The Psychological and Neural Mechanisms Underlying a New Model of Reinstatement of Responding to an Alcohol-Predictive Cue

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ABSTRACT

The Psychological and Neural Mechanisms Underlying a New Model of Reinstatement of Responding to an Alcohol-Predictive Cue

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Environmental stimuli that predict alcohol availability pose a significant threat to maintaining abstinence from alcohol use. Through Pavlovian conditioning, these stimuli can become cues that predict alcohol availability which can precipitate relapse in clinical populations and preclinical models. Preclinical research has largely focused on examining the immediate impact of alcohol-cues on the relapse-like return of responding for alcohol (i.e., reinstatement); however, the delayed impact of such cues on behaviour has rarely been investigated. In the current thesis, a new reinstatement model was developed to evaluate the delayed impact of re-exposure to alcohol on the return of responding for alcohol. The psychological and neural mechanisms that underlie delayed reinstatement in the new model were then investigated.

A Pavlovian conditioning task was used in which rats learned to respond to a conditioned stimulus (CS) that was paired with alcohol, followed by extinction of this response. Re-exposure to alcohol reinstated responding to the CS 24 h later, relative to responding during extinction. Additional procedures demonstrated that re-exposure to alcohol, and not another liquid made distinct from alcohol, reinstated responding to the alcohol-CS, indicating that preferential reinstatement was produced by re-exposure to alcohol compared to a control liquid.

Behavioural studies revealed that the delayed reinstatement of responding to the alcohol-CS was driven by an association that formed between alcohol and the context in which alcohol re-exposure was conducted. Reinstatement was prevented when the context that alcohol re-exposure occurred in was extinguished. Moreover, reinstatement was reduced when alcohol re-exposure was conducted in a context that differed from the test context.

Pharmacology studies revealed that μ-opioid receptors (MORs) are necessary for the delayed reinstatement of responding to the alcohol-CS. Systemically blocking MORs attenuated reinstatement, without affecting locomotor activity. Further, it was shown for the first time that

blocking MORs in the ventral hippocampus prevented reinstatement.

The novel delayed reinstatement model presented in this thesis helps establish a comprehensive understanding of how alcohol-cues can influence relapse. Moreover, a detailed understanding of the psychological and neural mechanisms underlying the delayed reinstatement model can inform the development of new behavioural and pharmacological treatment interventions against relapse.

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CONTRIBUTION OF AUTHORS

Chapter 2

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Mandy Rita LeCocq Contributed to all experiments, including conceptualizing all experimental designs, conducting Experiments 1, 3, and 4, supervising the undergraduate students conducting Experiments 2 and 4, administering injections, collecting blood samples, conducting blood alcohol concentration assays, analyzing data, and preparing figures.

Soraya Lahlou Contributed to Experiments 2 and 4, including conceptualizing experimental designs, conducting two behavioural experiments, collecting blood samples, and conducting blood alcohol concentrations.

Melanie Chahine Contributed to Experiment 4, including conducting a behavioural experiment, collecting blood samples, and conducting blood alcohol concentrations.

Lorraine Nadine Padillo Conducted a behavioural experiment included in supplementary materials.

Nadia Chaudhri Contributed to all experiments, including conceptualizing all experimental designs, and aiding in the analysis strategy and figure design.

Chapter 3

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Nadia Chaudhri Contributed to all experiments, including conceptualizing all experimental designs, and aiding in the analysis strategy and figure design.

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Nadia Chaudhri Contributed to all experiments, including conceptualizing all experimental designs, and aiding in the analysis strategy and figure design.

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List of Abbreviations

ANOVA..... analysis of variance
AP..... anterior-posterior
BLA..... basolateral amygdala
CS..... conditioned stimulus
CTAP..... D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
CTOP..... D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2
dHipp..... dorsal hippocampus
DV..... dorsal-ventral
g..... gram
GABA..... γ-Aminobutyric acid
h..... hour
ITI ..... intertrial interval
kg..... kilogram
mg..... milligram
ML..... medial-lateral
ml..... millilitre
MOR.....μ-opioid receptor
NAc.....nucleus accumbens
s..... seconds
US..... unconditioned stimulus
VP..... ventral pallidum
vHipp..... ventral hippocampus
VTA..... ventral tegmental area
µg..... microgram
µl..... microlitre

Chapter 1: General Introduction

1.1. Overview

Alcohol use permeates everyday life in a substantial portion of the world[1]. Many people enjoy a glass of their favourite alcoholic drink casually with a meal, to celebrate an achievement, or to relax after a stressful workday. For some individuals, however, the casual use of alcohol can develop into an alcohol use disorder (AUD). The diverse environmental stimuli that are present during periods of alcohol drinking can become alcohol-predictive cues that have particularly strong control over behaviour, such that they can evoke the craving to drink alcohol[2,3]. Importantly, the magnitude of this cue-evoked craving can predict the likelihood of relapsing in the future, thus demonstrating the important link between cue-evoked responses and propensity to relapse[4]. A major focus of preclinical research has been to model such real-world phenomena. The resulting body of research has similarly demonstrated that re-exposure to alcohol-predictive cues and alcohol itself can trigger a relapse-like return of behaviour[5]. These models highlight the damaging impact of these triggering stimuli on relapse-like behaviour; however, little is known about their long-term impact on behaviour. For example, questions like "would drinking one alcoholic beverage increase the risk for an alcohol-abstinent person to experience a full relapse in the future?" have, until now, remained unanswered. The aim of the current thesis is to develop a new animal model of relapse to understand the delayed impact of re-exposure to alcohol on the diminished responding for alcohol, and to identify the psychological and neural mechanisms that contribute to this effect.

1.2. Alcohol Use Disorder in Humans

1.2.1 The Alcohol Use 'Problem'

In 2012, 18.1% of the Canadian population aged 15 years and older met the criteria for an AUD at some point in their lifetime[6]. To put that percentage into perspective, that is approximately 6,700,000 people. It is evident that, despite the current culture of recreational alcohol use, AUD is rampant. AUD is clinically defined by a pattern of behavioural and physical symptoms, such as alcohol tolerance, withdrawal, and craving. Another cornerstone symptom is the failed attempts to control alcohol use[7]. Many people – up to 65%[8] – attempting to remain abstinent from alcohol use experience a chronic cycle of periods of abstinence followed by relapse, then a return to problematic use[9].

