The Role of Context on Responding to an Alcohol-Predictive Cue: Sex Differences and Dopamine D2 Receptors

Diana Segal

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By: Diana Segal

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Signed by the final Examining Committee:

Dr. Aaron Johnson _____ Chair

Dr. Andrew Chapman _____ Examiner

Dr. Andreas Arvanitogiannis _____ Examiner

Dr. Uri Shalev _____ Examiner

Dr. Nadia Chaudhri _____ Supervisor

Approved by: _____

Dr. Aaron Johnson

Chair of the Department or Graduate Program Director

_____ _____

Dr. Pascale Sicotte

Date: Dean of Faculty

ABSTRACT

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Physical contexts that are associated with alcohol can amplify responding to discrete, alcohol-predictive cues in males. Here, we examined the contextual modulation of responding to discrete alcohol-predictive cues as a function of biological sex and the role of dopamine neurotransmission at D2-like receptors. Male and female rats that have previously consumed alcohol (15%; v/v) were trained to associate an auditory CS (10 s; 15 trials per session) with alcohol delivery (0.2 ml/ CS), in a distinctive multi-modal context (alcohol context). During alternating sessions rats were exposed to a second context where they did not receive alcohol (neutral context). In Experiment 1, CS presentations occurred in both contexts without alcohol delivery at test. Rats then underwent extinction using repeated unreinforced presentations of the CS in both contexts. Next, an alcohol-primed reinstatement test was conducted, in which 0.2 ml of alcohol was presented both at the start of the session and during the first CS presentation, after which no alcohol was delivered. At both tests, CS-elicited responding was amplified in the alcohol-associated context compared to the neutral context in males. CS-elicited responding was similar in the alcohol-associated context and the neutral context in female rats. In Experiment 2, rats received administration (s.c.) of D2-like receptor antagonist eticlopride (10 μg/kg) 15 min prior to test. Eticlopride significantly attenuated CS-elicited responding in both sexes and contexts. These findings identify novel sex differences in the capacity of an alcohol-associated context to modulate responding to a discrete alcohol-predictive cue and suggests that dopamine neurotransmission at D2-like receptors is critical for cue-elicited responding.
Keywords: Alcohol, Sex Differences, Context, Cue, Pavlovian Conditioning, Dopamine, Eticlopride, D2-like Receptors

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CONTRIBUTION OF AUTHORS

Nadia Chaudhri designed the experiments and obtained funding. Diana Segal collected and analyzed data for experiments 1 and 2; and wrote the manuscript under the guidance of Nadia Chaudhri and Milan Valyear.

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Introduction

Overview

Learning plays a key role in alcohol use and the development of alcohol use disorder (AUD). Through Pavlovian learning, environmental stimuli become associated with the pharmacological effects of alcohol and serve as cues that predict alcohol (Valyear et al., 2017). Alcohol-predictive cues can elicit craving and can trigger relapse-like behaviour in humans and animals (Pina & Williams, 2016). Alcohol-predictive cues may be stimuli presented in close temporal proximity to alcohol (i.e., discrete cues) or may be configurations of stimuli that are present in the background during alcohol consumption (i.e., contextual cues). Discrete and contextual cues commonly co-occur and have been shown to invigorate alcohol-seeking behaviour (Nees et al., 2012). Previously in our laboratory we have shown that cue-elicited responding in male rats is amplified in an alcohol-associated context compared to a context in which alcohol was never consumed (Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2014, 2015; Valyear et al., 2020). However, the current literature has only assessed the interaction between alcohol-associated discrete and contextual cues in male subjects.

Accumulating evidence has reported that males and females differ in the etiology, maintenance, and health consequences of alcohol use disorder, as well as in triggers of relapse (Becker et al., 2017; Ceylan-isik et al., 2010; Miller et al., 2009; Walitzer & Dearing, 2006). Furthermore, sex differences have been reported in the acquisition of associative learning as well as context- and cue-induced conditioned responding (Dalla & Shors, 2009). Results from studies assessing context-induced renewal of responding to discrete cues have shown that responding to discrete cues is context-dependent in male rats but not in female rats (Anderson & Petrovich, 2017, 2018a, 2018b). These findings support the notion that males and females use different types of environmental information to guide their behaviour. Therefore, the current studies examined the amplification of responding to an alcohol-predictive cue by an alcohol-associated context using male and female rats.

The dopamine system has been implicated in the vulnerability to AUD, craving, and risk of relapse. It has been suggested that dopamine neurotransmission stimulated by alcohol consumption is increased in areas of the brain that govern the rewarding and reinforcing effects of alcohol (Chiara, 1997). Dopamine neurotransmission at D2-like receptors has been closely linked to alcohol use as individuals with AUD have a reduction in D2 receptor availability

compared to healthy individuals. Furthermore, down-regulation of D2 receptors during abstinence promotes craving, alcohol cue reactivity and increases the risk of relapse (Heinz et al., 2010; Hietala et al., 1994). The inhibition of dopamine neurotransmission at D2-like receptors attenuates responding to an alcohol-predictive cue in male rats suggesting that D2-like receptors mediate responding to discrete alcohol-predictive cues (Sparks et al., 2014; Valyear et al., 2020). The present study, therefore, examined the role of D2-like receptors on responding to a discrete alcohol predictive cue in an alcohol-associated context and a neutral context in both male and female rats. Findings from the current studies highlight potential sex differences in the neural and behavioural mechanisms of the contextual modulation of responding to discrete alcohol-predictive cues.

The Role of Environmental Cues on Alcohol Use

Alcohol consumption is prevalent worldwide, with almost half of the global population consuming alcohol regularly (World Health Organization, 2018). In Canada, the estimated total cost of alcohol-related harm is $14.6 billion per year, and the annual number of alcohol-related hospitalizations exceeding that for heart attacks (Canadian Centre on Substance Use, 2017). Alcohol use, misuse, and relapse can be influenced by the environment in which alcohol is consumed (Ludwig, 1986; Ludwig et al., 1974). During repeated use of alcohol, environmental stimuli gain the ability to predict the availability of alcohol through Pavlovian learning processes (LeCocq et al., 2018; Pina & Williams, 2016). Environmental stimuli become associated with the pharmacological effects of alcohol and can trigger physiological, psychological and behavioural responses (Field & Duka, 2002; Ludwig, 1986; Powell, 2006). These responses play a critical role in problematic alcohol use and relapse, as exposure to alcohol-predictive stimuli following abstinence can trigger craving and alcohol-seeking, which may lead to relapse of alcohol use (Pina & Williams, 2016).

Environmental stimuli can be broadly categorized as either discrete cues or contextual cues. Discrete cues are conditioned stimuli that are present in close temporal proximity to a drug (Remedios et al., 2014). Exposure to a discrete alcohol-predictive stimulus (e.g., sound, sight, smell or taste) can induce craving in humans (Litt & Cooney, 1999; Witteman et al., 2015) and can reinstate extinguished alcohol-seeking behaviour in rats (Bienkowski et al., 2000; Katner, 1999; Pina & Williams, 2016). Conversely, contextual cues or contexts are specific configurations of multimodal stimuli that are in the background during drug use (Janak, 2013;

Remedios et al., 2014). Environmental contexts that are associated with alcohol can facilitate craving in humans (Heinz et al., 2010; Ludwig et al., 1974; McCusker & Brown, 1990) and alcohol-seeking in rats (Chaudhri et al., 2009; Chaudhri, Sahuque, & Janak, 2008; Chaudhri, Sahuque, Cone, et al., 2008; Janak, 2013; Marchant et al., 2013; Powell, 2006; Valyear et al., 2017; Willcocks & McNally, 2011; Zironi et al., 2006). For example, studies comparing human alcohol consumption in either a laboratory setting, or a bar setting have found that the amount of alcohol consumed, and the drinking rate were greater in the bar setting compared to the laboratory setting (Strickler et al., 1979). Therefore, discrete cues and contextual cues associated with alcohol can greatly influence alcohol use and misuse.

Discrete alcohol-predictive cues are often embedded within alcohol-associated contexts and the interaction between these types of stimuli may play a role in the maintenance of problematic alcohol use and relapse. These cues commonly co-occur, which has been suggested to enhance the subjective and physiological effects of craving, when compared to the effects of the cues independently (LeCocq et al., 2018; Nees et al., 2012; Valyear et al., 2017). This notion is supported by studies using behavioural animal models of Pavlovian-conditioned alcohol-seeking. For instance, Remedios et al (2014) habituated male rats to drink alcohol in their home cages, after which they were trained in a distinct context to discriminate between two auditory conditioned stimuli, one that was paired with alcohol (conditioned stimulus; CS+) and the other that was presented without alcohol (CS-). Following training, rats were exposed to a different neutral context in which the conditioned stimuli and alcohol were never presented. Then, alcohol-seeking was tested by presenting the CS+ and CS- without alcohol in the alcohol-associated context, the neutral context, and a novel context. The results showed that responding to the CS+ was greater than responding to the CS- in all the contexts. Furthermore, responding to the CS+ was more robust in the alcohol-associated context than in the neutral and novel contexts. These results suggest that the combined experience of the discrete cue and the environment may be the strongest trigger for alcohol-seeking.

The interaction of an alcohol-associated context and a discrete alcohol-predictive CS has been extensively studied in our laboratory. Using a Pavlovian discrimination task, we have reliably found that CS-elicited responding is amplified in an alcohol-associated context compared to a neutral context in male rats (Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2014, 2015; Valyear et al., 2020). Most recently, Valyear et al (2020) acclimated male rats to

the taste and pharmacological effects of 15% ethanol across 12, 24-h sessions of intermittent access in their home cages. Then, rats were trained to discriminate between a distinct multi-modal context in which a discrete auditory CS was paired with alcohol delivery in a fluid port for oral intake (i.e., alcohol-associated context), and a second different multi-modal context in which a different auditory stimulus was presented without alcohol delivery (i.e., neutral context). Rats received a total of 24 sessions, in which the context alternated each day, resulting in 12 sessions in the alcohol-associated context and 12 sessions in the neutral context. Next, rats received two tests (1 in each context) in which the CS was presented without alcohol delivery, using a counterbalanced within-subjects design. Then, 8 repeated tests (4 in each context) were used to extinguish the CS-alcohol association, after which an alcohol-primed reinstatement test was conducted. During the reinstatement test, alcohol was delivered at the start of the session and during the first CS presentation, with no more alcohol delivered for the rest of the session, using a counterbalanced between-subjects design. The results showed that responding to the discrete alcohol-predictive CS was amplified in the alcohol-associated context, compared to the neutral context, during both tests. These findings suggest that the environmental context has a modulatory influence on responding to a discrete alcohol-predictive cue in male rats. The present experiments will build on the findings of Valyear et al (2020) by incorporating female rats in the experimental design, to determine whether the contextual modulation of responding to alcohol-predictive cues varies as a function of sex.

The Importance of Including Sex as a Biological Variable in Preclinical Research on Alcohol

Evidence from preclinical studies suggest that males and females differ in their propensity to use discrete and contextual cues to guide their behaviour**.** One hypothesis that can explain the differences in the context-dependency of behaviours across sex is that males and females use different types of environmental information to guide their behaviour. Evidence supporting this notion can be found in studies investigating spatial navigation strategies in humans and rodents. Spatial navigation tasks typically assess cue-use by manipulating landmark cues (i.e., key objects in the environment) and/or, Euclidean or geometric information (i.e., cardinal directions, exact distances, angles) used to navigate through environments (Boone et al., 2018). Virtual and real-world navigation tasks have shown that men typically use geometric strategies, while women typically use landmark strategies, and that performance of each gender

can be impaired if the other strategy is required to complete the task (Sandstrom et al., 1998; Saucier et al., 2002; Silverman & Choi, 2006). Similar sex differences in the use of environmental information have been shown in studies with rodents, in which the performance of male rats is disrupted by changes to the geometric properties of the environment, while the performance of females is disrupted by changes in landmark cues in radial-arm maze tasks and water maze tasks (Williams et al., 1990; Williams & Meck, 1991). More recently, Rodríguez et al (2011) examined sex differences in the interaction between landmark and geometry learning. Male and female rats were trained to find a platform in either a circular or triangular pool in which a visual landmark and/or a corner of the pool indicated the location of a platform. The results showed that in males the geometry learning overshadowed landmark learning, but that landmark learning did not overshadow geometry learning. However, in females landmark learning overshadowed geometry learning, but geometry learning did not overshadow landmark learning. These findings suggest that the salient or preferred cue overshadowed the less salient or less preferred cue, which was the geometric cue for males and the landmark cue for females. Thus, differences in the way that males and females learn, and associate contextual information may influence whether the environmental context modulates their conditioned responding to reward-predictive cues.

Studies assessing sex differences in context-dependent learning indicate that conditioned responding in females may be context-independent. For example, Anderson and Petrovich (2015) examined sex differences in the renewal of conditioned responding to food cues using an ABA renewal paradigm. Briefly, male and female rats were trained to associate an auditory CS with the delivery of food pellets in a distinct context (Context A). Next, the association between the CS and food was extinguished in a different context (Context B) by presenting the CS without food pellets. Lastly, context-induced responding to the discrete CS was tested by presenting the CS without the food pellets in the original acquisition context (Context A). Males and females in the control groups experienced the acquisition, extinction and test in the same context (i.e., AAA or BBB). At the test, males had greater CS-elicited responding (ABA renewal) compared to their control group. In contrast, CS-elicited responding did not differ between the females in the experimental group and the control group at the test. Therefore, in males, conditioned responding to the discrete cue was dependent on the context in which the discrete cue was presented, while in females the context did not impact conditioned responding

to the discrete cue. Notably, sex differences in context-induced renewal have been repeatedly replicated within the same laboratory (Anderson & Petrovich, 2017, 2018a, 2018b), suggesting that CS-elicited responding is context-dependent in males but may not be in females. This sex difference in context-induced renewal is consistent with sex differences shown in associative learning and contextual processing studies (Dalla & Shors, 2009). For instance, male rats have been shown to acquire aversive contextual associations faster than females, and present greater fear to conditioned contextual stimuli (Maren et al., 1994). It has also been suggested that males and females use different neural correlates and molecular mechanisms in the retrieval of context-associated memories (Colon & Poulos, 2020; Gresack et al., 2009; Keiser et al., 2017). Thus, contexts may modulate responding to discrete cues in males but not in females.

In addition to these aforementioned differences in how males and females use contextual cues to guide their behavior, there are also differences in the prevalence, duration, and consequences of alcohol misuse across gender. Women are more sensitive to the pharmacological effects of alcohol (Miller et al., 2009) and are at a greater risk for alcoholism-related cognitive and bodily impairments than men (Becker et al., 2017; Ceylan-isik et al., 2010). Although studies have shown that more men than women consume alcohol, in recent years there has been an increase of problematic alcohol use in women. In preclinical studies, presentation of alcohol-predictive cues has been shown to elicit alcohol-seeking in female rats, confirming that along with males, females are capable of forming associations between conditioned stimuli and alcohol (Cofresí et al., 2019). Furthermore, women have been reported to escalate from initial drug use to problematic use and substance use disorders more rapidly than men (Becker et al., 2017; Keyes et al., 2019) and are more likely to relapse after abstinence (Hudson & Stamp, 2011; Zlebnik, 2019). Similarly, preclinical studies have shown that female rats acquire the self-administration of drugs at a faster rate, escalate drug-taking more rapidly, and show greater reinstatement of drug-seeking, compared to males (Becker & Koob, 2016). Fundamental differences among men and women have also been found in relapse rates and triggers of drug use (Becker et al., 2017; Walitzer & Dearing, 2006). Literature assessing sex differences in the interaction of environmental cues associated with alcohol is limited, however, it is evident that there are critical sex differences in multiple aspects of alcohol use (Pina & Williams, 2016), associative learning, and contextual processing (Dalla & Shors, 2009). Therefore, sex differences in the behaviour and neural mechanisms underlying context modulated responding to alcohol-

predictive cues needs to be assessed. The current studies tested the hypothesis that responding elicited by a discrete alcohol-predictive cue is influenced by the environmental context in males but not in females

The Role of Dopamine in Alcohol Use

Alcohol intake stimulates dopamine synthesis in the mesolimbic dopamine system, increases dopamine release from neurons in the ventral tegmental areas (VTA) (Brodie et al., 1990), and increases dopamine concentrations in the nucleus accumbens (NAc), brain regions involved in reward-related learning and behaviours (Blanchard et al., 1993; Imperato & di Chiara, 1986; Wozniak et al., 1991; Yoshimoto et al., 1991). In contrast, alcohol withdrawal results in a decrease of dopamine output into the striatum and a reduction in extracellular dopamine concentrations (Rossetti et al., 1991). Dopamine binds to metabotropic G-protein coupled receptors that are classified into two families: the D1-like (i.e., D1 and D5 subtypes) and D2-like (i.e., D2, D3, D4 subtypes) (Chiara, 1997). The D1-like receptors are the most abundant dopamine receptors in the brain, are located on non-dopamine neurons, and stimulate excitatory effects; while D2-like receptors are located on dopamine neurons as autoreceptors and non-dopamine neurons, promoting inhibitory effects (Ford, 2014).

