tests did not differ (A). Female cHAPs elevated responding during the CS+ compared to their CS-. Males did not differ in responding between CSs (B). Mice assigned the click CS+ increased lever pressing during the CS+ periods compared to the CS-. Mice in the T+/C- group did not show this same effect (C). Elevation ratios were calculated by dividing CS+ lever presses by total CS lever presses; values greater than 0.5 indicate elevation of responding via the CS+. Female mice presented with a higher elevation ratio than male mice (D). Mice in the click CS+ group had a higher elevation ratio than mice in the T+/C- group (E). EtOH intake predicted 30.4% of the variance in PIT elevation ratio. ***$p<0.001$, **$p<0.01$, *$p<0.05$. ns= female C+/T- (11), female T+/C- (12), male C+/T(12), male T+/C- (11)

Shocked Pavlovian to Instrumental Transfer Test

Following two retraining days, the possible excitatory influence of Pavlovian stimuli on instrumental responding in the presence of negative consequences was evaluated via the sPIT test. Overall intake in the approximately 30-minute test averaged 1.855 g/kg ($SEM = 0.125$).

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There was no difference in intake between the PIT and sPIT tests, $F(1,42) = 0.088$, $p = 0.931$. As in the previous test, female intake ($M = 2.4$, $SEM = 0.136$) was greater than male intake ($M = 1.331$, $SEM = 0.146$), $F(1,45) = 28.079$, $p < 0.001$ (Figure 5A). CS assignment did not influence sPIT intake, $F(1,45) = 0.585$, $p = 0.448$.

Shocked Pavlovian to Instrumental Transfer
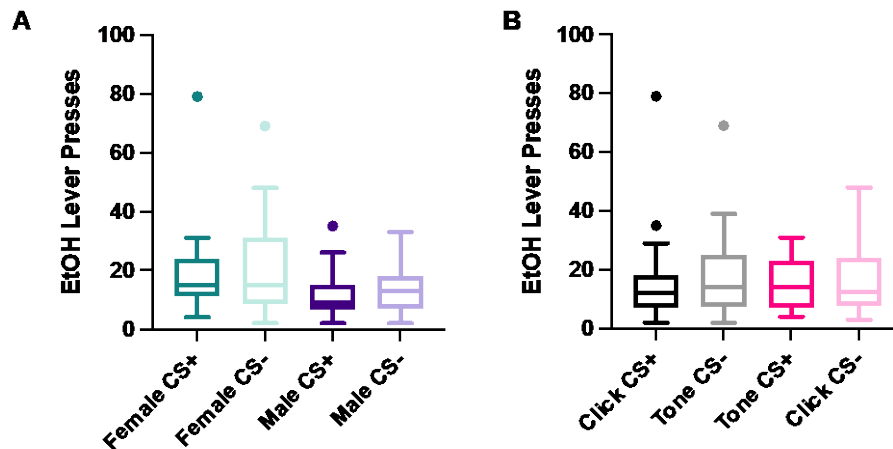
Figure 6. Shocked Pavlovian to Instrumental Transfer test (sPIT). Unlike the PIT test, there was no difference in reactivity to the CS+ between female or male mice (A). There was also no difference in responding during the CS periods between mice assigned the click CS+ and mice assigned the tone CS+ (B).

Unlike the PIT test, there was no difference between responses during the CS+ versus the CS- for males, $Z = -1.415$, $p = 0.157$, or females, $Z = -0.473$, $p = 0.637$ (Figure 6A). There was also no difference in sPIT responding for C+/T-, $Z = -1.269$, $p = 0.204$, or T+/C- mice, $Z = -0.442$, $p = 0.659$ (Figure 6B). Lever pressing on the non-reinforced lever also did not differ between CS+ and CS- presentations, $Z = -1.269$, $p = 0.205$.

The mean elevation ratio for the sPIT test was 0.495 ($SEM = 0.017$), indicating no elevation or suppression of responding by the CS+ as the value was close to 0.5. There was no difference between elevation ratios between the PIT and sPIT tests, $F(1,42) = 0.348$, $p = 0.559$ (Figure 7C). Neither Sex, $F(1,45) = 0.641$, $p = 0.428$, nor CS assignment, $F(1,45) = 0.448$, $p = 0.507$, had an effect on the sPIT elevation ratio. Finally, unlike the PIT test, sPIT intake was not correlated with the sPIT elevation ratio, $r(44) = 0.111$, $p = 0.463$.