Relapse episodes occur despite the far-reaching negative consequences associated with AUD such as loss of employment, damaged personal relationships, and in certain cases even death[1]. Beyond the consequences for the individual, AUD also has detrimental effects on society. In just 2017, the government of Canada spent roughly 16 billion dollars on alcohol-related healthcare, loss of job productivity, and criminal justice[10]. Given the individual and societal strain that AUD poses, there have been tremendous efforts to develop a variety of psychological[11,12] and pharmacological[13] treatment interventions. However, regardless of the treatment interventions available, rates of relapse to alcohol use in treatment-seeking individuals remain high. Nearly 65% of patients receiving psychological or pharmacological treatment for AUD relapsed to alcohol use at a 1-year follow-up[8]. This high prevalence of relapse, despite receiving treatment, implies that our current understanding of the mechanisms that drive AUD and relapse is incomplete.

1.2.2. Pavlovian Conditioning in Alcohol Use

One theory that imbues research on drugs of abuse is that environmental stimuli that are reliably present during drug use can eventually predict drug availability. These drug-predictive cues can gain the incentive motivational properties of the drug, and thus can promote continued drug use and relapse[14]. This capacity for drug-predictive cues to influence behaviour has widely been interpreted using a Pavlovian conditioning framework. In traditional Pavlovian conditioning paradigms[15], an unconditioned stimulus (US; e.g., an electrical foot-shock) elicits an unconditioned response (e.g., freezing). The US is paired with the presentation of an otherwise neutral stimulus (NS; e.g., clicker tone) which does not elicit any response. After repeated pairings with the US, the NS becomes a conditioned stimulus (CS), as it can evoke the freezing behaviour in the absence of the US, which is a conditioned response. Support for a similar associative learning process occurring with alcohol as an unconditioned stimulus has taken hold.

Pioneering research identified a collection of real-world environmental stimuli that were commonly associated with alcohol use. When asked to identify the "bells which trigger a craving for alcohol", alcohol-dependent participants largely reported environmental stimuli that were presented in close temporal proximity to drinking alcohol (e.g., the smell of beverage, wine list)[16]. Such environmental stimuli are commonly referred to as 'discrete cues'[17,18]. The effects of discrete alcohol-cues on human behaviour have since been systematically studied. Using a cue-

reactivity paradigm, alcohol-dependent participants are exposed to naturalistic alcohol-cues (e.g., smell, taste, picture of alcohol), and the conditioned response or 'reactivity' to the cue is measured. Such exposure to alcohol-cues reliably elicits psychological reactivity, such as craving for alcohol[19–21], as well as physiological reactivity, including increased skin conductance[22], heart rate[19,23], and salivation[20,21]. Interestingly, when alcohol was repeatedly delivered in a distinctly coloured and flavoured liquid, subsequent presentations of that liquid without alcohol induced greater craving for alcohol, skin conductance, and heart rate relative to a neutral liquid, in non-dependent participants[24]. Therefore, both naturalistic and artificial alcohol-cues can evoke reactivity.

Cue reactivity can have deleterious effects on individuals recovering from AUD. Within the Pavlovian conditioning framework, one would predict that exposure to an alcohol-predictive cue would evoke a conditioned response such as craving and consequently facilitate relapse. This relationship between cue reactivity and relapse has been reported, such that alcohol-dependent individuals that exhibited greater salivation triggered by the scent and sight of alcohol had greater levels of alcohol intake three months later at a follow-up[25]. Furthermore, greater craving for alcohol elicited by a personalized alcohol-predictive cue predicted a shorter time to relapse in treatment-seeking patients. This latter effect was so robust that every one-point increase in cue-evoked craving increased the likelihood of relapse by 16%[4]. Thus, it is evident that cue reactivity is closely linked to the propensity to relapse to alcohol use.

The relationship between cue reactivity and relapse to alcohol use is a promising line of research to better understand the mechanisms that precipitate relapse. Thus far, only a correlational relationship between cue reactivity and relapse has been established in humans. The ability to establish a causal relationship is limited by ethics and self-report data, which is an inherent limitation of research involving human participants[26]. Alternatively, animal models of relapse can determine causal links between alcohol-predictive cues and relapse-like behaviour. Moreover, animal models provide a way to precisely target and manipulate psychological and neural processes to better understand the mechanisms that underlie such relapse-like behaviour.

1.3. Animal Models of Relapse to Alcohol Use

1.3.1. Operant Models of Reinstatement

Various animal models have been developed to examine the relapse-like return of drug-

seeking behaviour[27]. These models are commonly used with operant conditioning tasks, in which a rodent must perform a specific response to self-administer a drug, and the continuation of this response in the absence of the drug is referred to as drug-seeking. The 'reinstatement model' is a particularly robust model that assesses the impact of various triggering stimuli on the return – or reinstatement – of drug-seeking[*]. Two distinct stimuli that are commonly used to elicit reinstatement are a discrete drug cue, and a priming dose of the drug, known as cue-induced and priming-induced reinstatement, respectively[28]. The reinstatement of operant drug-seeking produced by these models is used as a proxy to infer relapse-like behaviour.

The cue-induced reinstatement procedure demonstrates how discrete cues in one's environment can provoke relapse. In one of the earliest investigations of cue-induced reinstatement of drug-seeking, rodents learned an operant lever-pressing response to obtain delivery of morphine that was paired with the presentation of a discrete auditory cue. The response was then extinguished as lever pressing no longer resulted in the delivery of morphine or the discrete cue. Interestingly, reintroducing the discrete cue that was previously paired with morphine reinstated the extinguished drug-seeking response, despite the absence of response-contingent morphine delivery[29]. This cue-induced reinstatement procedure provided a foundation from which cue-evoked relapse-like behaviour could be assessed using various drugs of abuse, including alcohol. Similarly, reintroducing a discrete cue that was present during alcohol delivery reinstated both a lever pressing and nose poking response in rodents[30,31].

Priming-induced reinstatement, on the other hand, models how re-exposure to the drug itself can elicit relapse. For example, after the acquisition and extinction of a lever-pressing response for cocaine, rats that received a non-response-contingent priming dose of cocaine showed a reinstatement of the extinguished drug-seeking response[32]. Thus, the pharmacological effect of a drug, in the absence of discrete cues or an operant response, is sufficient to reinstate drug-seeking behaviour. This effect has also been demonstrated with alcohol, in which the priming dose of alcohol was delivered either by injection or oral gavage [33,34]. These priming doses of alcohol are sufficient to produce detectable levels of alcohol in the blood; thus, the pharmacological effects of alcohol can also reinstate the extinguished alcohol-seeking response.