The D2-like receptors are of particular interest due to their involvement in various aspects of alcohol use and misuse. For example, D2 receptor antagonists decrease alcohol consumption in rodents (Pfeffer & Samson, 1986) and humans (Ahlenius et al., 1973). It has been suggested that the density of D2 receptors is involved in the resistance and vulnerability to problematic alcohol use (Heinz et al., 2004; Hietala et al., 1994; Schellekens et al., 2012; Volkow et al., 1996). For instance, D2 receptor availability in humans is associated with a decrease in their sensitivity to the reinforcing effects of alcohol (Yoder et al., 2005). Similarly, in preclinical studies, increasing D2 receptor expression reduces alcohol self-administration in animals (Thanos et al., 2001) and selectively bred alcohol-preferring (P) rats (Thanos et al., 2004). Notably, reduced D2 receptor availability has been shown in individuals with AUDs in PET and fMRI imaging studies (Heinz et al., 2010; Hietala et al., 1994) and post-mortem brain evaluations (Tupala et al., 2001). Furthermore, it has been suggested that alcohol contributes to the down-regulation of D2 receptors in individuals with AUDs (Volkow et al., 1996). This down-regulation is prominent after detoxification and recovers during abstinence, however, delayed recovery is associated with higher risks of relapse in detoxified individuals (Dettling et

al., 1995; Heinz et al., 1996). Reduction in D2 receptor function mediates alcohol craving severity and increases alcohol-associated cue reactivity (Heinz et al., 2004). Thus, dopamine neurotransmission at D2-like receptors govern several aspects of alcohol use and its role in conditioned responding to alcohol-predictive cues needs to be assessed.

Dopaminergic signalling influences the motivational processes underlying the learning and execution of reward-related behaviours and is central to the development and maintenance of problematic substance use (Petersen & London, 2018). Accumulating evidence suggests that dopamine plays a key role in Pavlovian learning and behaviour elicited by reward-predictive cues. For example, craving induced by heroin-related stimuli is correlated with lower baseline D2 receptor availability and higher dopamine release within the striatum in opiate-dependent males (Zijlstra et al., 2008). Preclinical studies have also revealed that dopamine neurotransmission at D2-like receptors governs context- and cue-induced relapse-like behaviour (Crombag et al., 2002; Dias et al., 2010; du Hoffmann & Nicola, 2014; Liu & Weiss, 2002; Owesson-White et al., 2016).

We have previously shown that dopamine neurotransmission at D2-like receptors is required for alcohol-seeking and responding to discrete alcohol-predictive cues (Sparks et al., 2014; Valyear et al., 2020). For example, Valyear et al (2020) trained male rats to discriminate between an alcohol-predictive cue presented in an alcohol-associated context and a neutral stimulus presented in a neutral context. Next, responding to the alcohol-predictive cue was tested in the neutral context. Fifteen-minutes before each test rats received a systemic subcutaneous administration of vehicle, 10 μg per kg of D1-like receptor antagonist SCH23390, or 10 μg per kg of D2-like receptor antagonist eticlopride. The results showed that pre-treatment with the D2-like receptor antagonist significantly reduced responding to the alcohol-predictive cue in the neutral context compared to the D1-like receptor antagonist and vehicle. Pre-treatment with either dopamine receptor antagonist reduced responding to the cue. Thus, neurotransmission at both D1-like receptors and D2-like receptors are required for alcohol-seeking but alcohol-seeking elicited by alcohol-predictive cues may be governed by neurotransmission at D2-like receptors. Furthermore, pre-treatment with the D2-like receptor antagonist significantly reduced overall responding which may have been due to locomotor deficits, however the same dose of this antagonist was shown not to impact locomotor activity in an open field task (Cook & Beardsley, 2003; S. Y. S. Khoo et al., 2019). What remains unclear is whether neurotransmission

at D2-like receptors is selectively involved in cue-elicited responding or is also involved in the contextual modulation of cue-elicited responding, and whether the role of D2 receptors varies as a function of sex. To address this question, the present study assessed the impact of attenuating neurotransmission at D2-like receptors on responding to an alcohol-predictive cue in the alcohol-associated context and the neutral context in both male and female rats.

Research Aims of this Thesis

Based on the research examined above, two experiments were conducted with the following objectives. Experiment 1 tested the hypothesis that responding elicited by a discrete alcohol-predictive cue is influenced by the environmental context in males but not in females. Briefly, rats were acclimated to drinking alcohol in their home cage. Next, they were trained to associate an auditory CS with alcohol delivery for oral intake. Training occurred in a distinct multi-modal context and on alternating days rats were exposed to a second context in which a different auditory stimulus was presented, and alcohol was not delivered. To test the effect of context on responding to an alcohol-predictive cue, the CS was presented in both contexts without alcohol delivery. Then, rats underwent extinction of the CS-alcohol association using repeated unreinforced CS presentations in both contexts. An alcohol-primed reinstatement test was conducted in which alcohol was only delivered at the start of the session and during the first CS presentation, in both contexts. We predicted that males would show an amplification of CS-elicited responding in the alcohol-associated context compared to the neutral context during both tests. Furthermore, we predicted that CS-elicited responding would not differ between contexts during both tests in females. In Experiment 2, we examined the role of dopamine neurotransmission at D2-like receptors on context modulated CS-elicited responding. Rats received systemic administration of dopamine D2-like receptor antagonist eticlopride before the test in which the CS was presented in both contexts without alcohol delivery. We predicted that blocking dopamine neurotransmission at D2-like receptors would reduce responding to an alcohol predictive CS in both sexes, compared to vehicle. In addition, we predicted that responding would be lower in the neutral context compared to the alcohol-associated context in males, and similarly low in both the alcohol-associated context and the neutral context in females.

General Methods and Procedures

Animals

Thirty-one wild-type Cre-negative outbred Long-Evans rats (Experiment 1; n = 16 males, n = 15 females; bred in-house; ~240-420 g at the start of experiment) and thirty Long-Evans rats (Experiment 2; n = 15 males, n = 15 females; ~220-240 g on arrival; Charles River, QC) were single-housed in polycarbonate shoebox cages containing beta chip bedding (Aspen Sani chips; Envigo, Indianapolis, IN), a nylaboneTM chew toy (Nylabones, Bio-Serv, Flemington, NJ), a red plastic tunnel (Rat retreats, Bio-Serv, Flemington, NJ), and shredded paper for enrichment, with unrestricted access to standard rat chow (Rodent Diet, Charles River, St. Hubert, QC) and water. Male and female cages were housed side by side in a colony room held at 21 +/- 2°C and approximately 40-50% humidity on a 12-hour light-dark cycle (lights on at 0700 hours). All procedures were conducted in the light phase. Rats were given 1 week to acclimate and 1 week to be handled by the experimenter before starting behavioural testing. All experimental procedures were approved by the Animal Research Ethics Committee at Concordia University and complied with regulations provided by the Canadian Council on Animal Care.

Apparatus

Twenty-two conditioning chambers (ENV-009A) enclosed in fan-ventilated (72-80 dB background noise) sound-attenuating melamine cubicles (53.6 x 68.2 x 62.8 cm) from Med-Associates Inc (St. Albans, VT, USA) were used for behavioural training and testing. Conditioning chambers were made of stainless-steel bar floors (ENV-009A-GF), paneled aluminum sidewalls, and clear polycarbonate rear walls, ceilings, and front doors. Each chamber featured a white house-light (ENV-215M), and white noise (~8 dB above background; ENV-225SM) and clicker (5 Hz, ~8 dB above background; ENV-135M) generator on the upper left wall. The right wall of each chamber contained a dual fluid port (ENV-200R3AM). A syringe pump (PHM-100, 3.33 rpm) was located outside the cubicle that was used to deliver alcohol via a 20 ml syringe into the fluid well within the chamber. The entrance of the fluid port contained an infrared photobeam (ENV-205M) that when transected was counted and recorded as a port-entry on a PC computer. House light illumination, stimulus presentations, and fluid delivery were controlled by Med PC-IV software.

Solutions and Reagents

Ethanol (EtOH; 5, 10, and 15%; v/v) was prepared weekly by diluting 95 % EtOH in tap water. Lemon oil (used as the lemon odor; SAFC Supply Solutions, St-Louis, USA) or benzaldehyde (used as the almond odor; ACP Chemicals Inc., Montreal, Canada) were mixed in tap water to obtain 10% solutions (v/v). Eticlopride ($C_{17}H_{25}Cl\,N_2O_3$ HCl, Sigma Aldrich, #E101) was dissolved in sterile 0.9% saline to make 10 µg per ml solutions.

Home-Cage Ethanol Exposure

To acclimate rats to drinking ethanol, a total 15 EtOH sessions were conducted. Every other day (i.e., Monday, Wednesday, Friday) rats were given 24-hour access to 15% EtOH and water via a 100 ml graduated cylinder and 500 ml bottle, respectively, that were placed onto the home cage lids (Maddux et al., 2014; Simms et al., 2008; Wise, 1973). On Tuesday, Thursday, Saturday, and Sunday, EtOH cylinders were replaced with water bottles (procedure adapted from Sparks et al (2014)). The position (left or right) of the EtOH cylinders and water bottles were alternated to reduce the impact of side preference. At the end of each session containers were weighed. To control for spillage and evaporation, two empty control cages were set up on the highest and lowest shelves containing rats and were treated identically to the home cages. The average volume of fluid lost from the bottles in the cages was subtracted from the bottles in the home cages for each corresponding session. The grams of ethanol and water and the ingested dose of ethanol (g/kg; grams of ethanol consumed accounting for the density, per kg of body weight) were recorded for each rat in each 24-hour session.

Rats that consumed <1 g/kg of 15% EtOH averaged across sessions 5 and 6, were given access to 5% EtOH to encourage intake (Cofresí et al., 2017). When they reached ≥ 1 g/kg averaged across two consecutive sessions, they were given access to 10% EtOH until they once again reached ≥ 1 g/kg averaged across two consecutive sessions, after which they were given access to 15% EtOH. Rats that did not consume an average of ≥ 1 g/kg remained on the last given percentage of EtOH for the remainder of the experiment. The final number of males and female trained and tested on different EtOH concentrations, for both experiments is shown in Table S1.

Pavlovian Discrimination Training

<u>Habituation</u>

During the last week of the home-cage ethanol exposure, 3 habituation sessions were conducted on days that rats only had access to water. During the first session, rats were brought to the experimental room in their home cages, weighed, and left for 20 mins, to habituate them to the new environment. On the following 2 sessions, rats were habituated to the conditioning chambers in both contexts, Context A on the first day and Context B on the second day in a counterbalanced design (See Table S2 for a description of contexts). During these sessions, rats were weighed and placed in the conditioning chambers, and after a 1 min delay the house-light turned on for 20 minutes and entries into the fluid port were recorded in each session.

Training

Rats were counterbalanced across contexts and stimuli such that there were no differences in home-cage ethanol consumption. Conditioning chambers were designated based on sex so that both sexes were never placed into the same chamber. Rats were given one training session per day (Monday to Friday) until they had received 12 sessions in each context. Training sessions (73.5 mins duration) began with a two-minute delay, after which the house-light turned on and the first inter-trial interval (ITI) began. Sessions conducted in the alcohol context consisted of 15 trials of an auditory CS (10 s; continuous white noise or clicker) that occurred on a variable-time 240 s schedule. Before and after each CS interval was a 10 s PreCS and PostCS interval. Therefore, between the PostCS offset and subsequent PreCS onset, an ITI occurred for either 120, 240, or 360 s. Every CS presentation was paired with 0.2 ml of 15% ethanol dispensed into the fluid port over the last 6 s of the CS (total of 3 ml of ethanol per session). At the end of each session, all the ports were checked to ensure that the ethanol was consumed. Sessions conducted in the neutral context were similar to those in the alcohol context with the exception that a different auditory neutral stimulus (NS; continuous white noise or clicker) was presented to equate acoustic valence of the context, and no alcohol was dispensed into the fluid ports. Syringe pumps were activated on a similar schedule, but they did not contain any syringes. Rats underwent Pavlovian conditioning with context alternation between an alcohol context and a neutral context (Figure 1A) that occurred continuously from the first to the last session of the experiment.

Rats that made < 15 CS port-entries averaged across sessions 5 and 6, in the alcohol context (Experiment 1: male=4, female=2; Experiment 2: male=4, female=4), were given a 2% sucrose and ethanol solution during subsequent training sessions to encourage alcohol

consumption (Remedios et al., 2014). Once an average of ≥ 15 CS port-entries across two consecutive sessions was reached, rats were switched back to the ethanol solution without sucrose for the remainder of the experiment.

Experiment 1: Sex Differences in the Amplification of responding to an Alcohol-Predictive Cue by an Alcohol-Associated Context

Effect of Context on Responding to CS

Following the last training session, the effect of context on CS-elicited port-entries was tested using a counterbalanced within-subjects design (Figure 1B). Two test sessions were conducted using the same procedure as used during training, with the exception that all the rats received the CS without ethanol delivery. The test sessions were separated by 4 re-training sessions (2 in each context).

Alcohol-Priming Induced Reinstatement Test

Following the last test session, 4 re-training sessions were conducted (2 in each context). Next, a total of 8 repeated test sessions (4 in each context) were conducted to extinguish CS-elicited port-entries in the alcohol context and the neutral context. After the last repeated test session, an alcohol-priming induced reinstatement test was conducted using a between-subjects design (Figure 1C). The alcohol-primed reinstatement of conditioned responding was tested dispensing 0.2 ml of ethanol over 6 s into the fluid port, 30 s after the start of the session. Additionally, 0.2 ml of ethanol was dispensed over the first 6 s during the first CS trial, after which no ethanol was dispensed for the remainder of the session. The ethanol prime served as a reminder of the orosensory properties of the ethanol. The reinstatement test was conducted in the opposite context from that in the last repeated test session. Therefore, rats that completed the last repeated test session in their alcohol context received the reinstatement test in their neutral context (male = 8, female = 8) and vice versa (male = 8, female = 7).

Experiment 2: Sex Differences in the Attenuation of Responding to an Alcohol-Predictive cue by Systemic Administration of Dopamine D2-Like Receptor Antagonist Eticlopride

The Effect of the D2-like Antagonist Eticlopride on Responding to CS

All rats received a saline habituation injection (1 ml per kg; subcutaneous), 15 min before each of the last two training sessions. Following the last training session, the effect of the D2-like receptor antagonist eticlopride on CS-elicited port-entries was tested, using a counterbalanced

within-subjects design (Figure 1B). Four test sessions were conducted using the procedure as in training, with the exception that all the rats received the CS without ethanol delivery. Fifteen minutes before each test rats received a systemic administration (1 ml per kg) of vehicle or etriclopride (10 μg per kg). Test sessions were separated by 4 re-training sessions (2 in each context).

Statistical Analysis

Experiment 1

Five (2 = males, 3 = females) of the 31 rats that underwent Pavlovian discrimination training were excluded from the statistical analysis as they did not meet the acquisition criteria of 10 or more CS port-entries averaged across the last two training sessions in the alcohol context (Millan et al., 2015). A ΔCS port-entry (CS port-entries minus PreCS port-entries) variable was calculated for both tests to account for individual differences in baseline responding (Milan et al., 2015; Valyear et al., 2020). Responding at the reinstatement test was compared to the extinction baseline (average ΔCS across the last two extinction sessions).

The interquartile range method (Tukey, 1977) was used to identify and correct outliers by replacement with the median. Three data points were identified as outliers; the duration of CS port-entries from a female rat (session 7 of training; neutral context), the total duration of CS port-entries and number of CS port-entries from a female rat (session 4 of extinction; alcohol context).

Data were analyzed using mixed ANOVAs, with a Greenhouse-Geiser correction applied when Mauchly's test of sphericity was significant, with eta-squared is reported as the effect size. Our prior research has reliably found that male rats make more ΔCS port-entries at test in the alcohol context than in the neutral context (Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2015; Valyear et al., 2020). Therefore, planned comparisons were conducted to analyze ΔCS port-entries across contexts during the test assessing the effect of context on responding to an alcohol-predictive cue and the reinstatement test separately in both sexes, using paired- or independent- samples t-tests with Cohen's d reported as the effect size. Analyses were conducted with JASP Statistics version 0.11.1 (JASP Team, University of Amsterdam, NL) and graphs were created with Prism 8 (GraphPad Statistics, La Jolla, CA).