High frequency sPIT Test

An additional sPIT test with shocks occurring prior to every reinforcer was completed after all other experimental procedures, ensuring a sufficient challenge was present. Despite doubling the duration of the previous sPIT test, the median intake in the additional sPIT test was 0.456 g/kg, which was significantly lower than PIT, $Z = -5.144$, $p < 0.001$, and sPIT intake, $Z = -5.695$, $p < 0.001$ (Figure 7A). Overall EtOH responding did decrease with shock dosage between the PIT and sPIT tests, $Z = -3.376$, $p = 0.001$, and between the first and second sPIT tests, $Z = -4.835$, $p < 0.001$ (Figure 7B). However, in the second sPIT test there was no difference in responding for EtOH during CS+ or CS- periods for males, $Z = -0.272$, $p = 0.786$, or females, $Z = -1.439$, $p = 0.150$. There was also no effect of the CS+ for mice in either CS group, ps > 0.37. Mean elevation ratio for the additional sPIT test was 0.53 (SEM = 0.029), but elevation ratios between the three tests did not differ, $F(2,80) = 0.601$, $p = 0.551$ (Figure 7C).
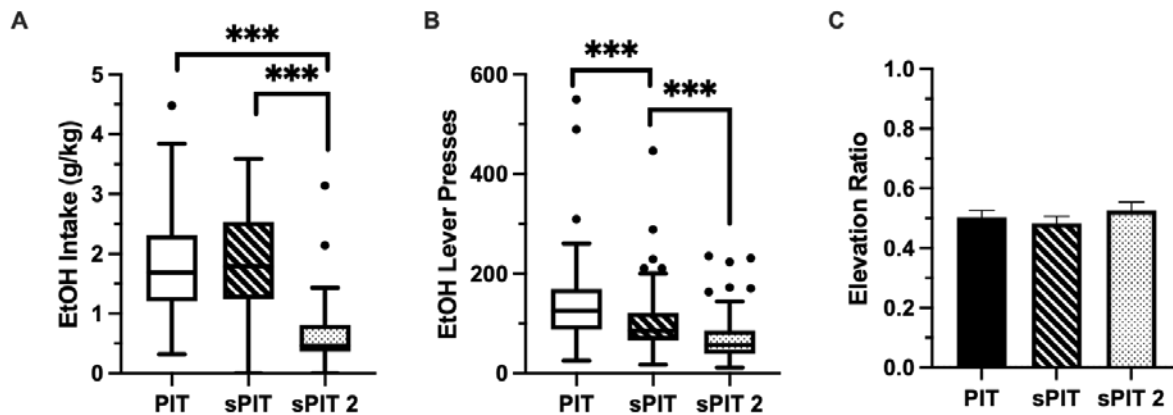
Figure 7.. Comparing PIT and sPIT tests. EtOH intake did not differ between the PIT and first sPIT test. Although the second sPIT test (sPIT 2) was double the duration, intake in this test was significantly reduced (A). Responding on the EtOH lever was reduced in both shocked PIT tests relative to the PIT test, and EtOH lever presses in the second sPIT test were reduced compared to the first (B). Although intake and responding were reduced in sPIT 2, there was no change in elevation ratio between any of the 3 tests (C).***p<0.001