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[*] Other animal models evaluate different methods to recover extinguished drug-seeking behaviour. The spontaneous recovery model delivers a passage of time between extinction and a test session[249]. The renewal model, also known as the context-induced reinstatement model, delivers a drug-associated context after extinction and is discussed in greater detail in subsequent sections. Both models evoke a return of diminished drug-seeking behaviours for a variety of drugs[27].

A unique aspect of alcohol is that when orally ingested, the orosensory properties (e.g., taste, smell) can act as discrete cues. The orosensory properties experienced from ingesting a small amount of alcohol, and not the pharmacological effects, also reinstate an extinguished lever pressing response for alcohol[35].

The cue-induced and priming-induced reinstatement procedures differ in terms of the modality of the alcohol-cues presented, the external environmental cues, or innate pharmacological and orosensory effects, respectively. Taken together, the reinstatement model illustrates that exposure to different drug cues can produce motivational states that influence behaviour, such as a relapse-like return of drug-seeking.

1.3.2. Pavlovian Models of Reinstatement

Reinstatement of responding for alcohol has, overwhelmingly, been studied using operant conditioning tasks in which the impact of alcohol-predictive cues is observed through an operant response[5]. While this technique has produced valuable findings, there are caveats. Notably, it is difficult to dissociate the contributions of Pavlovian conditioned responding from operant responding in the relapse-like return of behaviour. In operant tasks, rodents learn to perform an operant response, like lever pressing, to obtain delivery of alcohol which is paired with a stimulus such as a discrete cue[30,36] or discriminative stimulus complex[36]. Then, during extinction, the lever pressing response no longer results in alcohol delivery, nor are the alcohol-predictive cues presented. During this extinction training, the operant response for alcohol (i.e., Response-Outcome contingency) is extinguished, as well as the operant response paired with the presence of alcohol-cue (i.e., Response-Stimulus contingency). However, the association between the alcohol-cue and alcohol (i.e., Stimulus-Outcome contingency) is not explicitly extinguished[37]. Therefore, when the reinstating stimulus (i.e., discrete cue or discriminative stimulus) is reintroduced during the subsequent reinstatement test, the influence of this alcohol-cue on the return of the extinguished operant response for alcohol is examined. However, the reinstatement of the Pavlovian conditioned response to the alcohol-cue is not assessed, as it was never extinguished[27]. Thus, reinstatement models that use operant tasks do not provide clear insight into the relapse-like return of conditioning responding to alcohol-cues. This is an important distinction, because conditioned responses to alcohol-cues that have been systematically extinguished can return, and may contribute to relapse to alcohol use[38,39].

Reinstatement models that use Pavlovian conditioning tasks control for this caveat because the rodents do not have to perform an operant response to obtain alcohol at any point in the procedure. They are presented with CS-alcohol pairings throughout the acquisition of the task, followed by presentations of solely the CS during extinction[40,41]. The reinstating stimulus such as a non-response-contingent presentation of the scent of alcohol[40] or a small priming dose of ingested alcohol[41,42], is then delivered pseudorandomly, which reinstates extinguished responding to the alcohol-CS. Such tasks allow researchers to delineate the unique contribution of Pavlovian conditioned responding in the reinstatement of responding for alcohol. This assessment ultimately allows for a parsimonious assessment of the psychological and neural mechanisms underlying reinstatement evoked by alcohol-cues.

1.3.3. The Need for New Reinstatement Models

The established reinstatement models, using either operant or Pavlovian conditioning tasks, capture the capacity for alcohol-predictive cues to precipitate relapse through an animal analog. These models, however, typically only assess the immediate impact of drug cues on conditioned responding. The reinstating stimuli, like an alcohol-cue or a priming dose of alcohol, are presented and the impact on behaviour is immediately assessed[30,33]. Thus, these models provide insight into how alcohol-predictive cues to immediately precipitate relapse. Conversely, little is known about the delayed impact that reinstating stimuli has on relapse-like behaviour. For example, the capacity for the scent or taste of alcohol to reinstate responding to an extinguished alcohol-CS one or two days later is unknown.

The information gained from a model that evaluates the delayed impact of reinstating stimuli on conditioned responding is incredibly important because it would demonstrate how a lapse in alcohol use could influence relapse-like behaviour in the future. Such a relationship between a lapse in drug use and the risk of future relapse has been determined in nicotine-dependent individuals. A lapse in smoking, defined as a return to smoking tobacco for less than seven consecutive days, allowed participants to experience tobacco-predictive cues like the pharmacological effects and the orosensory properties of smoking. This lapse in smoking was a powerful predictor of future relapse, which was defined as smoking for seven or more consecutive days[43]. In fact, up to 70% of participants who experienced a lapse within 8 weeks of quitting had fully relapsed 4 months later. This is in stark contrast to a 30% relapse rate in

participants who did not experience a lapse[43]. Thus, transient drug use appears to be detrimental to the risk of relapse at a future timepoint. The lack of animal models investigating this effect necessitates the development of a novel relapse model.

An important reason for developing new animal models of relapse is to improve the face validity of these models[44]. A new reinstatement model, that evaluates the impact of a lapse in sobriety on relapse-like alcohol-seeking at a future timepoint, would complement the traditional reinstatement model by capturing a different aspect of human addiction, like the likelihood of a person relapsing days after having just one alcoholic beverage. Moreover, this new reinstatement model could elucidate unique psychological and neural mechanisms that contribute to relapse, which would ultimately inform the development of new therapeutic and pharmacological treatment interventions.

1.4. How Alcohol-Associated Contexts Contribute to Responding for Alcohol

Contexts in which alcohol use routinely occurs can also become conditioned stimuli that predict alcohol availability. Contexts can be defined as physical environments that are comprised of a constellation of stimuli that are present in the background, or a temporally distal manner, to an alcohol drinking event[17]. For example, a bar is a context in which a distinct set of smells, sounds and/or music, and visual décor are present. Exposure to such alcohol-predictive contexts encourages greater salivation, urge to drink, and more time spent in that context, relative to a neutral context in non-dependent individuals[38,45]. Contexts can also be interoceptive states such as a drug state (e.g., feeling 'buzzed'), anxiety, or malaise that exist in the background of alcohol drinking events and become associated with alcohol[46]. Accordingly, people report both physical and interoceptive contexts associated with alcohol use as common triggers for craving[16]. Thus, like discrete cues, alcohol-predictive contexts can be powerful triggers for relapse.