Experiment 2

Two males in of 30 rats that underwent Pavlovian discrimination training were excluded from the statistical analysis because they did not meet the acquisition criteria of 10 or more CS port-entries averaged across the last two alcohol sessions (Millan et al., 2015). A ΔCS port-entry variable was used for the test analysis.

Data were analyzed using mixed ANOVAs, with a Greenhouse-Geiser correction applied when Mauchly's test of sphericity was significant, with eta-squared is reported as the effect size. Analyses were conducted with JASP Statistics version 0.14.1 (JASP Team, University of Amsterdam, NL) and graphs were created with Prism 8 (GraphPad Statistics, La Jolla, CA).

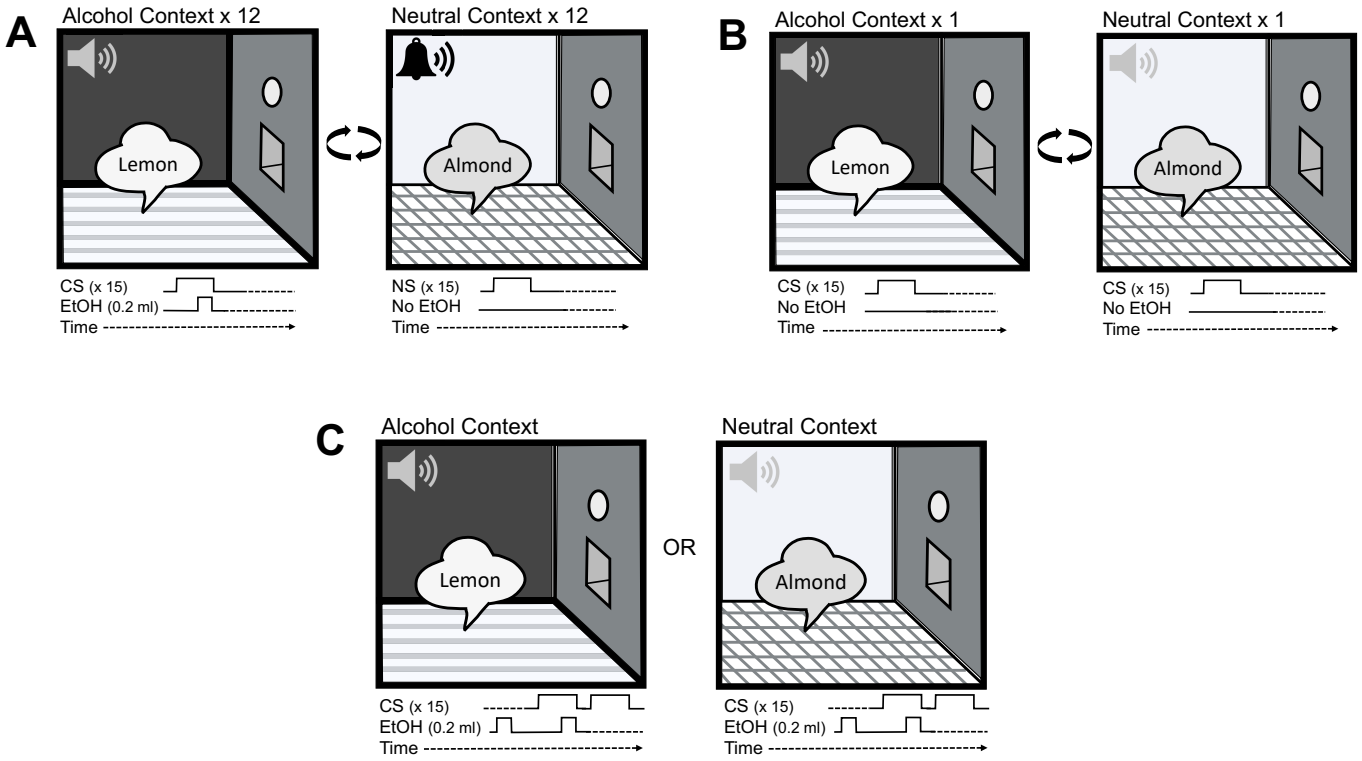

Figure 1. Schematic of Pavlovian discrimination paradigm. **(A)** During Pavlovian discrimination training, rats received 24 sessions (12 in each context), in which a discrete auditory conditioned stimulus (CS) was paired with 15% ethanol. On alternating days, rats were exposed to a neutral context where a neutral auditory stimulus (NS) was presented without ethanol. (**B**) At the test, the CS was presented in both contexts, but no ethanol was delivered. (**C**) Following extinction using repeated tests, a between-subjects alcohol-primed reinstatement test was conducted where the CS was presented in both contexts and 0.2 ml of ethanol was delivered at the start of the session and during the first CS presentation, after which no ethanol was delivered for the remainder of the session.

Results

Experiment 1

Home-Cage Ethanol Exposure. Males weighed more than females throughout home-cage ethanol exposure (Figure 2A). The weight (g) of males (n=14) and females (n=12) increased across sessions [Session, $F_{(1.54, 37.0)}$=263.45, p=< .001, η^2=.038] and was greater in males compared to females [Sex, $F_{(1,24)}$=146.92, p=< .001, η^2=.860; Session x Sex, $F_{(1.54, 37.0)}$=36.54, p=< .001, η^2=.005].

Analysis of fluid intake revealed that males drank more water (g) than females, whereas ethanol consumption (g) was similar in both sexes (Figure 2B). Overall, rats drank more water than ethanol [Fluid, $F_{(1,24)}$=88.28, p < .001, η^2=.451] and males consumed more fluid than females [Sex, $F_{(1,24)}$=25.03, p<.001, η^2 =.511]. The ANOVA revealed a significant Fluid x Sex interaction [$F_{(1, 24)}$=6.08, p=.021, η^2=.031]. Follow-up independent samples t-tests indicated no difference in ethanol intake in males (M=6.39 ± SE=.69) and females (M=5.77 ± SE=.864) [$t_{(24)}$=0.56, p=.582, d=0.22, 95% CI (-.556, .991)], but greater water intake in males (M=25.86 ± SE=1.66) than females (M=17.15 ± SE=1.78) [$t_{(24)}$=3.58, p=.002, d=1.41, 95% CI (.529, 2.263)].

Overall, fluid intake did not vary across sessions [Session, $F_{(1.33, 31.81)}$=0.78, p=.419, η^2=.003] in either sex [Session x Sex, $F_{(1.33, 31.81)}$=0.93, p=.370, η^2=.004]. Water and ethanol intake changed from the first to last sessions [Fluid x Session, $F_{(2.78, 66.82)}$=7.12, p < .001, η^2=.046] comparably in both sexes [Fluid x Session x Sex, $F_{(2.78, 66.82)}$=0.58, p=.617, η^2=.004]. Collapsed across sex, ethanol intake increased from session 1 (M=3.75 ± SE=0.58) to session 15 (M=8.44 ± SE=1.04) [$t_{(25)}$=-4.89, p < .001, d=-0.96, 95% CI (-1.419, -0.486)], whereas water intake remained similar from session 1 (M=24.76 ± SE=1.34) to session 15 (M=22.80 ± SE=5.84) [$t_{(25)}$=0.35, p=.731, d=0.07, 95% CI (-0.32, 0.45)].

Males and females did not differ in the overall ingested dose of ethanol throughout the home-cage ethanol exposure phase (Figure 2C; [Sex, $F_{(1,24)}$=2.12, p=.159, η^2=.081]. The ingested dose of ethanol varied as a function of session [Session, $F_{(4.77, 114.47)}$=9.00, p < .001, η^2=.085] similarly in both sexes [Session x Sex, $F_{(4.77, 114.47)}$=0.80, p=.550, η^2=.008]. Follow-up paired samples t-tests on the data collapsed across sex indicated that the ingested dose in session 15 (M=2.60 ± SE=.38) was higher than in session 1 (M=1.46 ± SE=0.25) [$t_{(25)}$=3.72, p=.001,

d=0.73, 95% CI (0.29, 1.16)]. Therefore, alcohol consumption did not differ between male and female rats.

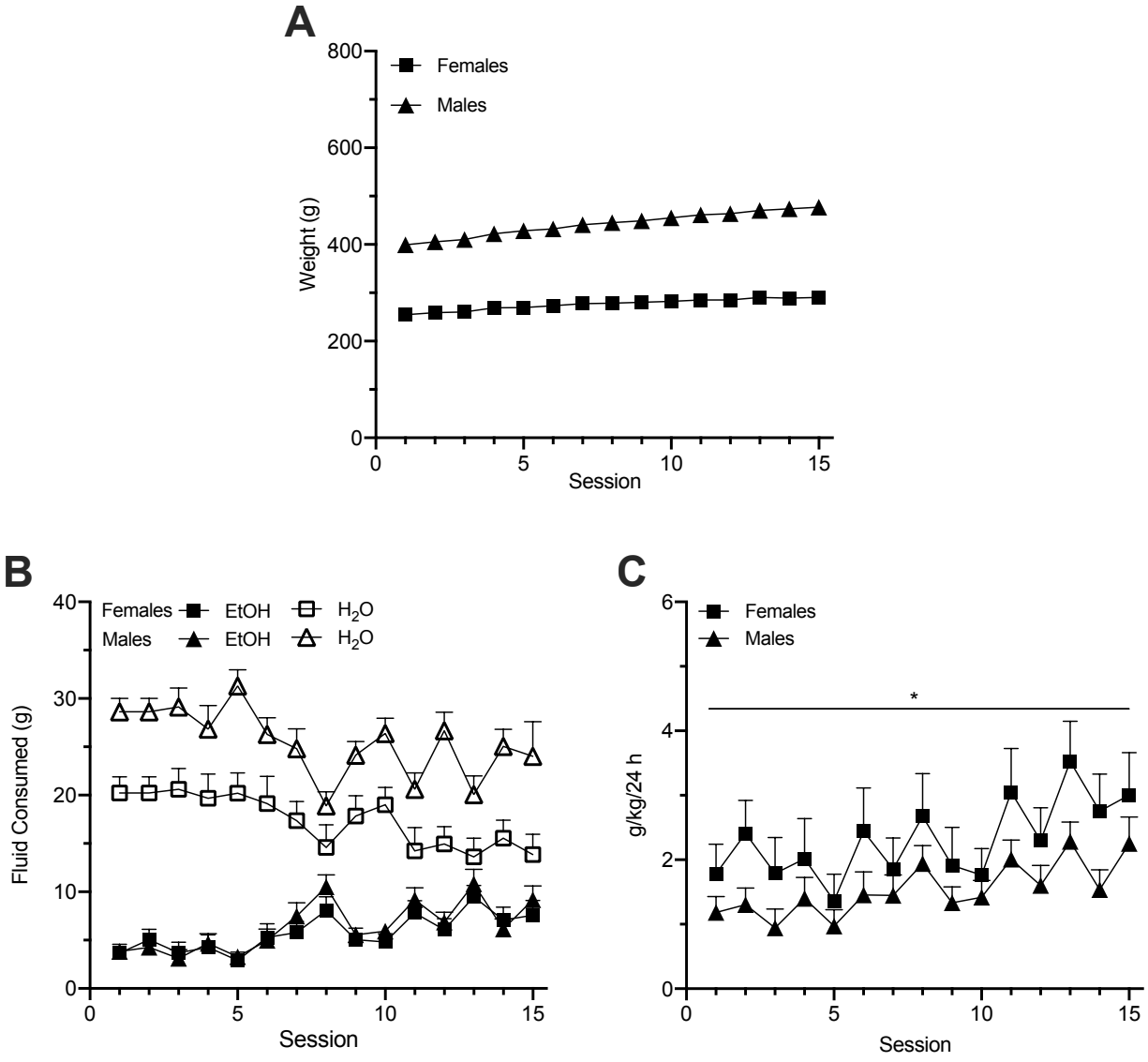

Figure 2. Intermittent access to alcohol and water in the home-cage in males (triangles) and females (squares). (**A**) Mean (± SEM) weight (g) of rats during the home-cage ethanol exposure. Males weighed more than females across sessions. (**B**) Mean (± SEM) grams of ethanol (filled shapes) consumed in each 24-h session were similar across sex, while mean (± SEM) grams of water (empty shapes) consumed in each 24-h session was greater in males compared to females. (**C**) Mean (± SEM) ingested dose of ethanol (grams of ethanol per kilogram of bodyweight accounting for the density of ethanol) per 24-h session was comparable in both males and females.

Pavlovian Discrimination Training. Both males and females learned to associate a CS with ethanol in the alcohol context, with females making more CS port-entries than males (Figure 3A). An ANOVA comparing port-entries made during the PreCS or PreNS and CS or NS intervals in the alcohol and neutral contexts indicated that overall, port-entries varied as a function of session [Session, $F_{(3.89, 93.27)}$=8.45, $p < .001$, η^2=.031], were higher in the alcohol context than in the neutral context [Context, $F_{(1,24)}$=130.86, $p < .001$, η^2=.145], were higher in females than males [Sex, $F_{(1, 24)}$=5.14, p=.033, η^2=.176] and varied as a function of interval [Interval, $F_{(1,24)}$=125.79, $p < .001$, η^2=.174]. Across sessions, CS port-entries increased, whereas port-entries during the NS, PreCS, and PreNS intervals remained low [Session x Interval, $F_{(4.28, 102.61)}$=14.98, p<.001, η^2=.040; Context x Session, $F_{(4.13, 101.23)}$=12.96, p<.001, η^2=.037; Context x Interval, $F_{(1,24)}$=140.85, p<.001, η^2=.102; Context x Interval x Session, $F_{(4.64, 111.44)}$=12.10, p<.001, η^2=.029]. A significant Context x Interval x Sex interaction [$F_{(1,24)}$=4.89, p=.037, η^2=.004] was found. Post hoc Bonferroni-corrected independent sample t-tests indicated that collapsed across sessions, in the alcohol context females made more CS port-entries than males [t=-4.15, p=.002, 95% CI (-6.00, -10.67)]. There was no sex difference in PreCS port-entries in the alcohol context [t=-0.80, $p > .999$, 95 CI (-5.82, 3.52)] or in NS [t=-0.49, $p >.999$, 95% CI (-5.372,3.965)] or PreNS [t=-0.36, $p >.999$, 95% CI (-5.19, 4.14)] port-entries in the neutral context. No other sex differences were found [Session x Sex, $F_{(3.89, 93.27)}$=0.77, p=.542, η^2=.003; Interval x Sex, $F_{(1,24)}$=2.98, p=.097, η^2=.004; Context x Sex, $F_{(1,24)}$=5.15, p=.033, η^2=.006; Session x Interval x Sex, $F_{(4.28, 102.61)}$=0.75, p=.571, η^2=.002; Context x Session x Sex, $F_{(4.22, 101.22)}$=0.84, p=.510, η^2=.002; Context x Interval x Session x Sex, $F_{(4.64, 11.44)}$=0.82, p=.532, η^2=.002]. No sex differences were observed for the total duration of CS port-entries, the latency to produce CS port-entries, or the port-entries during the ITI interval were found (see Figure S1).

Total port-entries remained high in the alcohol context, and decreased in the neutral context throughout training, with females making more port-entries than males (Figure 3B). Overall, total port-entries were greater in females than in males [Sex, $F_{(1,24)}$=4.35, p=.048, η^2=.034], varied across sessions [Session, $F_{(4.95, 116.34)}$=2.37, p=.046, η^2=.023], were greater in the alcohol context than in the neutral context [Context, $F_{(1,24)}$=104.06, p<.001, η^2=.290], and varied as a function of session in the neutral context but not in the alcohol context [Context x Session, $F_{(6.12, 146.92)}$=2.30, p=.036, η^2=.013]. Follow-up paired samples t-tests collapsed across

sex revealed that total port-entries in the alcohol context remained high [$t_{(25)}$=-1.81, p=.083, d=-0.35, 95% CI (-0.75, 0.05)] from session 1 (M = 85.35 ± SE = 8.20) to session 12 (M = 117.73 ± SE = 16.00), while total port-entries in the neutral context decreased [$t_{(25)}$=3.19, p=.004, d=0.63, 95% CI (0.20, 1.04)] from session 1 (M = 52.46 ± SE = 6.72) to session 12 (M = 26.69 ± SE = 6.27). No other sex differences were found [Context x Sex, $F_{(1,24)}$=0.21, p=.653, η^2<.001; Session x Sex, $F_{(4.85, 116.34)}$=0.92, p=.466, η^2=.009; Context x Session x Sex, $F_{(6.12, 146.92)}$=0.57, p=.756, η^2=.003]. Thus, both sexes were able to acquire the CS-alcohol association, but females produced more CS port entries than males.