Blood Ethanol Concentrations

An additional RR10 instrumental training day was completed following the first sPIT test. Immediately following, retro-orbital blood samples were taken. Responding on the EtOH lever did not differ between this day and mean RR10 responding prior to PIT testing, $Z = -.516$, $p = 0.606$. The mean BEC for this session was 99.23 mg/dL ($SEM = 8.174$). There was a main effect of Sex on BEC, with females ($M = 126.716$, $SEM = 10.208$) achieving a higher concentration than males ($M = 71.748$, $SEM = 10.281$), $F(1,46) = 14.031$, $p < 0.001$. A simple linear regression revealed that EtOH intake during this session significantly predicted 72.3% of the variance in BEC, $F(1,48) = 125.124$, $p < 0.001$. BEC increased by 55.26 mg/dL for every g/kg of EtOH consumed during the 60-minute session (Figure 8). Using this regression predicting BEC from one-hour EtOH intake, estimated BECs for Pavlovian and Instrumental conditioning were plotted (Figures 2 and 4A, respectively).
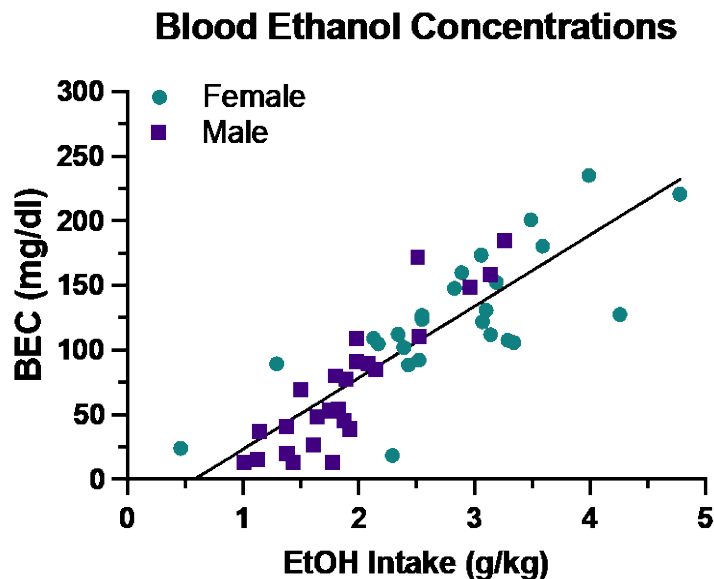
Figure 8. Blood Ethanol Concentrations. Female mice reached higher BECs following the instrumental session than male mice. EtOH intake significantly predicted BEC. In 60 minutes, cHAP mice achieved a mean BEC of 99.23 mg/dL

DISCUSSION

General Discussion

This study aimed to determine if mice selectively bred to drink high levels of alcohol would exhibit a CR to stimuli paired with alcohol, and if these appetitively motivating stimuli would invigorate alcohol responding in neutral and in punished conditions, to model compulsive drinking. As hypothesized, following 14 days of Pavlovian conditioning, cHAP mice spent more time near the EtOH sipper and exhibited more EtOH seeking responses during the sound paired with EtOH delivery. The mice readily learned to respond for EtOH, and females exceeded binge levels of intoxication (National Institute on Alcohol Abuse and Alcoholism, 2016) during instrumental conditioning. The CS+ caused a small elevation in responding for females under neutral conditions, but contrary to hypothesis, there was no excitatory effect of the alcohol-paired cues during a low or high frequency shock session.

Alcohol-Paired Cues Enhance Seeking

cHAP mice learned to approach and interact with the site of EtOH delivery following repeated presentations of a predictive cue. Pavlovian conditioning is traditionally tested in extinction so that the influence of the conditioned stimuli may be evaluated without the confound of the US's own motivating properties. However, in this study, extinction testing was avoided to preserve CRs for PIT testing. Importantly, the cued alcohol seeking responses here were only measured prior to delivery of the EtOH reinforcer, signifying the cues alone were sufficient to signal a seeking response. The production of this seeking response demonstrates these mice found the alcool rewarding, and stimuli associated with alcohol promoted seeking.

Mice in this experiment were trained to associate an auditory stimulus, either a clicker or a tone, with the delivery of alcohol. There was a stronger conditioning response to the clicker CS+ compared to the tone, which has been observed in previous experiments using these stimuli (Carron & Grahame, Unpublished data). The efficacy of the clicker stimulus could be due to the construction of the apparatus – while the tone was played via a speaker, the clicker was a mechanical device that produced a click each time two metal pieces connected. A tactile component was thus inherent to the clicker stimulus, producing a small vibration every time the

metal connected. The vibration may have felt similar to that produced with the descent of the sipper tube, easing the connection between the two. There may also be a greater evolutionary predisposition for forming associations between gustatory USs and tactile CSs than between gustatory USs and auditory CSs (Garcia et al., 1968). While the clicker CS+ paired more effectively than the tone CS+, we can be confident the effects seen in this experiment can be attributed to conditioning rather than an unconditioned effect of the clicker as mice in both CS assignments entered the magazine more during the CS+ than the CS-. Additionally, comparing sipper approach behaviors and magazine entries between day 1 and day 14 demonstrates mice increased seeking responses after conditioning, regardless of CS assignment.