1.4.1. Contextual Control Over Conditioned Responding for Alcohol

Animal models have captured the ability for drug-predictive contexts to influence behaviour. For example, rodents will readily develop conditioned place preference (CPP) to a distinct context that is associated with alcohol delivery. When given the choice, rodents will spend significantly more time in the context where alcohol was delivered over a neutral

context[†47,48]. Recently established models have demonstrated that alcohol-predictive contexts can also influence responding to discrete alcohol-cues. This has been elegantly demonstrated by the Pavlovian conditioning with context alternation task. This task reveals that an alcohol-CS presented in a neutral context evokes a baseline level of conditioned responding to the CS; however, presenting the CS in the alcohol-predictive context elevates this responding, illustrating an additive effect of an alcohol-associated context to invigorate responding to a discrete cue[42,49].

Drug-predictive contexts can similarly restore extinguished drug-seeking. This has been demonstrated using the renewal model, also known as context-induced reinstatement, based on a procedure established in the learning and memory field[50]. The renewal of drug-seeking was first demonstrated by Crombag and colleagues[51], in which rats learned to press a lever to obtain a heroin and cocaine mixture, referred to as a 'speedball'. This acquisition of drug self-administration occurred in a context ('A') that was comprised of distinct tactile, olfactory, and visual stimuli. Extinction of the response was then conducted in a separate context ('B') that was comprised of different stimuli and resulted in low levels of lever pressing. However, when returned to the initial acquisition context ('A'), the lever-pressing response was elevated in the absence of the drug delivery, indicating that the drug-predictive context was sufficient to prompt the relapse-like return of drug-seeking.

Renewal of responding for alcohol has also been reliably produced with operant[52] and Pavlovian[53,54] conditioning tasks in rodents. Interestingly, a modified procedure has demonstrated renewal of operant alcohol-seeking following a punishment-based abstinent period rather than extinction. After the acquisition of a lever pressing response for alcohol, a new contingency was introduced: lever pressing resulted in the delivery of both alcohol and a foot-shock, which produced a voluntary reduction in lever pressing. When the acquisition and the punishment-based abstinence period were conducted in different contexts, a return to the acquisition context renewed the lever pressing response in the absence of alcohol, while remaining in the punishment context did not[55]. This modified renewal procedure captures the voluntary reduction in alcohol use due to negative outcomes which humans often experience, an aspect that forced periods of abstinence like extinction procedures do not account for. Importantly, the capacity for alcohol-predictive contexts to renew responding for alcohol that was diminished by either forced or

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† It is important to note that conditioned place preference procedures are highly sensitive to the dose of alcohol administered. Higher doses of alcohol have the opposite effect, and conditioned place aversion can develop[250].

voluntary abstinence highlights the considerable influence that contexts have on relapse.

1.4.2. The Role of Context in the Reinstatement of Conditioned Responding

The capacity for contexts to exert control over an extinguished conditioned response has additionally been demonstrated with a unique reinstatement model developed in the learning and memory field. In this procedure, rats learned to associate a CS with a shock-US, followed by extinction of the conditioned fear response as the CS was presented in the absence of the US. When unsignalled re-exposure to the shock-US was delivered, conditioned responding was reinstated *24 hours later* in the presence of the CS[50,56]. This reinstatement model differs significantly from those commonly used in addiction research as the triggering stimulus, re-exposure to the US, is presented 24 hours before and not immediately before testing for reinstatement of responding.

The unique reinstatement of responding that is evoked 24 hours after US re-exposure is proposed to be driven by two separate associative processes that involve context: 1) US conditioning of the context, and 2) context-mediated CS-US conditioning. Early investigations into the psychological mechanisms that drive reinstatement discovered that a context-US association was required for reinstatement to occur. When the context that re-exposure to the US occurred in was extinguished, via repeated context exposure without US delivery, reinstatement did not occur[50]. Similarly, when US re-exposure was conducted in a novel context, and reinstatement was tested for in the initial training context, reinstatement did not occur[50,56]. These findings provide strong evidence that during US re-exposure an association is formed between the US and the context that the US is delivered in. It is posited that returning to the US-predictive context during the test session returns the subject to either the physical setting or emotional state that was present during the initial acquisition of the CS-US association, and this return evokes reinstatement[57].

However, a second role for context in reinstatement has more recently been proposed. Westbrook and colleagues[56] had similarly trained rats to acquire and extinguish a Pavlovian conditioned fear response. Interestingly, when US re-exposure was conducted in the context that extinction had occurred in, reinstatement of responding to the CS still occurred 24 h later when conducted in a completely novel context. Thus, when US re-exposure is conducted in the extinction context, reinstatement of responding to the CS occurs regardless of the context present

at test. This finding implies the existence of an additional process involved in evoking reinstatement, that of context-mediated conditioning of the CS. It is proposed that during extinction, the CS can become associated with the context, given the absence of the US. This context-CS association would allow exposure to the extinction context to activate a cognitive representation of the CS. Therefore, when returned to the extinction context during US re-exposure, the unsignalled US deliveries would be paired with the representation of the CS, as well as the context. In other words, the representation of the CS becomes reconditioned to the US, mediated through the common extinction context. Thus, the renewed link between the CS and US would reinstate responding to the CS regardless of the context present[57,58].

It is evident that contexts associated with alcohol can exert great control over responding to discrete alcohol-cues, as demonstrated through the Pavlovian conditioning with context alternation task and renewal of alcohol-seeking procedures[42,49,59]. Moreover, a unique reinstatement model has demonstrated additional ways in which context can govern responding to discrete cues, potentially through context conditioning and context mediated conditioning processes. The extent to which similar processes contribute to reinstatement of responding to an alcohol-cue, however, is unknown. Understanding the psychological mechanisms that contribute to relapse – like the associative processes involving contexts – provides invaluable insight into the etiology of human alcohol use.

1.5. The Role of μ-Opioid Receptors in the Reinstatement of Responding to Alcohol-Predictive Cues

AUD is characterized as a chronic, relapsing disorder[7]. The resulting prolonged alcohol use is linked to the perturbation of many neural systems that support the maintenance of problematic alcohol use and the precipitation of relapse[9]. While the neural systems involved are complex, there is evidence that one essential piece of the puzzle is the endogenous opioid system. The opioid system is implicated in all stages of addiction, including initiation, maintenance, craving, and relapse[60].