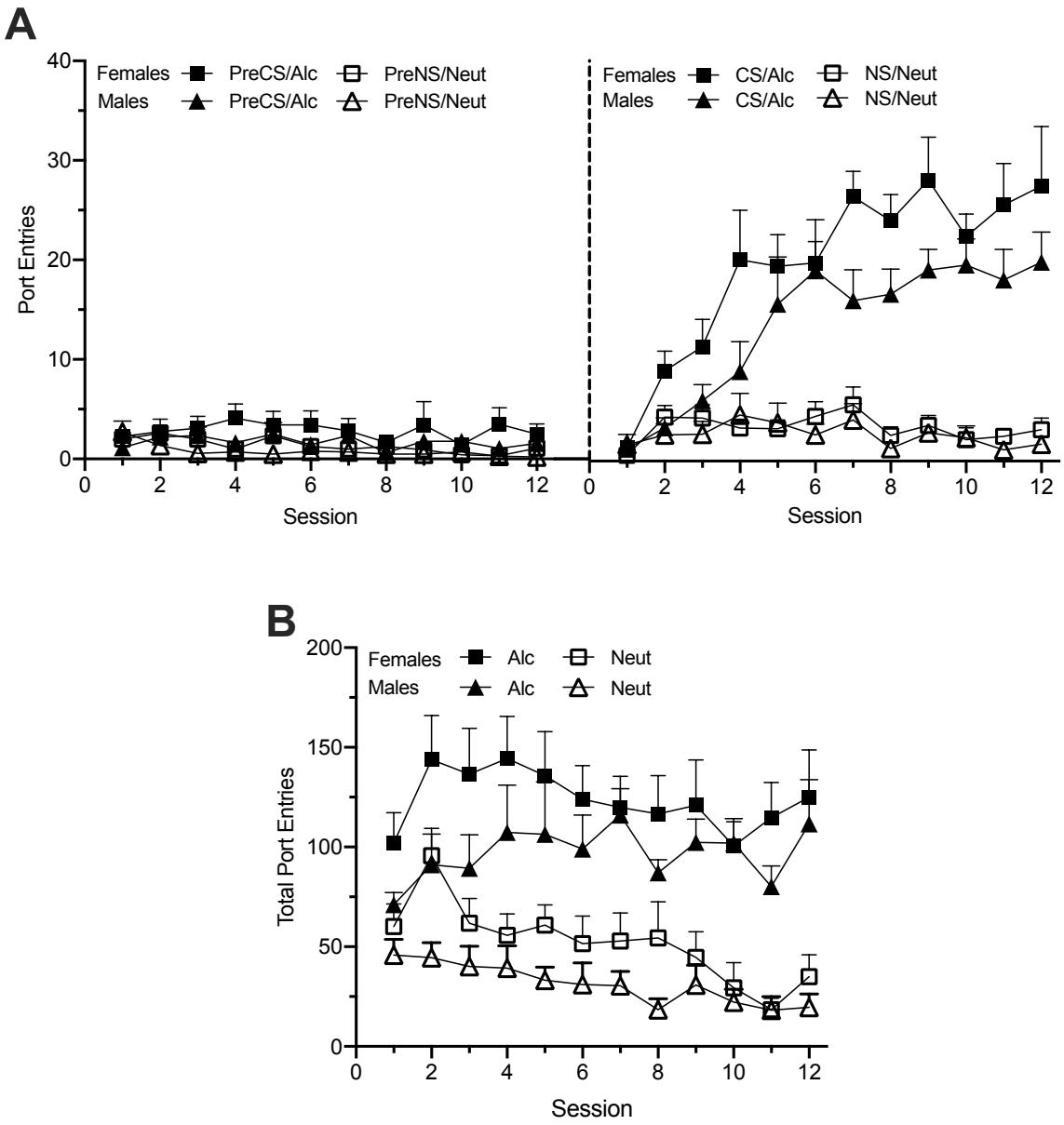

Figure 3. Acquisition of Pavlovian discrimination training in the alcohol context (filled shapes) and neutral context (empty shapes) in males (triangles) and females (squares). (**A**) Mean (± SEM) port entries during the PreCS or PreNS (left panel) and CS or NS (right panel) intervals. CS port entries were greater than NS, PreCS, and PreNS port entries in both sexes, with females making more CS port entries than males. (**B**) Mean (± SEM) total port entries (i.e., all the port entries in each session). Total port entries were greater in females than in males, remained high in the alcohol context, and decreased in the neutral context across sessions.

Effect of Context on Responding to CS. Following Pavlovian discrimination training, CS-elicited responding was tested in the absence of alcohol delivery in both the alcohol context and in the neutral context. ΔCS port-entries were higher in the alcohol context compared to the neutral context in males, while females responded similarly to the ΔCS in both contexts (Figure 4A). ANOVA was conducted to assess ΔCS port-entries at test in both contexts and sexes. Overall, there was no difference in the number of port-entries as a function of sex [Sex, $F_{(1,24)}$=0.52, p=.477, η^2=.015] or context [Context, $F_{(1,24)}$=0.55, p=.466, η^2=.006]. The Context x Sex interaction was not significant [$F_{(1,24)}$=3.14, p=.089, η^2=.036].

Our prior research has reliably found that male rats make more ΔCS port-entries at test in the alcohol context than in the neutral context (Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2015; Valyear et al., 2020). Visual inspection of Figure suggested that we replicated this result in males, but that females did not show a context-dependent modulation of ΔCS port entries. Planned comparison of ΔCS port-entries revealed that males made more ΔCS port-entries in the alcohol context (M = 22.00 ± SE = 4.07) than in the neutral context (M = 14.29 ± SE = 3.03) [$t_{(13)}$ =2.28, p=.040, d=.610, 95% CI (0.03, 1.17)], while ΔCS port-entries in females did not differ between the alcohol context (M = 20.00 ± SE = 4.07) and the neutral context (M = 17.54 ± SE = 5.06) [$t_{(11)}$ =-0.59, p=.566, d=-.17, 95% CI (-0.74, 0.40)]. Similar findings were observed for the total duration of CS port-entries and the latency to produce CS port-entries (Figure S2). Therefore, in males responding to an alcohol-predictive cue is context-dependent in males but may context-independent in females.

Alcohol-Primed Induced Reinstatement Test. Following the extinction of CS-elicited port entries in both contexts an alcohol-primed reinstatement test was conducted, in which 0.2 ml of ethanol was delivered at the start of the session and during the first CS presentation. Compared to the extinction baseline (Figure S3), CS-elicited port-entries were reinstated in both the alcohol context and neutral context, in males and females. However, CS port-entries at test were higher in the alcohol context compared to the neutral context in males but not females (Figure 4B). Overall, ΔCS port-entries were higher during the reinstatement test than the extinction baseline [Phase, $F_{(1,22)}$=81.48, p<.001, η^2=.527] and were greater in the alcohol context than in the neutral context [Context, $F_{(1,22)}$=7.87, p=.010, η^2=.073; Phase x Context, $F_{(1,22)}$=4.70, p=.041, η^2=.030]. No other differences were found [Sex, $F_{(1,22)}$=1.99, p=.173, η^2=.018; Phase x Sex, $F_{(1,22)}$=0.52 p=.477, η^2=.003; Context x Sex, $F_{(1,22)}$=.02, p=.891, η^2<.001;

Phase x Context x Sex, $F_{(1,22)}$=0.39, p=.538, η^2=.003]. Similar findings were observed for the total duration of CS port-entries and the latency to produce CS port-entries (Figure S4).

Our prior research found that male rats made more CS port-entries in the alcohol context than in the neutral context during reinstatement (Valyear et al., 2020). Planned comparisons revealed that males made more CS port-entries at test in the alcohol context (M = 20.29 ± *SE* = 2.78) than in the neutral context (*M* = 9.57 ± *SE* = 2.95) [$t_{(12)}$=-2.65, p=.021, d=-1.41, 95% CI (-2.58,-0.20)]. However, in females CS port-entries at test did not differ between the alcohol context (*M* = 21.00 ± *SE* = 4.26) and neutral context (*M* = 14.67 ± *SE* = 3.49) [$t_{(10)}$=-1.13, p=.283, d=-0.66, 95% CI (-1.81, 0.53)]. This suggests that in males, reinstatement of responding to an alcohol-predictive cue is context-dependent in males but may context-independent in females.

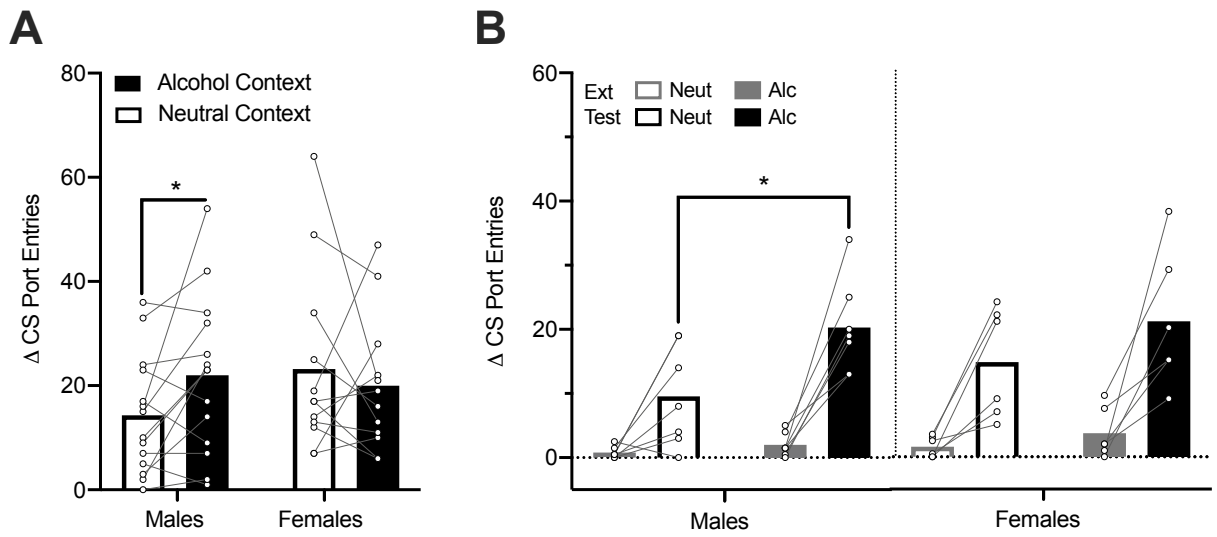

Figure 4. Mean ΔCS (CS minus PreCS port entries). (**A**) ΔCS port entries at test, in the neutral context (open bars) and alcohol context (filled bars) in the absence of alcohol delivery, in males and females. Circles depict data from individual rats. *$p < 0.05$. ΔCS port entries were significantly greater in the alcohol context than in the neutral context in males but not females. (**B**) ΔCS Port entries in the neutral context (empty bars) and alcohol context (filled bars) during alcohol-primed reinstatement test (black) compared to the extinction baseline (average of last two extinction sessions; grey) in males and females. Circles depict data from individual rats. *$p < 0.05$. ΔCS port entries at test were significantly greater in the alcohol context compared to the neutral context, in males.

Experiment 2

Home-Cage Ethanol Exposure. Throughout home-cage ethanol exposure, males weighed more than females (Figure 5A). The weight (g) of males (n=13) and females (n=15) increased across sessions [Session, $F_{(1.42, 36.9)}$=377.18, p=< .001, η^2=.130] and was greater in males compared to females [Sex, $F_{(1,26)}$=134.61, p=< .001, η^2=.695; Session x Sex, $F_{(1.42, 36.9)}$=89.84, p=< .001, η^2=.031].

Males drank more water (g) than females, whereas ethanol consumption (g) was similar in both sexes (Figure 5B). Overall, rats drank more water than ethanol [Fluid, $F_{(1,26)}$=91.39, p < .001, η^2=.493] and males consumed more fluid than females [Sex, $F_{(1,26)}$=77.33, p<.001, η^2 =.078; Fluid x Sex, $F_{(1, 26)}$=8.63, p=.007, η^2=.047]. Post hoc Bonferroni corrected t-tests indicated that males and females did not differ in the amount of ethanol consumed [$t_{(27)}$=0.79, p>.999, 95% CI (-3.33, 5.92)], but water intake was greater in males than females [$t_{(27)}$=6.18, p<.001, M_{diff}=10.23 , 95% CI (5.60, 14.85)]. Overall, fluid intake varied across sessions [Session, $F_{(5.51, 143.2)}$=43.77, p=<.001, η^2=.059] in both sexes [Session x Sex, $F_{(5.51, 143.2)}$=4.94, p=<.001, η^2=.007]. Water and ethanol intake changed from the first to last sessions [Fluid x Session, $F_{(4.75, 143.2)}$=23.43, p < .001, η^2=.100] comparably in both sexes [Fluid x Session x Sex, $F_{(4.75, 143.2)}$=1.47, p=.208, η^2=.006]. Collapsed across sex, ethanol intake increased from session 1 (M=3.21 ± SE=0.45) to session 15 (M=8.34 ± SE=1.01) [$t_{(27)}$=-5.57, p < .001, d=-1.05, 95% CI (-1.510, -0.582)], whereas water intake decreased from session 1 (M=22.42 ± SE=1.46) to session 15 (M=16.60 ± SE=1.46) [$t_{(75)}$=6.21, p<.001, d=1.17, 95% CI (0.68, 1.65)].

Males and females did not differ in overall ingested dose of ethanol throughout the home-cage ethanol exposure phase [Figure 5C; Sex, $F_{(1,26)}$=0.77, p=.387, η^2=.015]. The ingested dose of ethanol varied as a function of session [Session, $F_{(2.99, 77.79)}$=10.52, p < .001, η^2=.134] similarly in both sexes [Session x Sex, $F_{(2.99, 77.79)}$=0.58, p=.628, η^2=.007]. Thus, alcohol consumption did not differ between sexes.

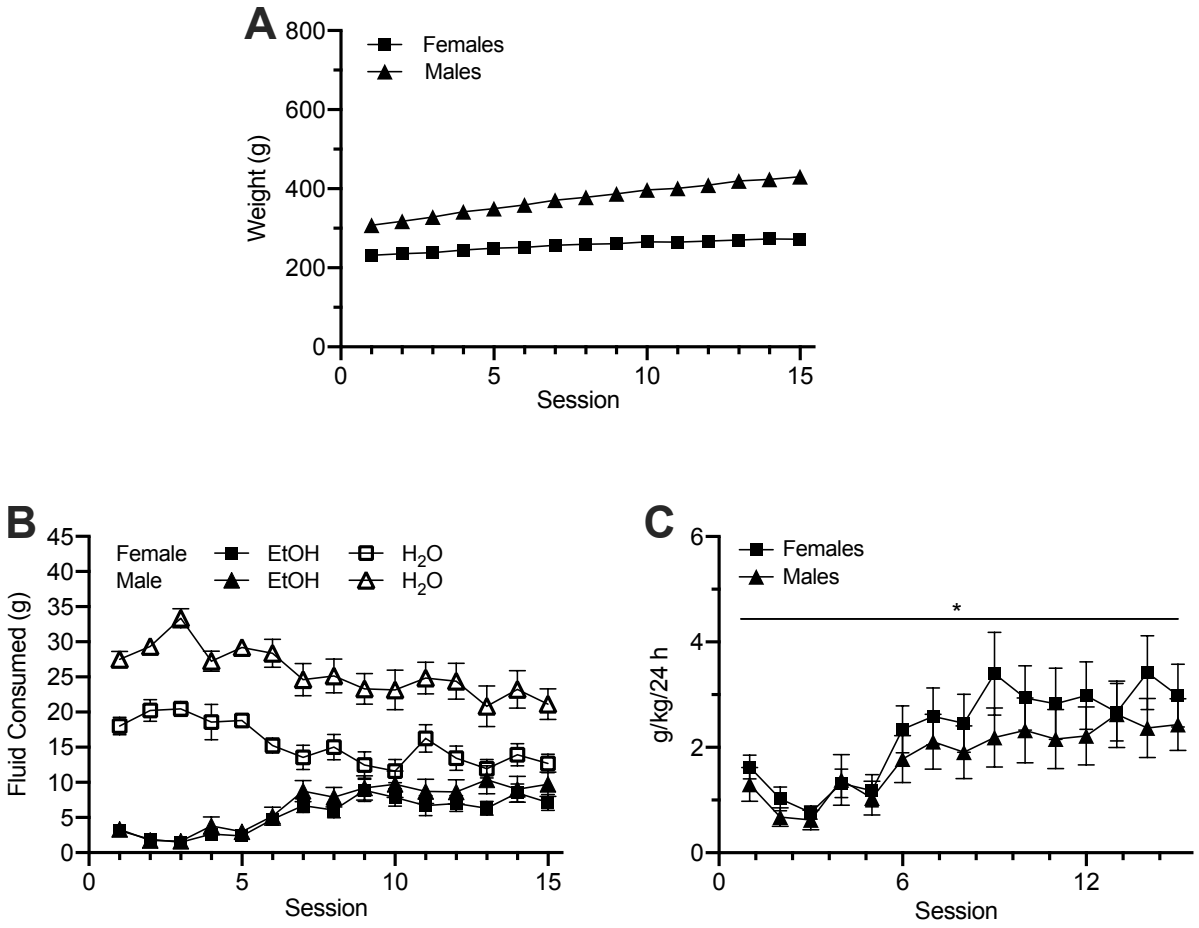

Figure 5. Intermittent access to alcohol and water in the home-cage in males (triangles) and females (squares). (**A**) Mean (± SEM) weight (g) of rats during the home-cage ethanol exposure. Males weighed more than females across sessions. (**B**) Mean (± SEM) grams of ethanol (filled shapes) consumed in each 24-h session were similar across sex, while mean (± SEM) grams of water (empty shapes) consumed in each 24-h session was greater in males compared to females. (**C**) Mean (± SEM) ingested dose of ethanol (grams of ethanol per kilogram of bodyweight accounting for the density of ethanol) per 24-h session was comparable in both males and females.