cHAP Mice Readily Self-Administer Alcohol

While the approach responses in this study are not necessarily novel, they are notable in that they were elicited without exposure to the reinforcer prior to conditioning, and without the addition of sweeteners to the alcohol. To conclude that EtOH is producing pharmacologically rewarding effects, intoxication in the model should closely resemble that seen in humans. Conditioned seeking responses similar to those in this experiment have been seen previously in rats (e.g. Cofresí et al., 2019), but it is unlikely these animals achieved relevant BECs, with estimates of only 5-15 mg/dL during conditioning. A sucrose fade procedure (Samson, 1986) is often utilized (e.g. Krank, 2003) to achieve pharmacologically relevant levels of intake. The addition of these sweeteners raises the possibility that CRs are related to those natural (Garcia et al., 1968) reinforcers instead of the alcohol itself. Similar conditioned seeking responses to those observed here, have been seen previously in rats (e.g. Cofresí et al., 2019), but it is unlikely these animals achieved intoxicating BECs, with consumption estimates of only 5-15 mg/dL during conditioning. Home cage preexposure to EtOH has also been utilized to achieve higher intakes, acclimating the animal to its taste and pharmacological effects (e.g. Millan et al., 2015). While these methods eliminate the need for natural reinforcers, they may also preclude unbiased measurement of conditioning, dampening response to the CS due to US preexposure effects (Randich & LoLordo, 1979). In our study EtOH intake was higher, with BEC estimates between 80-90 mg/dL (above the NIAAA binge definition) at the end of Pavlovian conditioning. The use of mice selectively bred for alcohol drinking allows us to achieve high levels of alcohol exposure without procedures that interfere with conditioning.

Following Pavlovian conditioning, mice completed 17 days of instrumental conditioning with no experimental Pavlovian stimuli present. The mice quickly learned to respond for EtOH, and their intakes exceeded those from the previous stage of training. While there was no effect of sex on intake during Pavlovian conditioning, when EtOH delivery was contingent on the animals' behavior, females drank more than males across all training sessions. Female self-administration in the High Alcohol Preferring lines of mice has exceeded that of males previously (Grahame et al., 1999; Matson et al., 2013; Oberlin et al., 2011). Female cHAPs tend to weigh less than males, suggesting they may drink the same amount of fluid, but when intake is adjusted for weight, female EtOH exposure is greater. Male cHAPs metabolize EtOH quicker than females, so the greater exposure of females likely leads to greater pharmacological effects as well (Matson et al., 2013). During Pavlovian conditioning, there was a fixed amount of time to consume EtOH, whereas instrumental training offered the mice more control over their exposure. Female mice may not have had adequate opportunities to reach their desired level of intake during Pavlovian conditioning, but instrumental conditioning provided additional chances to drink. Behavioral control over their exposure allowed them to exceed the intake of the males. Interestingly, the lack of sex effects on lever pressing during instrumental training compared to differences in intake indicates a sex difference in drinking efficiency, not necessarily on willingness to work for the EtOH reinforcer.

Male mice may not have chosen to drink from the sipper every time it descended, or they may not have been able to lick as efficiently as the females. Similarly, responding on the non-reinforced lever for both sexes was higher than has been seen in similar experiments using rats (e.g. Corbit & Janak, 2007), suggesting that the mice were able to reach their desired level of intoxication before the session time was complete. They could spend rest of the session time responding on the non-reinforced lever, as they did not need to devote the entire session time to work for EtOH. cHAP mice are highly active, even when compared to the C57BL/6J (B6) inbred strain, commonly used for their alcohol drinking (Ward et al., 2019). These off-target lever presses may serve as a measure of activity levels rather than an indication of impaired learning (Pickens & Thompson, 1968). Despite the high responding on the non-reinforced lever, a learned association between the reinforced lever and delivery of EtOH is supported by preference for this lever increasing with the demands of the instrumental schedule. If the mice were pressing both levers randomly, we would expect the rate of responding to increase equally between the

reinforced and non-reinforced lever. What we see instead is an increase in reinforced lever pressing when the number of responses required for reinforcement is increased – the mice need to devote more session time interacting with the reinforced level to achieve their preferred level of intake.