1.5.1. The Motivational Effects of μ-Opioid Receptors Activation

The opioid system consists of three receptors and three endogenous ligands. The endogenous ligands β-endorphin, enkephalin, and dynorphin exhibit preferential binding for the

mu (μ), delta (δ), and kappa (κ) receptors, respectively[‡][61]. Opioid receptors are inhibitory G-protein coupled receptors (GPCR), meaning that when they are bound with an endogenous or exogenous agonist, the excitability of the cell that they are bound to is reduced, resulting in an overall reduction in activity and neurotransmitter release[61]. Separate opioid receptors are commonly associated with distinct functions, where κ-opioid receptor activation produces aversive states like malaise, and δ- and μ-opioid receptors activation produce rewarding effects[62]. While all opioid receptors play an important role in drug and alcohol use (see Nutt, 2014 for a comprehensive review) the current thesis focuses on the μ-opioid receptor (MOR). We are interested in the neural processes that underlie the reinstatement of responding to alcohol-predictive cues, which most likely involve MORs given their role in reward processes.

The reinforcing effects of MOR activation are evidenced by animal models of self-administration and conditioned place preference[63]. Rodents voluntarily self-administer agonists that display a high affinity for MORs like morphine[64]. Moreover, repeated administration of either β-endorphin[65] or a MOR agonist[66] in a specific experimental context induces conditioned place preference (CPP) over a neutral context. The inverse effects are also demonstrated by blocking MORs with an antagonist like naloxone, such that repeated administration produces conditioned place aversion[66]. Therefore, it appears that reinforcement is mediated by activation of MORs, which is an effect that is particularly important in promoting alcohol use.

The distinct psychological experience of innate 'liking', or the hedonic value, of a substance is also regulated by the opioid system[67]. 'Liking' is commonly assessed using taste reactivity tests, in which either positive or aversive reactions to a substance are evaluated through reactions like rhythmic tongue protrusions and gapes, respectively[68]. Interestingly, morphine and other MOR agonists increase 'liking' reactions to a sucrose solution in rodents[69]. Moreover, this effect occurs through actions in hedonic hotspots located in the nucleus accumbens (NAc) and ventral pallidum (VP), as microinjections of MOR agonists delivered in these brain regions increase the 'liking' of sucrose rewards[70]. The 'liking' reaction has also been demonstrated in response to orally ingested alcohol in rats with a history of drinking alcohol[71], thus, MORs may also be involved in the 'liking', or the hedonic value, of alcohol.

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‡ Endogenous opioid ligands can bind to multiple receptors, however at a lower affinity. For example, the μ-opioid receptor has lower affinity for enkephalins, and the δ-opioid receptor has lower affinity for endorphins[201].

1.5.2. The Role of MORs in Alcohol-Seeking

The initiation of alcohol use is often credited to the reinforcing properties of alcohol. This is strongly supported by animal models, in which rodents voluntarily perform operant tasks to obtain alcohol[72]. These reinforcing effects are, in part, suggested to be attributable to the release of endogenous opioids. For example, both *in vitro* and *in vivo* studies demonstrate that acute alcohol intake in rodents increases levels of β-endorphin in the NAc[73] and ventral tegmental area (VTA), both brain regions that are heavily implicated in reward processing[74]. Similarly, in humans, Positron Emission Tomography (PET) imaging shows that after alcohol intake, there is reduced binding of the inert MOR binding molecule, [11C]carfentanil, in the NAc of both alcohol-dependent and healthy humans, indicating an increased release of endogenous ligands bound to MORs[75]. Conversely, when MOR activation is blocked with an antagonist, thereby reducing the binding of β-endorphins, rodents' alcohol intake is attenuated[76,77]. Not only has this been consistently observed in rodents, one of the few approved pharmacotherapies to treat AUD is the MOR antagonist naltrexone. Naltrexone's efficacy as a pharmacotherapy lies in its capacity to significantly reduce overall alcohol drinking[78] and prolong periods of abstinence[79]. Taken together, these findings lead to conclude that MORs have a significant role in the reinforcing effects of alcohol.

The reinforcing effects of alcohol are essential for the conditioning of environmental stimuli to alcohol. Environmental stimuli that are repeatedly paired with alcohol become alcohol-predictive cues that not only predict alcohol availability, but can also gain incentive salience (i.e., conditioned reinforcing properties). This property allows the cue to motivate conditioned responding, as demonstrated through conditioned reinforcement tasks, in which animals perform an operant task to obtain presentation of the cue in the absence of alcohol[80]. Given the relationship between MOR activity and the reinforcing effects of alcohol, a similar role for MORs in responding evoked by alcohol-predictive cues has been of great research interest. Accordingly, blocking MORs with naltrexone reduces cue-evoked craving for alcohol in human patients[3,81]. A similar reduction in reinstatement of alcohol-seeking evoked by alcohol-predictive cues has been convincingly demonstrated in animal models[34,36,82,83].

1.5.3. The Potential Neural Loci of MORs Involved in Reinstatement

MORs are expressed in brain regions that are highly implicated in associative learning

processes, like the hippocampus, and may be involved in responding to alcohol-cues[84]. Many studies have demonstrated that inhibiting the hippocampus disrupts the formation and expression of conditioned fear[85], and appetitive[86] responses evoked by contextual stimuli. The role of the hippocampus in associative learning has typically been linked to context-dependent learning; however, there is also evidence that the hippocampus is involved in associative learning with discrete cues. For example, inactivation of the hippocampus attenuates a conditioned freezing response to an aversive discrete cue[87] and expression of this response[88]. Given its diverse role in associative learning processes, the hippocampus has been highly implicated in cue-evoked drug-seeking. Moreover, and of importance to the current thesis, autoradiographic mapping of opioid receptors in the rat brain has demonstrated a high density of MOR expression throughout the hippocampus[89,90].

The dorsal subregion of the hippocampus is involved in conditioned responding to drug-predictive cues. Inhibition of this brain region impairs the acquisition and expression of conditioned place preference established with cocaine[91]. Furthermore, inhibition of the dorsal hippocampus (dHipp) attenuates renewal (i.e., context-induced reinstatement) of cocaine-seeking[92] and alcohol-seeking[93]. The converging evidence that MORs populate the dHipp and that this brain region is involved in cue-evoked drug-seeking suggests that this may be a neural locus for MORs that govern responding to alcohol-cues.