Pavlovian Discrimination Training. Both males and females learned to associate a CS with ethanol in the alcohol context (Figure 6A). An ANOVA comparing port-entries made during the PreCS or PreNS and CS or NS intervals in the alcohol and neutral contexts indicated that overall, port-entries varied as a function of session [Session, $F_{(4.61, 118.58)}$=9.36, $p < .001$, η^2=.031], were higher in the alcohol context than in the neutral context [Context, $F_{(1,26)}$=141.76, $p < .001$, η^2=.165], and varied as a function of interval [Interval, $F_{(1,26)}$=114.37, $p < .001$, η^2=.143]. Across sessions, CS port-entries increased, whereas port-entries during the NS, PreCS, and PreNS intervals remained low [Session x Interval, $F_{(3.98, 103.44)}$=22.60, p<.001, η^2=.056; Context x Session, $F_{(5.23, 136.05)}$=14.21, p<.001, η^2=.038; Context x Interval, $F_{(1,26)}$=119.30, p<.001, η^2=.109; Context x Interval x Session, $F_{(4.92, 127.91)}$=16.83, p<.001, η^2=.038]. No sex differences were found [Sex, $F_{(1, 26)}$=0.02, p=.896, η^2<.001; Session x Sex, $F_{(4.61, 119.73)}$=0.80, p=.540, η^2=.003; Interval x Sex, $F_{(1,26)}$=0.14, p=.716, η^2<.001; Context x Sex, $F_{(1,26)}$=0.03, p=.857, η^2<.001; Session x Interval x Sex, $F_{(3.98, 103.44)}$=1.20, p=.315, η^2=.003; Context x Session x Sex, $F_{(5.23, 136.05)}$=0.58, p=.726, η^2=.002; Context x Interval x Sex, $F_{(1, 26)}$=0.01, p=.942, η^2<.001; Context x Interval x Session x Sex, $F_{(4.92, 127.91)}$=1.17, p=.328, η^2=.003]. Similar findings were observed for the total duration of CS port-entries and the latency to produce CS port-entries, no main effect of sex or interactions with sex were found during the ITI interval (see Figure S5).

Total port-entries remained high in the alcohol context and decreased in the neutral context throughout training in both males and females (Figure 6B). Overall, total port-entries varied across sessions [Session, $F_{(4.16, 108.10)}$=5.52, p<.001, η^2=.048], were greater in the alcohol context than in the neutral context [Context, $F_{(1,26)}$=215.90, p<.001, η^2=.356; Context x Session, $F_{(6.38, 165.74)}$=2.99, p=.007, η^2=.016]. Follow-up paired samples t-tests collapsed across sex revealed that total port-entries in the alcohol context though remained high, decreased [$t_{(27)}$=2.54, p=.017, d=0.48, 95% CI (0.08, 0.87)] from session 1 (M = 128.29 ± SE = 14.95) to session 12 (M = 82.96 ± SE = 8.18), while total port-entries in the neutral context remained low and decreased [$t_{(27)}$=4.96, p<.001, d=0.94, 95% CI (0.49, 1.38)] from session 1 (M = 54.50 ± SE = 6.85) to session 12 (M = 18.07 ± SE = 3.23). No sex differences were found [Sex, $F_{(1,26)}$=0.74, p=.397, η^2=.004; Context x Sex, $F_{(1,26)}$=0.99, p=.328, η^2=.002; Session x Sex, $F_{(4.16, 108.10)}$=0.69, p=.604,

η^2=.006; Context x Session x Sex, $F_{(6.38, 165.74)}$=1.01, p=.420, η^2=.006]. This suggests that both males and females were able to acquire the CS-alcohol association.

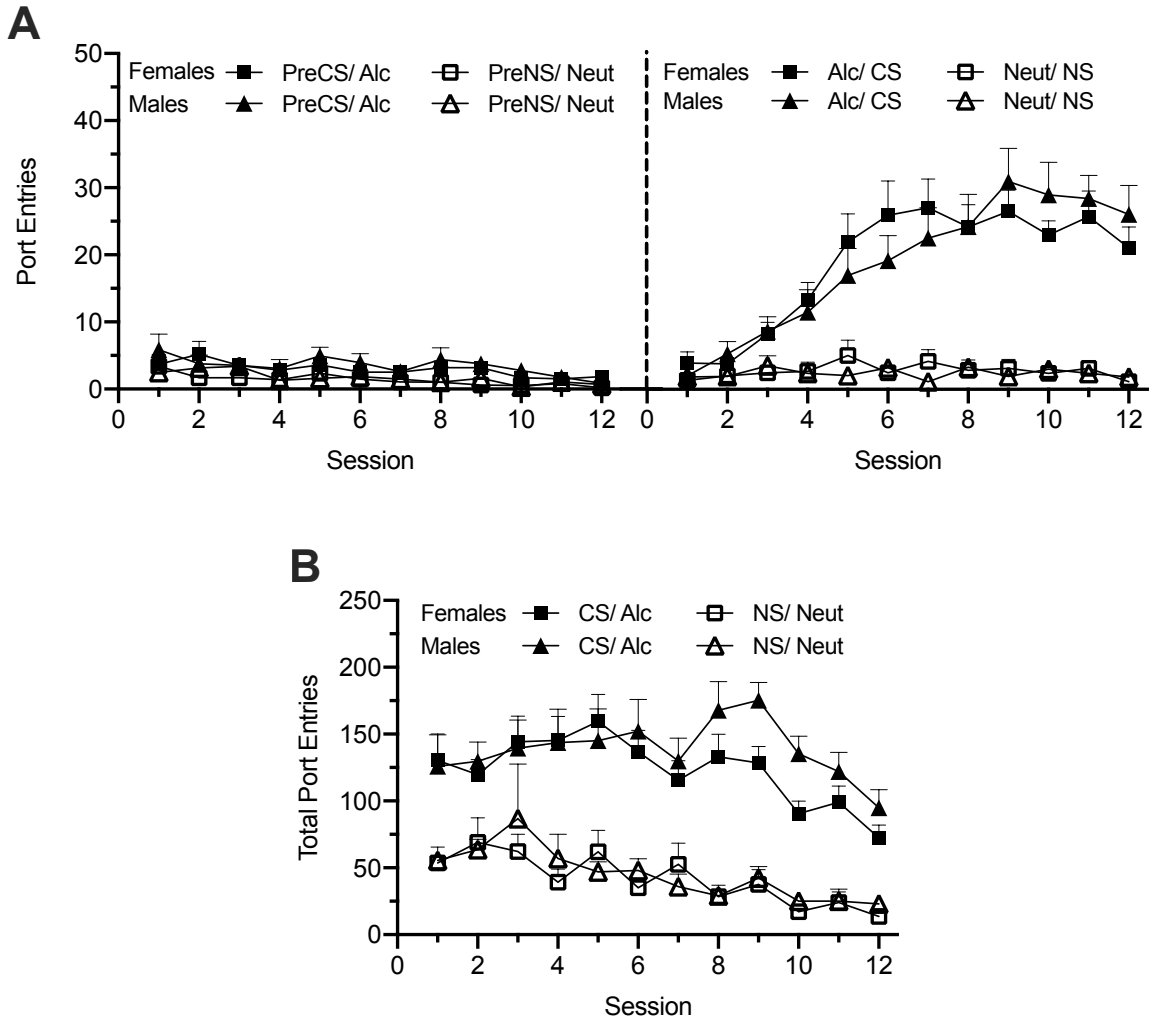

Figure 6. Acquisition of Pavlovian discrimination training in the alcohol context (filled shapes) and neutral context (empty shapes) in males (triangles) and females (squares). (**A**) Mean (± SEM) port entries during the PreCS or PreNS (left panel) and CS or NS (right panel) intervals. CS port entries were greater than NS, PreCS, and PreNS port entries in both sexes. (**B**) Mean (± SEM) total port entries (i.e., all the port entries in each session). Total port entries were greater in the alcohol context compared to the neutral context.

Effect of Eticlopride on Context on Responding to CS. Following Pavlovian discrimination training, the effect of systemic administration of dopamine D2-like antagonist eticlopride on CS-elicited responding was tested, in the absence of alcohol delivery in both the alcohol context and in the neutral context. ΔCS port-entries were higher in the alcohol context compared to the neutral context and were greater in the vehicle drug group compared to the drug group (Figure 7A). ANOVA was conducted to assess ΔCS port-entries at test in both contexts and sexes. Overall, more port-entries were made in the alcohol-associated context compared to the neutral context [Context, $F_{(1,26)}$=5.98, p=.022, η^2=.050], in both sexes [Sex, $F_{(1,26)}$=0.62, p=.438, η^2=.006; Context x Sex, $F_{(1,26)}$=0.10, p=.757, η^2<.001]. Furthermore, eticlopride reduced port entries compared to vehicle [Drug, $F_{(1,26)}$=13.61, p=.001, η^2=.083], and this effect did not differ across context [Drug x Context, $F_{(1,26)}$=0.03, p=.875, η^2,.001] or sex [Drug x Context x Sex, $F_{(1,26)}$=1.97, p=.172, η^2=.015]. Similar findings were observed for the total duration of CS port-entries and the latency to produce CS port-entries (Figure S6). Port-entries during the ITI time interval did not vary by drug group, sex, or context (Figure 7B) [Drug, $F_{(1,26)}$=2.79, p=.107, η^2=.030; Sex, $F_{(1,26)}$=0.41, p=.530, η^2=.007; Context, $F_{(1,26)}$=2.68, p=.114, η^2=.012; Drug x Sex, $F_{(1,26)}$=0.65, p=.429, η^2=.007; Context x Sex, $F_{(1,26)}$=0.11, p=.746, η^2<.001; Drug x Context, $F_{(1,26)}$=0.34, p=.565, η^2=.001; Context x Drug, $F_{(1,26)}$=1.76, p=.196, η^2=.006]. This suggests that eticlopride attenuated responding to an alcohol-predictive cue in both context in male and female rats.

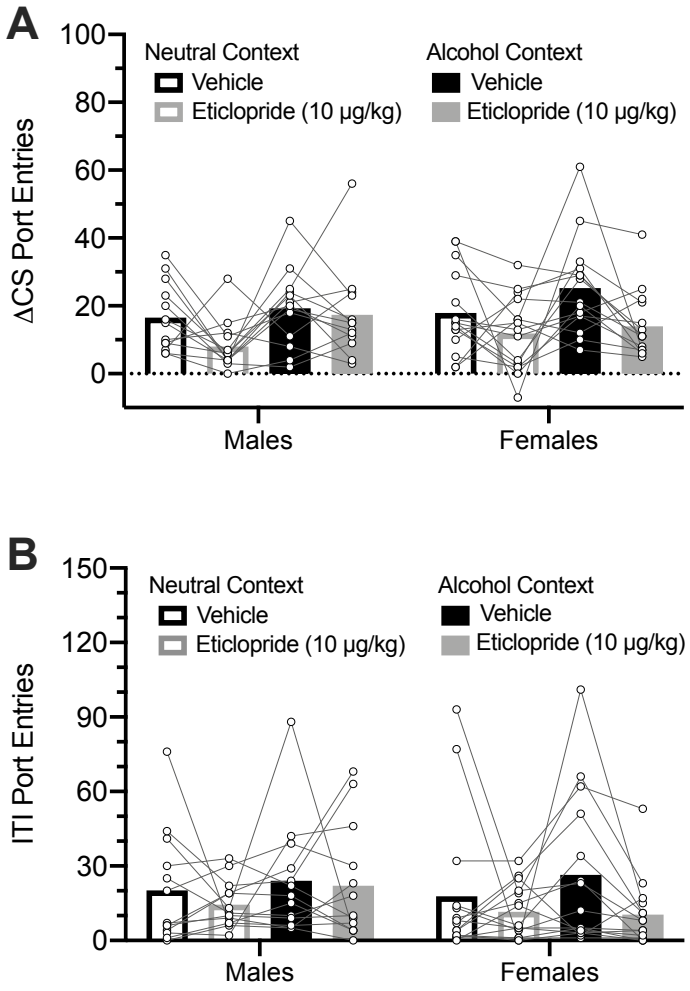

Figure 7. Port entries at test following the systemic administration of vehicle (black bars) or dopamine D2-like receptor antagonist eticlopride (grey bars, in the neutral context (open bars) and alcohol context (filled bars) in the absence of ethanol delivery, in males and females. Circles depict data from individual rats. (**A**) Mean (± SEM) ΔCS (CS minus PreCS port entries) port entries were greater in the alcohol context than in the neutral context and were attenuated following administration of eticlopride compared to vehicle in both contexts and sexes. (**B**) ITI Port entries did not differ across sex, context, or drug groups.

General Discussion

The current research investigated potential sex differences in the influence of an alcohol-associated context on responding to a discrete alcohol-predictive cue. The role of dopamine D2-like receptors in the modulation of responding to a discrete alcohol-predictive cue by an alcohol-associated context was also investigated. In Experiment 1, unreinforced presentations of a discrete alcohol-predictive CS-elicited greater responding in an alcohol-associated context compared to a neutral context in males. However, in females CS-elicited responding did not differ between contexts. Similarly, reinstatement of responding to a discrete alcohol-predictive cue was greater in the alcohol-associated context compared to a neutral context in males, while in females, reinstatement of responding to a discrete alcohol-predictive cue did not differ between contexts. In Experiment 2, overall CS-elicited responding was greater in the alcohol context than in the neutral context in both sexes. Systemic administration of the dopamine D2-like receptor antagonist eticlopride reduced CS-elicited responding in both contexts and both sexes. The results form Experiment 1 build on previous findings that an alcohol-associated context plays an important role in modulating responding to a discrete alcohol-predictive cue in males and suggests that context might not modulate responding to a discrete alcohol-predictive cue in females. Furthermore, findings from Experiment 2 provide evidence that dopamine neurotransmission at D2-like receptors governs responding to alcohol-predictive cues in both male and female rats, in a context-independent manner.

The Effect of Context on Responding to an Alcohol-Predictive Cue

To assess the effect of context on responding to the alcohol-predictive cue, the CS was presented in the alcohol-associated context and the neutral context, in the absence of alcohol. In Experiment 1, CS-elicited responding was amplified in the alcohol-associated context compared to the neutral context in males. Males were also faster to initiate CS port-entries and the total duration of their CS port-entries were longer in the alcohol-associated context compared to the neutral context. Thus, an alcohol-associated context modulated conditioned responding to an alcohol-predictive CS in males. These findings replicate previous results from our laboratory using male rats (Sciascia et al., 2015; Valyear et al., 2020) and support the notion that the context in which a drug is consumed can influence responding to a discrete drug predictive CS in males (Chaudhri et al., 2009; Conklin, 2006; Crombag & Shaham, 2002; Janak, 2013; Remedios et al.,

2014; Valyear et al., 2020; Zironi et al., 2006). In females, the number of ΔCS port-entries, total latency of CS port-entries, and total duration of CS port-entries were similar in both contexts. Thus, females responded to a discrete alcohol-predictive CS in a context-independent manner at test. As both males and females were able to acquire the CS-alcohol association, the observed sex difference was not due to the incapability to associate the CS with alcohol availability. These findings are supported by studies assessing sex differences in the context-induced renewal of food cues, in which males displayed context-induced renewal, while females did not (Anderson & Petrovich, 2015, 2017, 2018a, 2018b). Therefore, responding to a discrete alcohol-predictive cue in the absence of alcohol is context-dependent in male rats but may be context-independent in female rats.