As more work was required in the same time frame by requiring more lever presses per reinforcer, there was a reduction in reinforcers delivered. However, even though the mice were earning fewer opportunities to drink, their intake remained consistent throughout instrumental training. One explanation is the mice learned to drink more efficiently, by licking more quickly or obtaining more fluid per lick, as more demand was placed on them. Another possibility is that when demand was low, the cHAPs baseline activity was still high and resulted in pressing the EtOH lever even past achieving their preferred intake. When the number of lever presses required for reinforcement increased, these excessive rewards disappeared, and the mice needed to drink each time EtOH was delivered to maintain their intake.

Pavlovian to Instrumental Transfer is Related to EtOH Intake

Following instrumental training, the excitatory influence of EtOH cues was assessed via a PIT test, and an elevation ratio was calculated to better understand the effect of the Pavlovian conditioned stimuli. This ratio was positively related to alcohol intake during the PIT test, such that mice with higher elevation ratios also had higher PIT intakes. This relationship could be interpreted in two ways. The first indicates that alcohol intake is predictive of PIT responding. That is, mice that are high drinkers exhibit a greater excitatory response to the EtOH-paired stimuli. In experiments investigating PIT in human subjects, similar effects have emerged. Behavioral PIT effects are stronger in high-risk drinkers compared to low-risk drinkers, defined by average EtOH consumption on drinking occasions within the last year (Garbusow et al., 2019). Similarly, alcohol-dependent patients exhibit more robust PIT effects than healthy controls (Garbusow et al., 2016). These results suggest that previous alcohol consumption influences PIT responding. Yet in our experiment, PIT elevation ratio was not correlated with EtOH intake during either Pavlovian or instrumental conditioning, nor was it related to measures of Pavlovian CRs, which suggests previous high drinking behavior is not the cause of higher PIT elevation ratios, in these animals.

A second, alternative, explanation for the relationship between PIT intake and elevation ratio is that PIT response is predictive of intake. In other words, mice that show more excitation in their instrumental responding after presentation of an alcohol-paired cue will subsequently drink more alcohol. It is possible the motivational influence of the EtOH-paired stimulus not only affects responding for EtOH, but also drinking behaviors after the reinforcer has been delivered. Previous experiments with animals and EtOH PIT are all conducted in extinction, therefore no effects of intake during the test can be evaluated. Furthermore, few of these experiments report intakes at all, even during Pavlovian and instrumental conditioning phases, making it difficult to draw conclusions about drinking behaviors relation to CS reactivity. However, immediately after exposure to alcohol cues, human subjects not only report greater craving for alcohol, they also actually consume more alcohol than those exposed to neutral cues (Jones et al., 2013). Moreover, the strength of PIT effects in human alcohol drinkers predict relapse and subsequent consumption (for review see Doñamayor et al., 2021) While greater reactivity to alcohol cues may explain higher alcohol intakes, a better understanding of PIT responding as a whole is necessary to draw these conclusions.

Elevated Responding in PIT Relies on Multiple Factors

Enhanced instrumental responding in the presence of EtOH-paired cues indicates these cues modulate motivated behaviors and have their own incentive value. In this PIT test, we only observed modest elevation of responding in females and those in the C+/T- group. These effects did not carry over to the subsequent sPIT tests. One explanation of these results is that training with EtOH simply does not produce robust PIT responses in cHAP mice. Additionally, the aversive consequences present in the sPIT tests could have occluded the elevated responding seen in the first test. However, enhanced instrumental responding for EtOH has been observed in rats (Corbit & Balleine, 2011; Corbit & Janak, 2007; Glasner et al., 2005; Krank, 2003; Milton et al., 2012), a species that has shown mixed results on how rewarding they find EtOH (Green & Grahame, 2008) and that often drinks and prefers alcohol to a lesser degree than mice (Tordoff & Bachmanov, 2003). It is therefore unlikely that EtOH is not sufficiently rewarding to produce similar effects in a mouse population bred to drink EtOH, especially after EtOH was sufficiently rewarding to produce a CR in these mice. As the click CS+ was more effective during Pavlovian conditioning, and females were exposed to more EtOH during instrumental training, it is possible

only the mice in these groups experienced the appropriate conditions to produce a PIT effect. Greater EtOH exposure, either by using a higher EtOH concentration, longer session times, or additional conditioning sessions, may result in elevated male responding. Similarly, optimizing Pavlovian conditioning to ensure equal effectiveness of both CS+ could also balance these results.