Studies have demonstrated, through correlation, that MORs in the dHipp are involved in the reinstatement of alcohol-seeking evoked by various alcohol-cues. Reinstatement induced by a discriminative stimulus increased neuronal activity, as measured by c-Fos expression, in the dHipp. Moreover, systemic naltrexone administration attenuated this reinstatement and reversed the c-Fos expression[94]. Context-induced reinstatement of alcohol-seeking was also associated with increased c-Fos mRNA counts in the dHipp, both of which were reversed following systemic naltrexone treatment[95]. These findings suggest that cue-evoked reinstatement of alcohol-seeking recruits neuronal activity in the dHipp that is attenuated by blocking MORs, implying that this neuronal activity is MOR-dependant. However, directly inhibiting MORs in the dHipp with naloxone did not affect context-induced reinstatement of alcohol-seeking[96]. Thus, although the dHipp *may* be involved in reinstatement, the effects of MORs on reinstatement likely occur by blocking receptors outside of the dorsal hippocampal subregion. Therefore, other subregions of the hippocampus may be more promising regions for this effect.

Burgeoning research has provided evidence for a role of the ventral subregion of the hippocampus in the reinstatement of responding for various drugs of abuse. Functional inactivation of the ventral hippocampus (vHipp) attenuated context-induced reinstatement of cocaine-[97] and heroin-seeking[98]. Additionally, inactivating ventral hippocampal structures also inhibited cue- and prime-induced reinstatement of cocaine-seeking[99,100]. These findings indicate that the vHipp is involved in the reinstatement of drug-seeking that is evoked by a diverse set of cues. Importantly, autoradiographic analyses demonstrate that the vHipp appears to express a greater number of MORs than the dHipp[89].

Taken together, the current literature depicts MORs as a critical component in responding to alcohol-predictive cues. Moreover, there is strong evidence that the vHipp is a neural locus that contains MORs that modulate cue-evoked responding for alcohol. Despite this converging evidence, the recruitment of MORs in the ventral hippocampus in responding for any drug of abuse has not yet been tested. Investigating how MORs in different brain regions contribute to cue-evoked alcohol-seeking is critical as it will further the understanding of diverse neural mechanisms that contribute to this behaviour, which is vital for the development of new pharmacotherapies to treat AUD.

1.6. Thesis Experiment Rationales and Hypotheses

This thesis presents a novel reinstatement model that assesses the delayed impact of re-exposure to alcohol on responding to an alcohol-predictive cue in rats, and an examination of the psychological and neural processes underpinning this delayed reinstatement effect. Experiments in Chapter 2 establish the novel delayed reinstatement model that was used throughout the thesis. Through Pavlovian conditioning, rats learned to associate a CS with the delivery of alcohol that could be ingested. After rats acquired this association, conditioned responding to the CS was extinguished by repeatedly presenting the CS in the absence of alcohol. Next, rats were re-exposed to alcohol in the absence of CS presentation. 24 h later, delayed reinstatement of responding was tested in the absence of alcohol. Additionally, the capacity for re-exposure to i) a water control, ii) a control liquid made more distinct from alcohol, or iii) alcohol delivered by intraperitoneal injection, to evoke reinstatement was examined.

Experiments in Chapter 3 implemented a behavioural approach to determine the psychological processes underlying delayed reinstatement of responding to an alcohol-CS.

Specifically, the involvement of a context-alcohol association in facilitating reinstatement was examined. First, the effects of extinguishing the context-alcohol association formed during alcohol re-exposure on reinstatement were examined. Second, the effects of conducting alcohol re-exposure in a context that differed from the subsequent test context on reinstatement were examined. These two distinct manipulations theoretically would prevent a context-alcohol association from being present at test. Therefore, we predicted that if a context-alcohol association was involved in reinstatement, then reinstatement would be prevented following each behavioural manipulation.

Experiments in Chapter 4 implemented a pharmacological approach to elucidate the neural processes underlying the delayed reinstatement of responding to an alcohol-CS. First, the necessity of μ-opioid receptors (MORs) in the reinstatement of responding to an alcohol-CS, or a sucrose-CS was examined. Rats received systemic administration of the MOR antagonist, naltrexone, before a reinstatement test. Next, the necessity of ventral hippocampal MORs for reinstatement of responding to an alcohol-CS was examined. Rats received localized administration of the MOR antagonist, CTAP, into the ventral hippocampus before a reinstatement test. We predicted that if MORs were involved in reinstatement, then blocking them systemically and specifically in the ventral hippocampus would attenuate reinstatement.

The experiments presented in the current thesis examined the psychological and neural mechanisms underlying a new model of delayed reinstatement of responding to an alcohol-predictive cue. These data validate the relapse model, which illustrates the enduring effects of exposure to alcohol on responding to alcohol-cues. Moreover, these data provide evidence for novel psychological and neural processes involved in reinstatement, and provide new avenues for future research.

Chapter 2: Modeling Relapse to an Alcohol-Predictive Cue in Rats Using a Reinstatement Paradigm

2.1. Abstract

Animal models are critical for studying causal explanations of relapse. Using a Pavlovian conditioning procedure with alcohol, we examined relapse after extinction triggered by either re-exposure to alcohol (reinstatement). Male, Long-Evans rats were acclimated to 15% alcohol in the home-cage using an intermittent- access 2-bottle choice procedure. Next, they received Pavlovian conditioning sessions in which an auditory-conditioned stimulus (CS; 20 second white-noise; 8 trials/session; variable time 240 seconds) was paired with 15% alcohol (0.3 ml/CS; 2.4 ml/session) that was delivered into a fluid port for oral ingestion. In subsequent extinction and test sessions, CS presentations occurred as before, but without alcohol. In experiment 1, exposure to either alcohol or water in the fluid port following extinction reinstated CS-elicited port entries at test 24 hours later. In a follow-up study using the same procedure (experiment 2), reinstatement was more robustly stimulated by alcohol, compared to a familiar lemon-flavored liquid. In experiment 3, systemic alcohol injections (0, 0.5, or 1.0 g/kg, intraperitoneal) administered either 24 hours or 15 minutes before test did not reinstate CS-elicited alcohol-seeking. Importantly, enzymatic assays in experiment 4 revealed detectable levels of alcohol in the blood following oral alcohol intake or intraperitoneal injection, suggesting that a pharmacological effect was likely with either route of administration. The reinstatement and spontaneous recovery effects revealed herein provide evidence of viable new behavioral paradigms for testing interventions against relapse.

2.2. Introduction

Animal models are critical for uncovering the causal processes that lead to relapse[5], which is a highly probable outcome for individuals recovering from alcohol use disorder[28,101,102]. Relapse models based on instrumental conditioning, in which subjects perform an operant response that is reinforced by alcohol, are abundant and have contributed greatly to our overall understanding of mechanisms underlying alcohol-seeking and use[103–105]. However, Pavlovian conditioning also plays a vital role in the development and maintenance of alcohol use disorder and in relapse[2,16,24,28,106,107]. Preclinical studies show that conditioned alcohol-seeking elicited by cues that predict alcohol can be renewed by exposure to an alcohol-associated context following extinction in a different context[18,53,54,108]. When conditioning, extinction, and test are conducted in the same context, cue-elicited alcohol-seeking can be reinstated by the scent of alcohol[40]. These findings indicate that re-exposure to distal and proximal environmental cues that predict alcohol can prompt a return of Pavlovian-conditioned alcohol-seeking after extinction.