A similar sex difference was found during the alcohol-primed reinstatement test, in which alcohol was only presented at the start of the session and during the first CS presentation, in both contexts. In males, CS-elicited responding was reinstated in both contexts, and was amplified in the alcohol-associated context compared to the neutral context. This finding replicates our previous work showing that reinstatement of responding to a discrete alcohol-predictive CS is amplified in a context associated with alcohol in male rats (Valyear et al., 2020). Males in the alcohol-associated context were also faster to initiate CS port-entries and the total duration of CS port-entries was longer in the alcohol-associated context compared to the neutral context, suggesting that in the alcohol-associated context rats were more motivated to produce port-entries during the CS. In females, CS-elicited responding was also reinstated in both contexts, but ΔCS port-entries, latency of CS port-entries, and duration of CS port-entries did not differ across the contexts. Given that no sex differences were found during extinction and responding in both sexes significantly decreased throughout the sessions in both contexts (Figure S3), these effects cannot be explained by a lack of extinction learning. These results align with prior published work suggesting that drug-associated contexts do not impact reinstatement of cue-elicited responding in females. For example, in context-driven reinstatement of methamphetamine self-administration, males responded more to the methamphetamine-paired lever than to the inactive lever, while females reinstated to a lesser extent than males and responded similarly to both levers, in the drug-associated context (Takashima et al., 2018). Thus, the context may modulate alcohol-priming induced reinstatement in males but not in females.

The results found during the tests in Experiment 1, may because males and females use different types of environmental information to guide their behaviour. This notion is supported by findings that females are more likely to generalize responding to novel contexts (Asok et al., 2019; Keiser et al., 2017; Lynch et al., 2013). Lynch et al (2013) examined sex differences in contextual fear generalization. Male and female rats were trained in passive avoidance and tested at different retention intervals (i.e., 1 day, 3 days, 5 days, and 7 days) in the training context or a novel context. The results showed that male rats displayed context discrimination at all the intervals, while females exhibited fear avoidance in the novel context at the 5-day and 7-day intervals. Thus, females may process contextual information differently and males may be more efficient in forming contextual representations than females. However, whether these processes contributed to the sex differences found in the current study remains to be tested. Unfortunately, studies investigating how environmental contexts influence alcohol use in men and women are limited. However, it is suggested that the environmental context can influence alcohol craving in individuals with AUDs (Heinz et al., 2004). Recently, it was shown that both men and women with AUD report high levels of alcohol craving in alcohol-associated contexts (e.g., a bar, a pub and parties), but women also reported higher levels of cravings in contexts not commonly associated with alcohol (e.g., the workplace, bedroom and supermarket) (Ghiţă et al., 2019). Thus, alcohol craving in men may be more influenced by on the environmental context compared to women.

The sex differences found during the tests in Experiment 1 were obtained using planned comparisons, that compared CS-elicited responding between the alcohol-associated context and the neutral context in males and females. Uncorrected planned comparisons have been proposed as a valid analysis to address specific apriori research hypotheses, when the number of comparisons does not exceed the number of degrees of freedom associated with the overall treatment mean square (Keppel, 1991). Based on prior research that has reliably found that male rats make more ΔCS port-entries at test in the alcohol context than in the neutral context (Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2015; Valyear et al., 2020), we used planned comparisons to address our hypothesis that responding elicited by a discrete alcohol-predictive cue is influenced by the environmental context in males but not in females.

In Experiment 2, the role of dopamine neurotransmission at D2-like receptors on responding to an alcohol-predictive cue was investigated by administering D2-like receptor

antagonist eticlopride before the test. Overall CS port entries were greater in the alcohol-associated context compared to the neutral context, indicating that CS-elicited responding was context-dependent in both males and females. These findings in females do not align with those in Experiment 1, in which CS-elicited responding was context-independent. Previous studies have successfully demonstrated context-induced renewal in female rats (Bouton & Peck, 1989; Brooks & Bouton, 1993; Woods & Bouton, 2008), however, these studies typically included pre-training in which food-pellets were delivered in the contexts prior to conditioning. The amount of exposure to the contexts may influence context discrimination in females. For instance, studies assessing sex differences in contextual fear generalization have shown that following context preexposure, context discrimination increases in females (Asok et al., 2019; Keiser et al., 2017; Lynch et al., 2013; Wiltgen et al., 2001). Notably, rats in Experiment 2 had more exposure to the contexts due to the greater number of tests and retraining sessions. Therefore, greater exposure to the contexts may have aided females in forming contextual associations. Future studies could consider adopting a between-subjects design for the test to reduce repeated exposure to the contexts.

Dopamine Neurotransmission at D2-like Receptors are Needed for Responding to an Alcohol-Predictive Cue

In Experiment 2, the systemic administration of eticlopride before the test resulted in an overall reduction in CS port-entries compared to the vehicle across both contexts and sexes. These findings suggest that dopamine neurotransmission at D2-like receptors is needed for conditioned responding elicited by discrete cues that predict alcohol, regardless of the context in which these cues are experienced. These findings align with previous studies conducted in our laboratory, showing that eticlopride reduced CS-elicited responding in a neutral context compared to vehicle (Sparks et al., 2014; Valyear et al., 2020) and findings from alcohol operant self-administrations studies showing that eticlopride reduces the reinstatement of conditioned responding to alcohol-predictive cues (Liu & Weiss, 2002). Notably, previous evidence has shown that in the presence of alcohol eticlopride does not reduce CS port entries when compared to antagonists of other dopamine receptors (e.g., D1 receptor antagonists) (Sparks et al., 2014), suggesting that D2 receptors may be selectively involved in the expression of responding to alcohol-predictive cues in the absence of alcohol. Similarly, D2 receptor availability is associated with alcohol craving and alcohol cue reactivity during abstinence in individuals with AUD

(Heinz et al., 2004). Future studies could assess the role of D2 receptors in responding to discrete alcohol-predictive cues in both the presence and absence of alcohol to determine whether D2 receptors are needed for conditioned responding driven by the memory of the CS-alcohol association.

Reductions in CS port entries by eticlopride were likely not due to locomotor effects as port-entries during a non-CS time interval were not impacted by eticlopride. Open field assessments have shown that a 10 μg/kg dose of eticlopride, as was used in the present study, does not impact locomotor activity (Cook & Beardsley, 2003; S. Y. S. Khoo et al., 2019). Locomotor deficits due to eticlopride are usually observed at higher doses such as 20 μg/kg or greater (Bardo et al., 1999; Bevins et al., 2001). Although, it is unlikely that the systemic administration of eticlopride impaired locomotor activity during test, future studies could examine locomotor activity as a control measure during the manipulation.

Although eticlopride attenuated responding to a discrete alcohol-predictive cue similarly in both males and females in Experiment 2, the neural circuitry involved in this behavioural outcome may still vary as a function of biological sex (Becker & Koob, 2016). Previous literature has shown that dopaminergic projections from the VTA to the NAc are needed for the contextual modulation of responding to a discrete alcohol-predictive cue in male rats (Valyear et al., 2020). Specifically, chemogenetic inhibition of dopaminergic projections from the VTA to the NAc core attenuates cue-elicited responding irrespective of context, while inhibition of dopaminergic projections from the VTA to the NAc shell selectively attenuates the amplification of cue-elicited responding by an alcohol-associated context (Valyear et al., 2020). Thus, future studies could use a chemogenetic manipulation of the dopaminergic projections from the VTA to the NAc in male and female rats to further assess the role of dopamine in the contextual modulation of responding to a discrete alcohol-predictive cue.

Ethanol and Water Consumption

In both of the present experiments, males and females did not differ in ethanol consumption. These results differ from prior published studies in which females have commonly been shown to consume more ethanol per kg of bodyweight compared to males in this procedure. These differences between the present results and prior studies could be related to the strain of rat used, drinking conditions, and experimental procedures (Hilderbrand & Lasek, 2018; Priddy et al., 2017; Simms et al., 2008). Although both sexes consumed similar amounts of ethanol,

males consumed more water than females. As males weighed significantly more than females it may be the case that females experienced greater pharmacological effects during this procedure. In the present studies, the doses of ethanol ingested in males align with previous findings from our laboratory using the same rat strains (Supplementary Material; Valyear et al 2020). In humans, women have been shown to reach higher peak blood alcohol concentrations and are more susceptible to alcohol's pharmacological effects compared to men after consuming the same doses (Mumenthaler et al., 1999). Furthermore, in female rats increased cue-elicited responding is associated with increased ethanol consumption and greater blood alcohol levels (Cofresí et al., 2019). Therefore, future studies should measure the blood alcohol concentrations of male and female rats following intermittent access to ethanol, as it can provide insight into potential sex differences in the relationship between alcohol consumption and blood-ethanol levels (Juárez & de Tomasi, 1999).

Acquisition of the CS-Alcohol Association

Reinforced CS presentations in the alcohol-associated context resulted in the acquisition of a CS-alcohol association in male and female rats. In the present studies, port entries during the PreCS interval and the NS presentations remained low throughout training in both sexes, while port entries during the CS increased from the first to the last training session. Previous work from our laboratory has consistently shown a similar pattern of responding during acquisition using a Pavlovian discrimination task (Chaudhri et al., 2009; Chaudhri, Sahuque, & Janak, 2008; Chaudhri, Sahuque, Cone, et al., 2008; S. Y. Khoo et al., 2019; Remedios et al., 2014; Sciascia et al., 2014; Sparks et al., 2014; Valyear et al., 2020). The total duration of CS port-entries and the latency to produce CS port-entries did not differ across sex in the present studies, suggesting that both males and females were equally motivated to make port-entries during the CS. Our findings support the notion that the CS gained the ability to predict the availability of alcohol, following repeated pairings with alcohol delivery (Cofresí et al., 2017, 2018, 2019).

Notably, in Experiment 1, females entered the fluid port during the CS more than males, however, no sex differences were found during a non-CS time interval such as the ITI. Similar findings have been exhibited in eye-blink conditioning and fear conditioning studies, which have indicated that females acquire and retain CS-unconditioned stimulus associations more effectively than males (Dalla & Shors, 2009). Additionally, females have also been shown to make more entries into a dipper containing alcohol compared to males during operant self-

administration (Nieto & Kosten, 2017). Thus, females may have acquired the CS-alcohol association more effectively than males. However, in Experiment 2, a sex difference in the amount of CS port entries made during acquisition was not found. One explanation for this difference could be because of where the rats in both experiments originated. In Experiment 1 rats were bred in-house using Long Evans sires form Charles River as part of our transgenic rat colony. However, in Experiment 2, Long Evans rats were purchased from Charles River. Differences in associative learning have been shown to vary across rat strain (Flores-Bonilla & Richardson, 2019; Pryce et al., 1999; Simms et al., 2008) and rat vendors (Sparks et al., 2014). Therefore, the acquisition of the CS-alcohol association should be replicated in rats of different origin and strain, as this may influence CS-elicited responding.

Future Directions

Our current findings highlight potential sex differences in the contextual modulation of responding to alcohol-predictive cues, however, the question remains as to why the effect of context on cue-elicited responding varies across sex. Previous literature has shown that male and female sex hormones influence alcohol intake in humans and rats. For example, high levels of testosterone are associated with binge drinking and alcohol dependence in men, while high estradiol levels are associated with high drinking rates in women (Lenz et al., 2012). Sex hormones have also been shown to influence associative learning in rats. For example, females in proestrus (i.e., high estrogen levels) have been shown to acquire conditioned associations at a faster rate compared to females with low estrogen levels in eyeblink conditioning paradigms, while high levels of testosterone, in males has been shown to facilitate extinction during conditioned taste aversion (Dalla & Shors, 2009). The effect of sex hormones has also been shown to influence context-dependent learning in females. In females, estrogen has been shown to facilitate context-induced renewal in response to reward-predictive cues (Anderson & Petrovich, 2015) and spatial learning (Tropp & Markus, 2001), but has been shown to attenuate aversive context discrimination (Lynch et al., 2013). Therefore, male and female sex hormones may play a role in the effect of context on responding to alcohol-predictive cues and should be assessed in future studies. Furthermore, sex hormones have been shown to influence the activity of the hippocampus activity, a brain region central in context-dependent learning (Anderson & Petrovich, 2018b; Tropp & Markus, 2001). For instance, during spatial tasks hippocampal activity is increased in female rats with high estrogen levels compared females with low estrogen

levels (Tropp & Markus, 2001). Conversely, frontal pathways projecting to the hippocampus have been shown to be under-activated in female rats during context-dependent learning, compared to males (Anderson & Petrovich, 2017, 2018b). Thus, the hippocampus may be a potential neural target for future studies assessing sex differences in the role of context on responding to reward-predictive cues.

In conclusion, these findings suggest that the modulatory influence of an alcohol-associated context on the effects of a discrete alcohol-predictive cue varies as a function of biological sex. In Experiment 1, we replicated the finding that in males CS port-entries were amplified in the alcohol-associated context compared to the neutral context, both at test and during an alcohol-primed reinstatement test. Females made a similar number of CS port-entries in both the alcohol-associated context and the neutral context at both tests, suggesting that their responding was not modulated by context. While this behavioral result was not replicated in Experiment 2, these differences could be explained by the origin of rats or the training conditions used in both experiments, suggesting that more work is needed to establish the validity of the sex difference observed in Experiment 1. Furthermore, administration of a dopamine D2-like receptor antagonist attenuated CS port-entries in the alcohol-associated context and the neutral context in both sexes. These data indicate potential sex differences in context use and processing and suggest that dopamine neurotransmission at D2-like receptors is needed for responding to alcohol-predictive cues. Due to the large discrepancy between men and women in the physiological, behavioural, and neurobiological factors involved in alcohol use, sex differences should be taken into account in the assessment and treatment of alcohol use disorders.

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Supplementary Materials

Table S1. Final number of male and female rats trained and tested with ethanol concentrations for experiments 1 and 2.

	Experiment 1		**Experiment 2**	
Ethanol Con.	Males (n=14)	Females (n=12)	Males (n=13)	Females (n=15)
5% EtOH	n = 1	n = 2	n = 3	n = 2
10% EtOH	n = 2	n = 1	n = 1	n = 1
15% EtOH	n = 11	n = 9	n = 9	n = 11

Table S2. Description of contexts used for Pavlovian discrimination training.

Modality	Context A	Context B
Visual	Black cardboard covered walls Brown paper in waste tray	Clear plexiglass walls Brown paper in waste tray
Tactile	Clear polycarbonate floor	Wire-grid floor
Olfactory[1]	Lemon odour	Almond odour

[1] Sprayed (3 sprays) onto petri dish placed in the waste tray beneath the chamber.

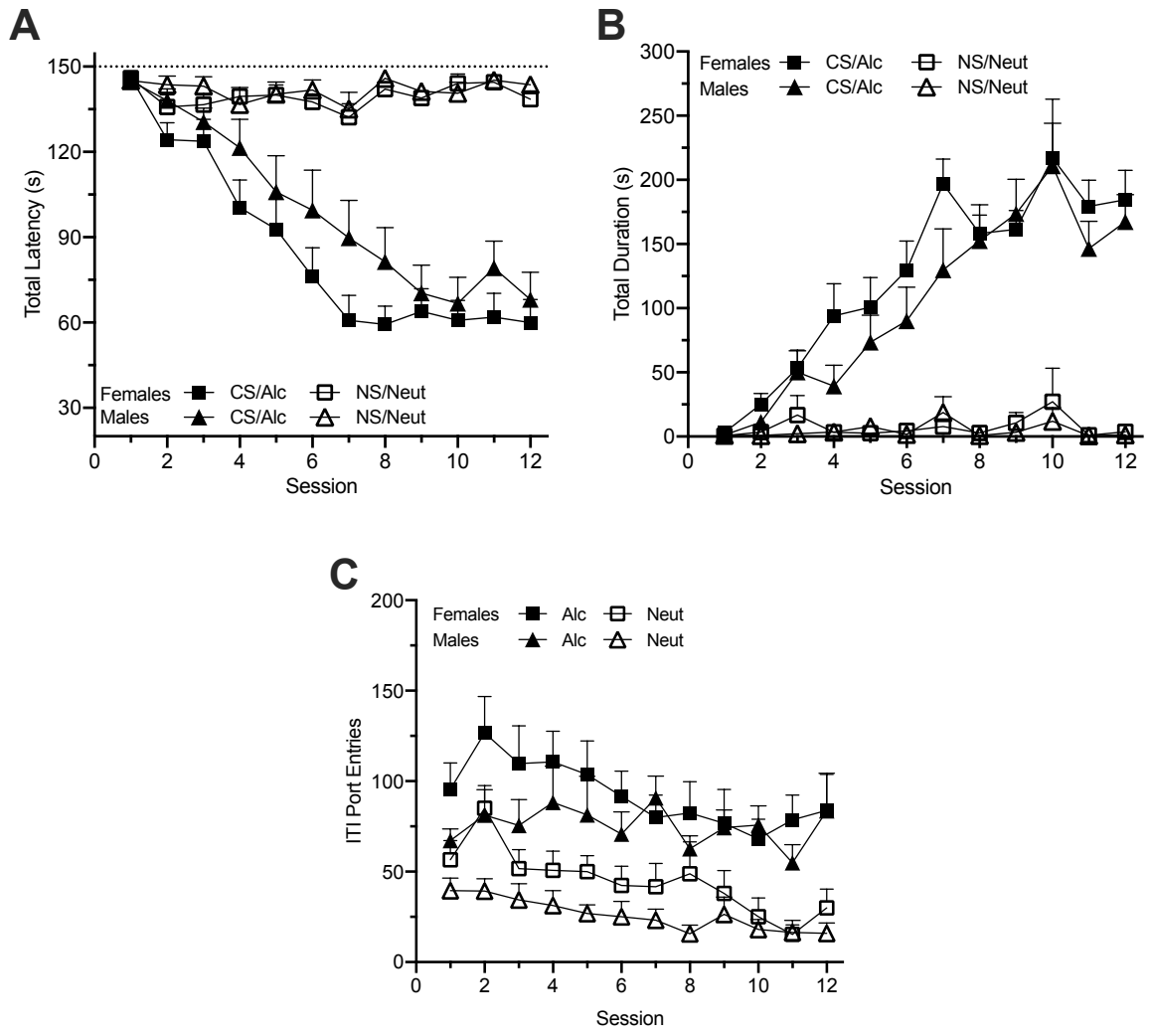

Figure S1. Acquisition of Pavlovian discrimination training in the alcohol context (filled shapes) and neutral context (empty shapes) in males (triangles) and females (squares). **(A)** Mean (± SEM) total latency (s) to initiate port entries during the CS or NS interval. The total latency to initiate a port-entry after stimulus onset was shorter for the CS in the alcohol context compared to the NS in the neutral context in both males and females. Overall the total latency to initiate a CS or NS port-entry varied as a function of session [Session, $F_{(4.45,\ 106.68)}$=36.88, p<.001, η^2=.127] in the alcohol context but not in the neutral context [Context, $F_{(1,24)}$=109.77, p<.001, η^2=.261; Context x Session, $F_{(5.35,\ 128.42)}$=43.43, p<.001, η^2=.124]. Follow-up paired samples t-tests collapsed across sex revealed that the latency to make a CS port-entry in the alcohol context decreased from session 1 (M=145.53 ± SE=1.25) to session 12 (M=64.35 ± SE=6.29) [$t_{(25)}$=12.33, p < .001, d=2.42, 95%