There are a number of other reasons as to why the PIT effects seen in this study were not as robust as those in other PIT experiments. First, the elevation of responding seen in PIT is often transient; as testing goes on the association between the CS+ and the US is disrupted (Matell & Della Valle, 2018). This transience could explain why CS-induced invigoration of responding was not seen in the sPIT tests – the retraining days between tests may not have been sufficient to reestablish this associative relationship. A sPIT test could be completed in a separately trained cohort of animals, without the PIT test, in order to mitigate this effect.

As previously noted, PIT testing is normally completed in extinction, which allows the researcher to evaluate the motivational properties of the conditioned stimuli in isolation, without interference from those of reinforcer. In this study, however, tests were conducted with the reinforcer present, as it was probable mice would stop responding altogether once they were shocked without reinforcement in the sPIT test. In order to compare PIT and sPIT results, the PIT test was thus also reinforced. While these methods may have ensured responding after shock, reinforcement may have also precluded effective measurement of the motivational properties of the CS+. Once EtOH was delivered irrespective of the CS timing, the US could have been become the sole driver of behavior, overshadowing any motivational influence the CS+ may have exerted alone. A possible solution that addresses cessation of responding upon footshock without overshadowing the influence of the CS would be training mice with a shock signal and conducting compulsion testing without the shock itself (Vanderschuren & Everitt, 2004). The presence of a shock stimulus should be enough to reduce responding when presented alone but may allow sufficient behavioral variability to measure CS incentive motivation.

While there were concerns about the intensity of the aversive stimulus ablating instrumental responding during the sPIT testing, there is also the possibility PIT effects were occluded by the excessive responding of cHAPs. Lamb and colleagues (2016, 2020) discuss the possibility of a ceiling effect in experiments investigating drug-induced PIT – that it may be impossible for a CS to elevate responding for drugs of abuse because these rewards already bring

response rates to a maximum. If responding for EtOH is elevated, and the high activity levels of cHAP mice increase reinforced and non-reinforced response rates, there may have been no room for the CS+ to enhance responding further. In experiments that have found a PIT effect with an EtOH reinforcer (Alarcón & Delamater, 2019; Corbit et al., 2016; Corbit & Janak, 2007, 2016; Glasner et al., 2005; Krank, 2003; Krank et al., 2008; Milton et al., 2012) rats were tested in extinction, which reduced response rates. In contrast, response rates were not reduced in our PIT test, and while lever presses decreased slightly in the first sPIT tests, intakes persisted. High responding in the PIT test may have resulted in a ceiling effect to prevent excitation caused by the CS+.

The consistency in intake between the unshocked and first shocked test implies the mice were behaving compulsively, drinking the same amount of EtOH despite the aversive consequence of footshock. However, both the possible ceiling effect and the transient nature of PIT impeded our ability to fully evaluate our hypothesis and determine if the appetitive stimulus contributed to this compulsive responding. Though EtOH lever presses were reduced in the first sPIT test, we added the second, more intense, shock test to ensure the mice were being adequately challenged. This test resulted in reduced intake and reduced lever presses. The lack of PIT effects observed in this second test could indicate that the motivation provided by alcohol cues is not sufficient to promote compulsive responding for alcohol. However, at the point of this second shock test, mice had already experienced two tests prior, which likely disrupted the CS-US association. Furthermore, this second test may have reduced responding and intake too much, again preventing the necessary variance required to test for CS-induced changes. To confirm that classically conditioned cues are not involved in compulsive responding, the shock test must first be optimized to ensure the measurement of compulsion. Only animals with an untarnished conditioning history should subsequently be tested in this optimized procedure. If this test still results in equal responses between CS+ and CS- timepoints, it could be concluded these stimuli had no effect on compulsive responding in this paradigm.