Interestingly, alcohol seeking in rats can also be reinstated by pretest exposure to a small quantity of orally ingested alcohol[35], which provides access to the smell and taste of alcohol but does not produce pharmacological effects. This stimulus has also been used to augment reinstatement produced by response–contingent presentations of a discrete nondrug cue following extinction[109,110] and by re-exposure to an alcohol context[53,108]. Reinstatement of instrumental responding for alcohol has also been reported after alcohol was administered via oral intubation or systemic injection; however, the effect was modest and occurred at an alcohol dose of 0.48 g/kg, but not at 0.24 g/kg[33]. Thus, re-exposure to either the orosensory properties of alcohol or a pharmacologically relevant alcohol prime can trigger relapse.

Overwhelmingly in these studies, the reinstating stimulus is presented immediately before a test session in which instrumental or Pavlovian alcohol-seeking responses are assessed in the absence of alcohol delivery. This sequence models the immediate impact of relapse triggers on alcohol-seeking but does not address the potential delayed impact that a lapse in drinking may have on future alcohol-seeking. To model the latter scenario, we adapted an established reinstatement paradigm that was developed in aversive Pavlovian conditioning procedures[15,50,111–113] for use with alcohol. In this reinstatement paradigm, after Pavlovian conditioning and extinction, subjects receive a single session of re-exposure to the unconditioned stimulus (US) by itself, followed 24 hours later by a test session that assesses conditioned responding elicited by

the conditioned stimulus (CS). Using this reinstatement paradigm, we determined whether exposure to a pharmacologically effective dose of alcohol would reinstate CS-elicited alcohol-seeking 24 hours later.

In experiment 1, after completing Pavlovian conditioning and extinction, separate groups of rats received alcohol or water (as control liquid) for oral ingestion, delivered according to the same schedule of access as during prior Pavlovian conditioning sessions but without the CS. Because equivalent levels of reinstatement were observed in both groups 24 hours later, experiment 2 was conducted to test the prediction that making the control liquid discernably different from alcohol would produce an alcohol-specific reinstatement effect. Following Pavlovian conditioning and extinction, separate groups of rats received alcohol or a familiar lemon-flavored liquid, followed by a reinstatement test 24 hours later. In experiment 3, we investigated the ability of alcohol administered via an intraperitoneal injection (based on Lê and colleagues 1998[33]) to reinstate CS-elicited alcohol-seeking 24 hours later. Separately, in experiment 4 we conducted enzymatic assays to confirm that alcohol delivered via the distinct routes of administration used in experiments 1 to 3 would result in detectable levels of alcohol in the blood.

2.3. Material and Methods

2.3.1. Subjects

Male, Long-Evans rats (Envigo, Indianapolis, IN) weighing 220 to 240 g on arrival were used in all experiments. Rats were pair-housed for 3 days after arrival and then individually housed in polycarbonate shoebox cages containing beta chip bedding (Aspen Sani chips; Envigo, Indianapolis, IN) and a nylabone toy (Nylabones; Bio-Serv, Flemington, NJ). Cages were held in a temperature (21.0°C) and humidity (40 to 50%) controlled colony room on a 12hour light/dark cycle (lights on at 0700 hours). All experimental procedures were conducted during the light phase. Rats had constant unrestricted access to water and rat chow (Purina Agribrands; Charles River, St. Hubert, QC, Canada) and were handled and weighed for 3 days before experimental procedures began. All procedures were conducted following the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

2.3.2. Apparatus

Behavioral training was conducted in 12 conditioning chambers (ENV-009A; Med Associates Inc., St. Albans, VT) that were enclosed in sound-attenuating, ventilated melamine cubicles (made in-house). Chambers had a plexiglass ceiling, back-wall, and front door, as well as stainless steel, paneled left and right sidewalls, and a metal bar floor (ENV-009A-GF). A white-noise generator (ENV225SM, 80 to 85 dB; background noise 72 to 80 dB) and a white house light (ENV-215M; 75W, 100 mA) were mounted on the upper left chamber wall. A dual-cup fluid port (ENV-200R3AM) was located off-center on the right wall, 2 cm above the chamber floor. A 20 ml syringe mounted on a syringe pump (PHM 100 [Med Associates]; 3.33 revolutions per minute) was located outside the melamine cubicles and delivered liquid into a well within the port via polyethylene tubing. Port entries were measured by interruptions of an infrared beam that crossed the entrance of the fluid port. Two cue lights (ENV-221M) were mounted on the upper right chamber wall but were not illuminated during Pavlovian conditioning procedures. Experimental chambers used in experiments 2 and 3 also included 2 retractable levers (ENV-112BM) located 6 cm above the chamber floor and 5 cm from either side of the liquid receptacle; however, these remained retracted during experiments using Pavlovian conditioning procedures. House light illumination, stimulus presentations, and liquid delivery were controlled by Med-PC IV software on a PC computer.

2.3.3. Solutions

Alcohol solutions for oral ingestion (5, 10, and 15%; v/v) were prepared by diluting 95% alcohol in tap water. A 25% v/v alcohol solution used for intraperitoneal injections was prepared by diluting 95% alcohol in 0.9% sterile saline. A 2% v/v lemon-flavored liquid was prepared by diluting concentrated lemon juice (Mott's ReaLemon, Plano, TX) in tap water.

2.3.4. Home-Cage Alcohol Exposure

Before behavioral training, rats received 15 sessions of intermittent access to 15% alcohol and water in the home-cage to induce high levels of alcohol intake[114–116]. Briefly, rats were weighed and then given separate, pre-weighed bottles containing alcohol or water (Monday, Wednesday, Friday) which were replaced 24 hours later with 2 water bottles (Tuesday, Thursday, Saturday, Sunday). Bottle placement randomly alternated between the left and right sides of the cage to mitigate the impact of side preferences on drinking. Alcohol bottles were weighed after

each 24-hour session to calculate intake. Spillage was accounted for by subtracting the average amount of alcohol and water lost from bottles placed on 2 empty cages from drinking data obtained in the corresponding session. Starting on session 5, in rats that drank less than 1 g/kg for 2 consecutive sessions the alcohol concentration was reduced to 5% to encourage drinking. Once intake had increased to 1 g/kg for 3 sessions, the alcohol concentration was increased to 10%, and then, when rats attained 1 g/kg for 2 sessions, alcohol concentration increased to 15%. Any rats that completed this phase while drinking 10% alcohol also received 10% alcohol during subsequent Pavlovian conditioning sessions (see Table S1).