CI (1.64, 3.18)], whereas latency to make a port-entry following NS onset in the neutral context remained stable and high [$t_{(25)}$=1.59, p=.124, d=.31, 95% CI (-0.09, 0.70)] from session 1 (M = 145.46 ± SE = 1.76) to session 12 (M = 141.28 ± SE = 2.04). No differences across sex were found [Sex, $F_{(1,24)}$=1.75, p=.198, η^2=.068; Session x Sex, $F_{(4.45, 106.68)}$=1.05, p=.390, η^2=.004; Context x Sex, $F_{(1,24)}$=1.60, p=.217, η^2=.005; Context x Session x Sex, $F_{(5.35, 128.42)}$=1.06, p=.387, η^2=.003]. **(B)** Mean (± SEM) total duration of port entries initiated during the CS or NS interval. The total duration of port-entries initiated during the CS was longer and increased over time in the alcohol context, while the total duration of port-entries initiated during the NS in the neutral context remained long, in both males and females. Overall, total duration of port-entries initiated during the CS or NS varied across sessions [Session, $F_{(2.90, 69.55)}$=26.06, p<.001, η^2=.137], was greater in the alcohol context than in the neutral context [Context, $F_{(1,24)}$=111.50, p<.001, η^2=.324] and varied as a function of session in the alcohol context but not in the neutral context [Context x Session, $F_{(2.88, 69.01)}$=20.82, p<.001, η^2=.107]. Follow-up paired samples t-tests collapsed across sex revealed that the total duration of CS port-entries in the alcohol context increased from session 1 (M = 2.05 ± SE = .85) to session 12 (M = 175.31 ± SE = 15.27) [$t_{(25)}$=-11.31, p<.001, d=-2.22, 95% CI (-2.03, -1.49)], while the total duration of NS port-entries in the neutral context remained low [$t_{(25)}$=-1.53, p=.139, d=-0.30, 95% CI (-0.69, 0.10)] from session 1 (M = 0.80 ± SE = .45) to 12 (M = 2.44 ± SE = .95). No differences across sex were found [Sex; $F_{(1,24)}$=1.18, p=.289, η^2=.005; Context x Sex, $F_{(1,24)}$=0.70, p=.413, η^2=.002; Context x Session x Sex, $F_{(2.88, 69.01)}$=1.24, p=.301, η^2=.006]. **(C)** Mean (± SEM) port entries produced during the ITI interval. ITI port entries varied as a function of session [Session, F(5.098, 122.346) = 4.257, p = .001, η^2 = .046] and were greater in the alcohol context than in the neutral context [Context, F(1,24) = 67.462, p < .001, η^2 = .205]. No other interactions or sex differences were found [Context x Session; F(6.338, 152.100) = 0.891, p = .508, η^2 = .006, Context x Sex; F(1,24) = 0.06, p = .808, η^2 < .001, Session x Sex; F(5.098, 122.346) = 1.451, p = .210, η^2 = .016, Context x Session x Sex; F(6.338, 152.100) = .735, p = .629, η^2 = .005, Sex; F(1,24) = 3.525, p = .073, η^2 = .029].

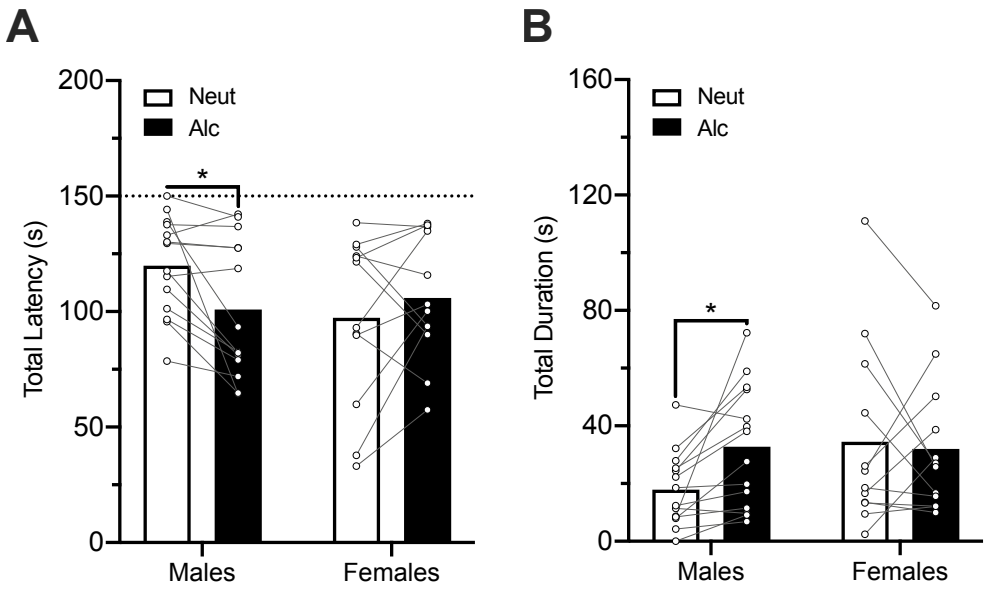

Figure S2. Responding at test, in the alcohol context (filled bars) and neutral context (open bars) in the absence of ethanol delivery, in males and females. Circles depict data from individual rats. *$p < 0.05$. **(A)** Mean (± SEM) latency to alcohol CS port entries. The total latency to initiate port-entries after CS onset was shorter in the alcohol context compared to the neutral context in males but was similar across contexts in females. Overall latency did not vary across contexts [Context, $F_{(1,24)}$=1.01, p=.326, η^2=.008] or sex [Sex, $F_{(1,24)}$=0.75, p=.396, η^2=.030]. However, latency of port-entries initiated during the CS varied as a function of context across sex [Context x Sex, $F_{(1,24)}$=6.97, p=.014, η^2=.055]. Planned comparisons using paired samples t-tests revealed that males were faster to initiate CS port-entries in the alcohol context (M = 100.939 ± SE = 7.927) compared to the neutral context (M = 119.84 ± SE = 5.68) [$t_{(13)}$=-3.00, p=.011, d=-.80, 95% CI (-1.39, -0.18)]. However, the time it took in females to initiate the CS port-entries did not differ between the alcohol context (M = 105.86 ± SE = 7.89) and the neutral context (M = 97.37 ± SE = 10.59) [$t_{(13)}$=1.01, p=.335, d=.291, 95% CI (-0.29, 0.86)]. **(B)** Mean (± SEM) total duration of the port-entries initiated during the CS was greater in the alcohol context compared to the neutral context in males, while in females the total duration of CS port-entries did not differ across contexts. Overall, the total duration of CS port-entries did not differ across context or sex [Context, $F_{(1,24)}$=1.91, p=.180, η^2=.018; Sex, $F_{(1,24)}$=1.01, p=.325, η^2=.040; Context x Sex, $F_{(1,24)}$=3.73, p=.065, η^2=.035]. Planned comparisons using paired samples t-tests found that in males the total

duration of CS port-entries was greater in the alcohol context (M = 32.794, SE = 5.688) than in the neutral context (M = 17.94 ± SE = 3.43) [$t_{(13)}$=3.11, p=.008, d=.831, 95% CI (0.21, 1.43)], whereas in females the total duration CS-elicited port-entries was similar in the alcohol context (M = 31.99 ± SE = 6.62) and the neutral context (M = 34.46 ± SE = 9.29) [$t_{(11)}$=-0.31, p=.762, d=-.090, 95% CI (-0.66, 0.48)].

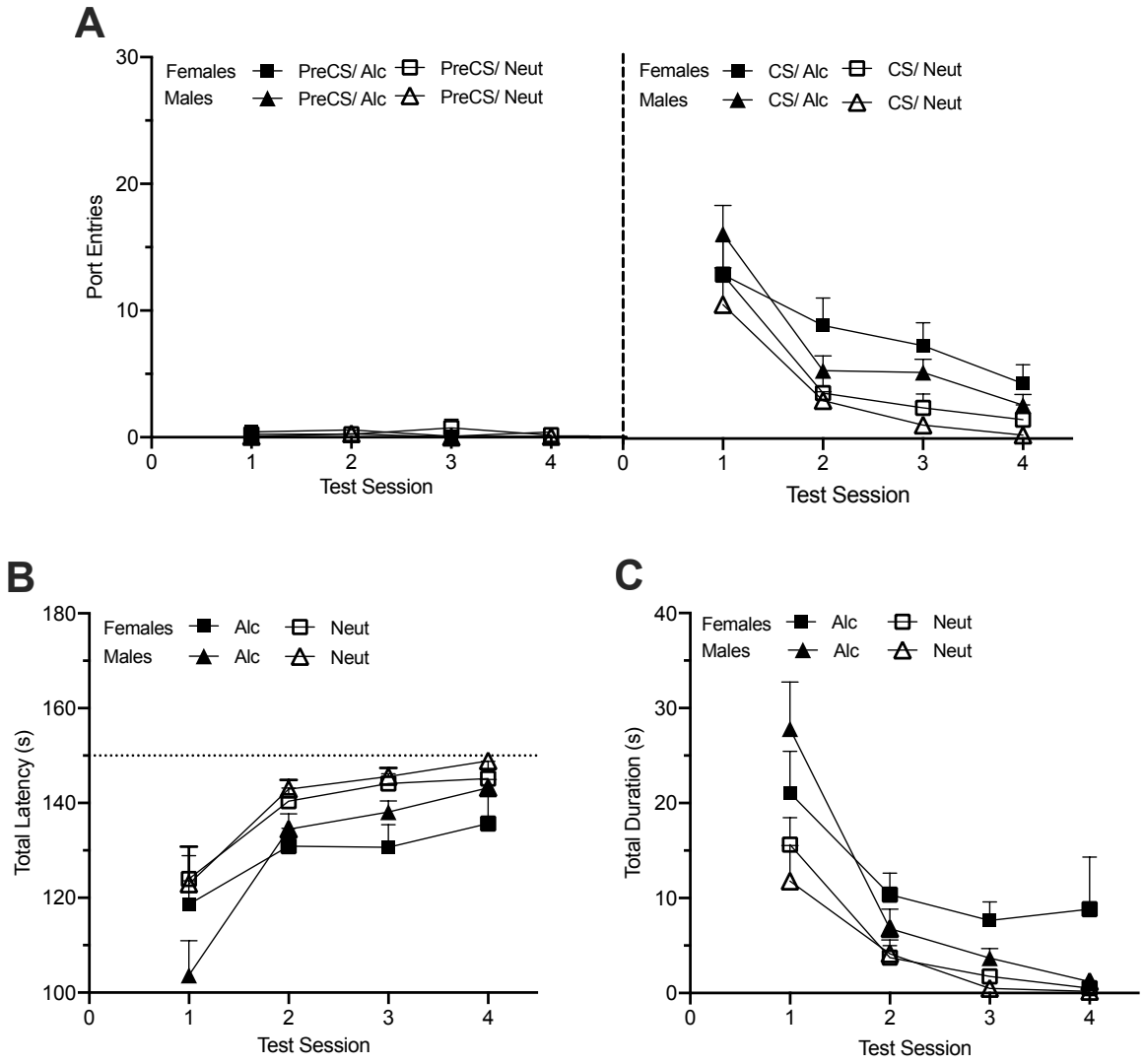

Figure S3. Extinction of CS port entries in the alcohol context (filled shapes) and neutral context (empty shapes) in the absence of alcohol delivery during repeated tests, in males (triangles) and females (squares). (**A**) Mean (± SEM) port entries during the PreCS (left panel) and CS (right panel) intervals in the alcohol and neutral contexts. Port entries varied as a function of session [Session, $F_{(1.98, 47.4)}$=41.13, p<.001, η^2=.120], were higher in the alcohol context than in the neutral context [Context, $F_{(1,24)}$=16.94, p<.001, η^2=.020], and varied as a function of interval [Interval, $F_{(1,24)}$=117.31, p<.001, η^2=.239; Interval x Context, $F_{(1,24)}$=17.30, p<.001, η^2=.020]. Across sessions, CS port entries decreased, whereas PreCS port entries remained low [Interval x Session, $F_{(1.89, 45.35)}$=47.95, p<.001, η^2=.120] with no difference as a function of context [Context x Session, $F_{(1.74, 41.73)}$=0.22, p=.777, η^2<.001]. The three-way Interval x Context x Session interaction was not

significant [$F_{(1.92, 46.11)}$=0.41, p=.776, η^2=.001]. No sex differences were found [Sex, $F_{(1,24)}$=1.59, p=.220, η^2=.004; Interval x Sex, $F_{(1,24)}$=.71, p=.408, η^2=.001; Session x Sex, $F_{(1.98, 47.39)}$=0.65, p=.525, η^2=.002; Context x Sex, $F_{(1,24}$ =0.03, p=.876, η^2<.001; Interval x Session x Sex, $F_{(1.89, 45.35)}$=0.63, p=.531, η^2=.002; Interval x Context x Sex, $F_{(1,24)}$=0.06, p=.807, η^2<.001; Interval x Context x Session x Sex, $F_{(1.92, 46.11)}$=1.30, p=.283, η^2=.005]. (**B**) Mean (± SEM) total latency (s) to initiate port entries during the CS in the alcohol and neutral contexts. The total latency to initiate a CS port entry varied as a function of session [Session, $F_{(1.57, 37.57)}$=36.44, p<.001, η^2=.284] and was greater in the alcohol context than in the neutral context [Context, $F_{(1,24)}$=25.64, p<.001, η^2=.068] with no difference across sessions [Context x Session, $F_{(2.06, 49.47)}$=0.26, p=.779, η^2=.002]. No sex differences were found [Sex, $F_{(1,24)}$=0.17, p=.682, η^2=.007; Session x Sex, $F_{(1.57, 37.57)}$=2.67, p=.094, η^2=.021; Context x Session x Sex, $F_{(2.06, 49.47)}$=1.27, p=.291, η^2=.011]. **(C)** Mean (± SEM) total duration of port entries initiated during the CS in the alcohol and neutral contexts. The total duration of CS port entries varied across sessions [Session, $F_{(1.63, 39.23)}$=34.42, p<.001, η^2=.304] and was greater in the alcohol context than in the neutral context [Context, $F_{(1,24)}$=21.21, p<.001, η^2=.066]. The two-way Context x Session interaction was not significant [$F_{(1.99, 47.83)}$=1.40, p=.256, η^2=.012]. No sex differences were found [Sex, $F_{(1,24)}$=1.29, p=.267, η^2=.005; Session x Sex, $F_{(1.63, 39.23)}$=0.79, p=.438, η^2=.007; Context x Sex, $F_{(1,24)}$=0.10, p=.757, η^2<.001; Context x Session x Sex, $F_{(1.99, 47.83)}$=2.32, p=.110, η^2=.020].