One of the major challenges in animal models of compulsion is finding this experimental balance: the test must present a sufficient challenge, ensuring the animals find the consequences truly aversive, but not challenging the subjects so extremely that behavioral variability is eliminated. Further complicating things are the inter-species (Tordoff & Bachmanov, 2003), inter-strain (Halladay et al., 2017; Houck et al., 2019), and inter-individual variability (Spoelder

et al., 2015) in drinking behaviors and response to aversive stimuli. These differences create difficulties when translating between studies, and care must be taken when designing experiments to adequately account for this variability. As such, developing this paradigm investigating compulsive drinking and PIT requires trial and error, and careful consideration of experimental parameters.

To our knowledge, this is the first study of PIT using EtOH as a reinforcer in mice, so parameters will require further testing for optimization. For example, Combag et al. (2008) found that short-duration CSs (10 seconds) facilitated conditioned reinforcement, where a Pavlovian CS is able to reinforce acquisition of a novel instrumental response. However, these short cues were not optimal for producing PIT. Longer CS (2 minutes) produced the opposite effect: robust PIT but no conditioned reinforcement. Extending the duration of our CS presentations in the Pavlovian conditioning stage may therefore result in stronger PIT effects. While changing the CS duration may influence PIT, Lamb et al. (2017) had difficulty obtaining PIT with long or short CSs of either visual or auditory modalities. They suggest other factors, such as strain and induction of EtOH drinking determine if PIT is observed. However, a meta-analysis by Holmes and colleagues (2010) demonstrates that shorter CS durations like that used in this experiment (between 10 and 30 seconds) have been used successfully in PIT procedures.

This same meta-analysis suggests difficulties in observing PIT using drug rewards may be related to particularly strong Pavlovian responses to the CS, which effectively mask the target instrumental response. In other words, competition between Pavlovian conditioning and instrumental responding is a key determinant of the strength of the observed PIT response, and there is an optimal level of Pavlovian and instrumental conditioning required to produce the desired elevation in responding (Holmes et al. 2010). In this study, the duration of instrumental training needs to be lengthened in order to compete with the Pavlovian response. Furthermore, the spatial configuration of the operant boxes used for PIT testing, with levers and auditory stimuli on opposite sides of the box as the US delivery, may have decreased transfer effects.

Tomie (1995, 1996) explicates a model in which the placement of the drug taking implement, in our case the sipper tube, will either facilitate or impede drug taking based on its proximity to the response manipulandum. In situations when the cue and response manipulandum are located proximally, or are even the same implement (i.e. autoshaping), drug seeking and taking is heightened to the point of compulsivity. Conversely, when the cue and

response manipulandum are distanced from one another, response competition can interfere with drug taking behaviors. In our experiment, the drug seeking manipulandum, the EtOH lever, as well as the origin of the drug-predictive auditory stimuli, were located on the opposite side of the box as the drug taking implement, the sipper tube. According to Tomie's model, this placement would interfere with drug taking, as competition would arise between seeking responses (sign tracking or interacting with the lever and speaker) and taking responses (goal tracking or approaching the sipper). Spatial limitations with our equipment prevented optimal placement of levers, sippers, and speakers when using a two-sipper configuration. Ideally, all three would be located on the same side of the operant box to limit response competition, in turn improving PIT.

Implications and Conclusions

These experiments demonstrate that measuring CS-induced elevation of responding via PIT relies not only on the motivational properties of the CS, but also the parameters set within the experimental design. Seeking responses were elicited not only by EtOH itself, but via cues predictive of the reinforcer. EtOH reinforcement was sufficient to produce increased seeking responses, and mice drank to intoxicating levels regardless of if EtOH delivery was contingent on their behavior. cHAP mice also drank high amounts of alcohol through some footshock, although when shocks were increased their drinking subsided. Despite these results indicating that EtOH and EtOH-paired cues motivate behavior, we were unable to observe the expected interaction between Pavlovian cues and instrumental responding. Experimental parameters such as testing order, CS length, and apparatus configuration all may have impeded measurement of PIT. Future experiments should first focus on optimizing training and testing PIT in this population. Subsequent testing for compulsivity should also be adjusted to ensure the challenge is aversive while still allowing enough variability in behavior to measure possible effects. These experiments demonstrate that EtOH-paired cues can become incentive motivators but did not support this motivation playing a role in compulsive drinking. To confirm these findings, PIT and compulsivity testing must be reliable and there should be no disruption of CS-US relationships prior to these tests.

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