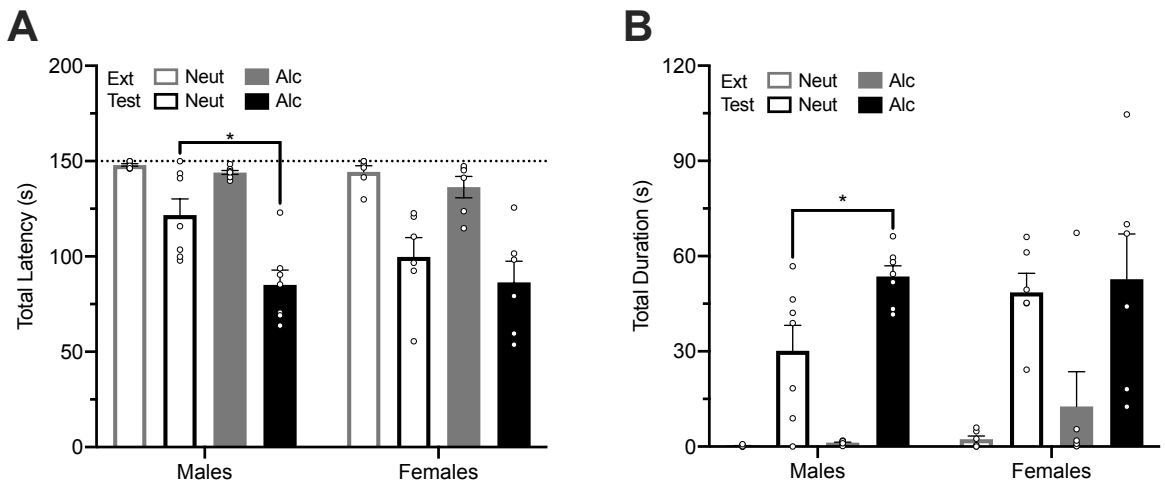

Figure S4. Port entries in the neutral context (empty bars) and alcohol context (filled bars) during alcohol-primed reinstatement test (black) compared to the extinction baseline (average of last two extinction sessions (grey), in males (left panels) and females (right panels). Circles depict data from individual rats. *$p < 0.05$. **(A)** Mean (± SEM) total latency to initiate port-entries following CS onset was shorter in the test than the extinction baseline and was shorter in the alcohol context compared to the neutral context in males, but not in females. Overall total latency was shorter during the test compared to the extinction baseline [Phase, $F_{(1,22)}$=105.11, p<.001, η^2=.570], was shorter in the alcohol context compared to the neutral context [Context, $F_{(1,22)}$=8.28, p=.009, η^2=.067; Phase x Context, $F_{(1,22)}$=4.69, p=.041, η^2=.025]. No sex differences were found [Sex, $F_{(1,22)}$=2.22, p=.151, η^2=.018; Phase x Sex, $F_{(1,22)}$=0.29, p=.593, η^2=.002; Context x Sex, $F_{(1,22)}$=0.79, p=.384, η^2=.024; Context x Phase x Sex, $F_{(1,22)}$=2.41, p=.135, η^2=.013]. However, planned comparisons indicated that the total latency to initiate CS port-entries at test was shorter in the alcohol context (M = 85.15 ± SE = 7.66) than the neutral context (M = 121.71 ± SE = 8.53) in males [$t_{(12)}$=3.19, p=.008, d=1.71, 95% CI (0.35, 2.80)]. In contrast, the total latency to initiate CS port-entries at test did not differ between the alcohol (M = 86.35 ± SE = 11.19) and the neutral (M = 99.74 ± SE = 10.17) contexts in females [$t_{(10)}$=0.89, p=.397, d=0.511, 95% CI (-0.69,1.61)]. **(B)** Mean (± SEM) total duration of port-entries initiated during the CS was greater during the test compared to the extinction baseline and was greater in the alcohol context than in the neutral context in males, but not females. Overall, the total duration of CS port-entries at test was greater than at the extinction baseline [Phase, $F_{(1,22)}$=93.30, p<.001, η^2=.630]. No other effects were found [Context, $F_{(1,22)}$=0.79, p=.383, η^2=.007; Phase x Context, $F_{(1,22)}$=0.21, p=.650, η^2=.001; Sex,

$F_{(1,22)}$=0.91, p=.350, η^2=.008; Phase x Sex, $F_{(1,22)}$=0.06, p=.806, η^2<.001; Context x Sex, $F_{(1,22)}$=0.07, p=.790, η^2<.001; Phase x Context x Sex, $F_{(1,22)}$=0.77, p=.389, η^2=.005]. Planned comparisons revealed that the total duration of CS port-entries at test were greater in the alcohol context (M = 53.59 ± SE = 3.34) relative to the neutral context (M = 30.21 ± SE = 8.01) in males [$t_{(8.03)}$=-2.70, p=.020, d=-1.44, 95% CI (-2.55, -0.09)]. In females, the total duration of CS port-entries at test was similar in both the alcohol (M = 52.76 ± SE = 14.25) and neutral (M = 48.57 ± SE = 6.00) contexts [$t_{(10)}$=-0.27, p=.792, d=-0.16, 95% CI (-1.29, 0.98)].

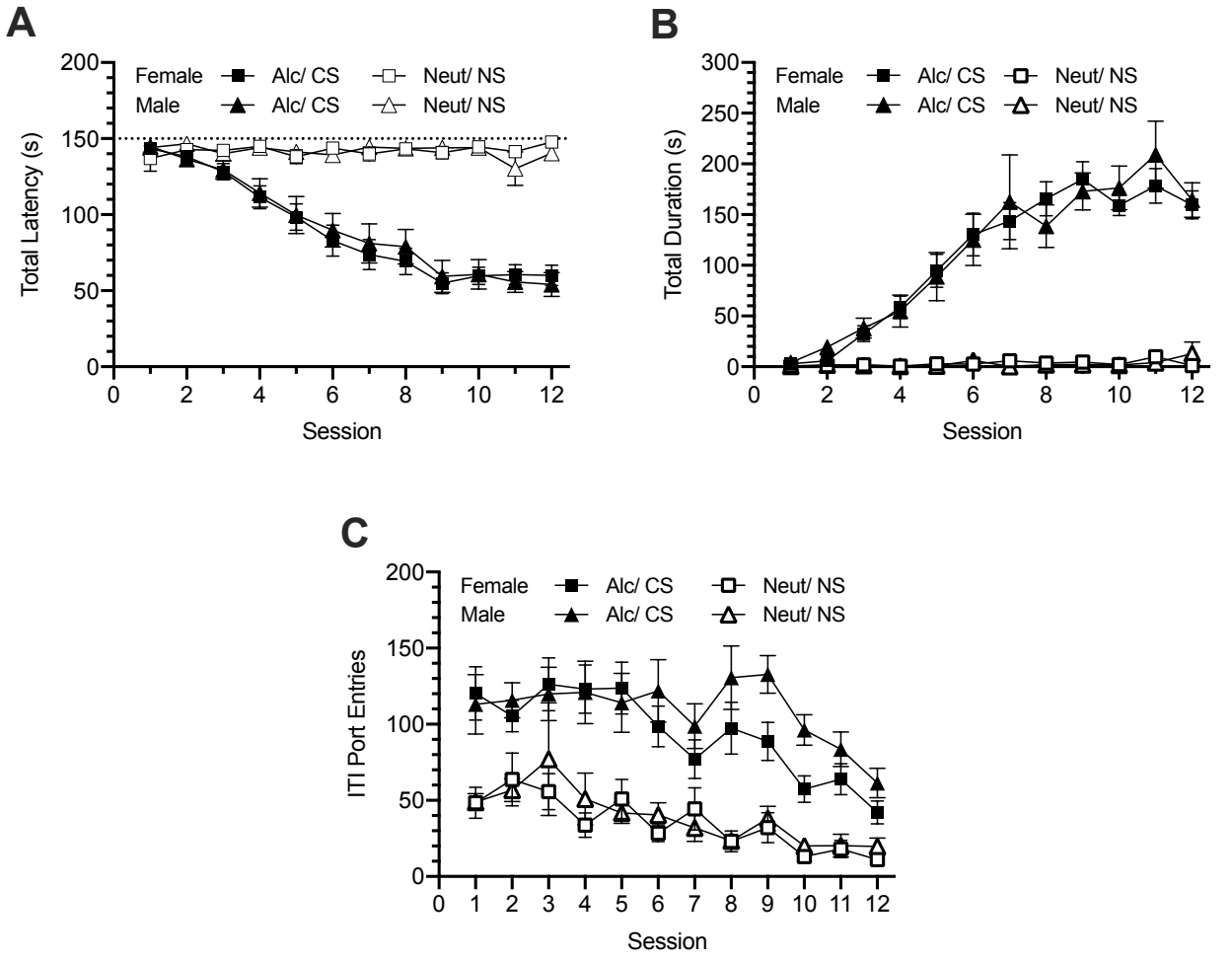

Figure S5. Acquisition of Pavlovian discrimination training in the alcohol context (filled shapes) and neutral context (empty shapes) in males (triangles) and females (squares). **(A)** Mean (± SEM) total latency (s) to initiate port entries during the CS or NS interval. The total latency to initiate a port-entry after stimulus onset was shorter for the CS in the alcohol context compared to the NS in the neutral context in both males and females. Overall the total latency to initiate a CS or NS port-entry varied as a function of session [Session, $F_{(4.78,\ 123.31)}$=44.96, p<.001, η^2=.149] in the alcohol context but not in the neutral context [Context, $F_{(1,26)}$=197.54, p<.001, η^2=.388; Context x Session, $F_{(5.30,\ 137.78)}$=49.70, p<.001, η^2=.146]. Follow-up paired samples t-tests collapsed across sex revealed that the latency to make a CS port-entry in the alcohol context decreased from session 1 (M=144.15 ± SE=1.33) to session 12 (M=57.34 ± SE=5.05) [$t_{(27)}$=17.59, p < .001, d=3.32, 95% CI (2.36, 4.28)], whereas latency to make a port-entry following NS onset in the neutral context remained stable and high [$t_{(27)}$=-0.87, p=.393, d=-.16, 95% CI (-0.54, 0.21)] from session 1 (M

=140.25 ± *SE* = 4.50) to session 12 (*M* = 144.35 ± *SE* = 1.32). No differences across sex were found [Sex, $F_{(1,26)}$=0.03, *p*=.872, η^2<*.001*; Session x Sex, $F_{(4.78,\ 124.31)}$=0.76, *p*=.576, η^2=.003; Context x Sex, $F_{(1,26)}$=0.11, *p*=.749, η^2<.001; Context x Session x Sex, $F_{(5.30,\ 137.78)}$=0.32, *p*=.912, η^2<.001]. **(B)** Mean (± SEM) total duration of port entries initiated during the CS or NS interval. The total duration of port-entries initiated during the CS was longer and increased over time in the alcohol context, while the total duration of port-entries initiated during the NS in the neutral context remained long, in both males and females. Overall, total duration of port-entries initiated during the CS or NS varied across sessions [Session, $F_{(3.77,\ 97.92)}$=47.04, *p*<.001, η^2=.151], was greater in the alcohol context than in the neutral context [Context, $F_{(1,26)}$=159.20, *p*<.001, η^2=.397] and varied as a function of session in the alcohol context but not in the neutral context [Context x Session, $F_{(3.76,\ 97.76)}$=43.59, *p*<.001, η^2=.138]. Follow-up paired samples t-tests collapsed across sex revealed that the total duration of CS port-entries in the alcohol context increased from session 1 (*M* = 3.45 ± *SE* = 1,13) to session 12 (*M* = 161.80 ± *SE* = 10.63) [$t_{(27)}$=-15.12, *p*<.001, *d*=-2.86, 95% CI (-3.69, -2.01)], while the total duration of NS port-entries in the neutral context remained low [$t_{(27)}$=-1.14, *p*=.264, d=-0.22, 95% CI (-0.59, 0.16)] from session 1 (*M* = 0.50 ± *SE* = .14) to 12 (*M* = 6.61 ± *SE* = 5.38). No differences across sex were found [Sex; $F_{(1,26)}$=0.02, *p*=.877, η^2<.001; Context x Sex, $F_{(1,26)}$=0.04, *p*=.849, η^2<.001; Context x Session x Sex, $F_{(3.76,\ 97.76)}$=0.72, *p*=.575, η^2=.002]. **(C)** Mean (± SEM) port entries produced during the ITI interval. ITI port entries varied as a function of session [Session, $F_{(4.31,\ 112.09)}$=8.85, *p* < .001, η^2=.083] and were greater in the alcohol context than the neutral context [Context, $F_{(1,26)}$ = 158.38, *p* < .001, η^2=.270; Context x Session; $F_{(6.74,\ 175.16)}$=2.36, *p*=.027, η^2=.014]. No sex differences were found [Context x Sex; $F_{(1,26)}$=1.28, *p*=.269, η^2=.002, Session x Sex; $F_{(4.31,\ 112.09)}$=0.69, *p*=.613, η^2=.006, Context x Session x Sex; $F_{(6.74,\ 175.16)}$=1.15, *p*=.337, η^2=.007, Sex; $F_{(1,26)}$=0.96, *p*=.335, η^2=.006].

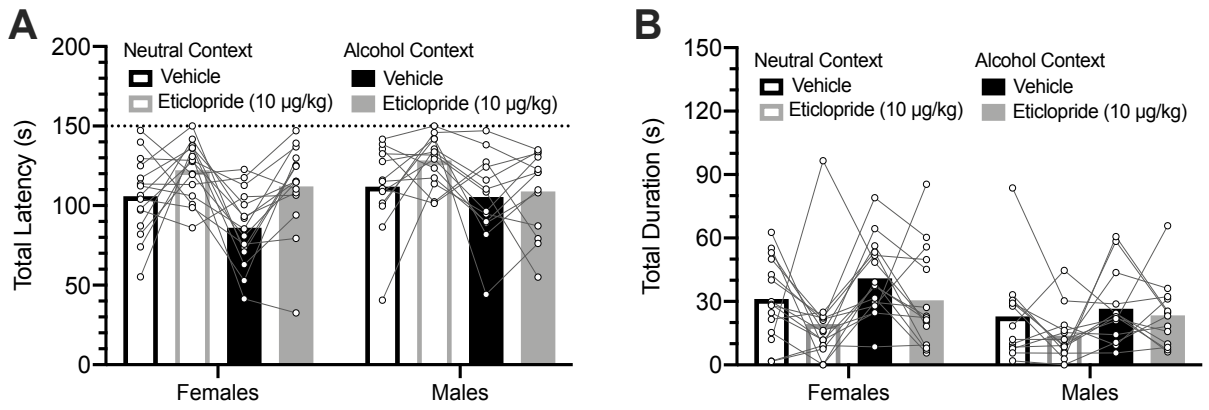

Figure S6. Port entries at test following the systemic administration of vehicle (black bars) or dopamine D2-like receptor antagonist eticlopride (grey bars), in the neutral context (open bars) and alcohol context (filled bars) in the absence of ethanol delivery, in males and females. Circles depict data from individual rats. **(A)** Mean total latency to initiate port-entries after CS onset was shorter in the alcohol context compared to the neutral context and was longer in the drug group compared to vehicle, in males and females. Overall latency varied across context [Context, $F_{(1,26)}$=9.01, p=.006, η^2=.073] and drug groups [Drug, $F_{(1,26)}$=16.28, p<.001, η^2=.088]. No other interactions or sex differences were found [Context x Drug, $F_{(1,26)}$=0.04, p=.847, η^2=.012; Sex, $F_{(1,26)}$=1.64, p=.212, η^2=.018; Context x Sex, $F_{(1,26)}$=0.04, p=.838, η^2<.001; Drug x Sex, $F_{(1,26)}$=2.13, p=.156, η^2=.012; Context x Drug x Sex, $F_{(1,26)}$=1.96, p=.173, η^2=.012]. **(B)** Mean total duration of the port-entries initiated during the CS was longer in the alcohol context compared to the neutral context, and was shorter in the drug group compared to vehicle, in males and females Overall, the total duration of CS port-entries varied across context [Context, $F_{(1,26)}$=7.04, p=.013, η^2=.045] and drug [Drug, $F_{(1,26)}$=6.41, p=.018, η^2=.046]. No other interactions or sex differences were found [Context x Drug, $F_{(1,26)}$=0.27, p=.610, η^2=.002; Sex, $F_{(1,26)}$=4.28, p=.049, η^2=.048; Context x Sex, $F_{(1,26)}$=0.36, p=.555, η^2=.002; Drug x Sex, $F_{(1,26)}$=0.52, p=.476, η^2=.004; Context x Drug x Sex, $F_{(1,26)}$=0.09, p=.761, η^2<.001].