2.3.5. Behavioral Procedures

Habituation. During the last week of home-cage alcohol exposure, 2 habituation sessions were conducted on days that rats had only access to water. During the first session, rats were wheeled into the experimental behavior room in their home-cages, weighed, and left in their home-cages for 20 minutes. During the second session, rats were wheeled into the experimental behavior room, weighed, and then loaded into designated conditioning chambers. Chamber house lights were illuminated for 20 minutes, and the number of port entries made during this time was recorded.

*Pavlovian Conditionin*g. Pavlovian conditioning sessions occurred daily (Monday to Friday; 44 to 47 minute sessions). Initiation of the Med-PC program was followed by a 2-minute delay, after which house lights were illuminated to signal the start of the session. In each session, 8 trials of a 20 second white-noise CS were presented. Ten seconds after CS onset, the fluid pump was activated for 10 seconds, resulting in the delivery of 0.3 ml of alcohol into the fluid port. The CS and alcohol delivery co-terminated, but rats had unrestricted access to the fluid port and could ingest the alcohol at any point during the session. Intertrial intervals occurred on a variable time 240 second schedule (possible intervals of 120, 200, 210, 280, 310, and 320 seconds), not including 20 second pre- and post-CS intervals. Fluid ports were checked at the end of each session to ensure that alcohol was ingested.

Extinction and Test. Following Pavlovian conditioning, extinction sessions occurred daily (Sunday to Saturday). Parameters during extinction sessions were identical to Pavlovian

conditioning, except that the 20 second white-noise CS was paired with the 10 second activation of an empty syringe pump (i.e., no alcohol was delivered). Test sessions were identical to extinction sessions, unless specified.

2.3.6. Experiment 1. Reinstatement Triggered by Alcohol or Water (Oral)

Based on a reinstatement paradigm developed using aversive Pavlovian conditioning procedures[50,111], we sought to trigger reinstatement by exposing rats to alcohol in the fluid port 24 hours before a test session. Naïve rats (414 to 552 g at test) underwent 12 Pavlovian conditioning sessions and then 8 extinction sessions. At 24 hours after the last extinction session, separate groups received a single fluid exposure session in which either 2.4 ml of alcohol or water was delivered into the fluid port according to the same US delivery schedule as during Pavlovian conditioning, but without the CS. A test session (test 1) occurred 24 hours later.

Because reinstatement was observed in both groups, we conducted a second test using the same rats to determine whether reinstatement in the water group may have been the result of water in the fluid port being experienced as a novel stimulus. After 3 additional Pavlovian conditioning sessions and 5 additional extinction sessions, each group received 3 consecutive daily fluid exposure sessions, in which either 2.4 ml of alcohol or water was delivered into the fluid port. Here too, fluid delivery occurred on the same schedule of access as during prior Pavlovian conditioning, but without the CS. We reasoned that increasing the number of fluid exposure sessions would habituate rats to receiving water in the fluid port. A test session (test 2) was conducted 24 hours after the last fluid exposure session. Experimental groups and parameters were identical to the previous test.

2.3.7. Experiment 2. Reinstatement Triggered by Alcohol or Lemon-Flavored Liquid (Oral)

Alcohol and water may have both triggered reinstatement in experiment 1 because similarities in the color, texture, and location of delivery of these liquids made them difficult to differentiate during fluid exposure sessions. To test this hypothesis, we compared reinstatement produced by alcohol or a familiar lemon-flavored liquid. Naïve rats (426 to 601 g at test) were acclimated to a lemon-flavored liquid during two 24 hour sessions of access to separate pre-weighed bottles of lemon-flavored liquid and water, with 1 session occurring before and 1

session occurring after the 14th home-cage alcohol exposure session. There was no difference in the amount of ingested water or lemon-flavored liquid averaged across these 2 sessions, suggesting that the lemon-flavored liquid was neither inherently aversive nor appetitive (Figure S1). Rats then received 12 Pavlovian conditioning sessions. The last 5 sessions alternated daily with a session in which the lemon-flavored liquid was delivered into the fluid port on the same schedule used for Pavlovian conditioning, but without CS presentations. This procedure was carried out in order to acclimate rats to receiving lemon-flavored liquid in the behavioral chambers. After Pavlovian conditioning, rats received 8 extinction sessions, followed by 1 fluid exposure session during which separate groups received 2.4 ml of alcohol or lemon-flavored liquid according to the same US delivery schedule as Pavlovian conditioning, but without the CS. A test session was conducted 24 hours later.

2.3.8. Experiment 3. Reinstatement Triggered by Intraperitoneal Alcohol Injection

24 Hours Before Test. A prior study showed that systemically injected alcohol (0.48 g/kg) produced a modest reinstatement of responding in an instrumental model of alcohol-seeking[33]. Here, we examined whether a systemic injection of alcohol delivered following extinction would reinstate CS-elicited alcohol-seeking 24 hours later. Naïve rats (402 to 503 g at test) received 12 Pavlovian conditioning sessions followed by 8 extinction sessions. Before extinction session 6, rats received a sham injection with an empty syringe containing no needle. Before extinction sessions 7 and 8, rats received 1 ml/kg and 1 ml injections of saline (intraperitoneal), respectively. At 24 hours after the last extinction session, a US exposure session was conducted in which separate groups received a systemic injection of alcohol (25% v/v; 0.5 or 1.0 g/kg, intraperitoneal) or saline (volume based on the average volume of the 0.5 and 1.0 g/kg injections). Fifteen minutes later, rats were placed into the conditioning chambers for a session during which the house lights were illuminated as before; however, no other stimuli were presented, and port entries were recorded (44 to 47 minutes). A test session (test 1) was conducted 24 hours later.

Immediately Before Test. Using the same rats (402 to 504 g at test), we examined whether a systemic injection of alcohol administered immediately before test would reinstate Pavlovian-conditioned alcohol-seeking (based on Lê and colleagues 1998[33]). Following test 1, rats received

1 extinction session to verify low levels of responding. At 24 hours later, rats received identical doses of saline or alcohol via intraperitoneal injection as per their original group assignments. A second reinstatement test (test 2) was conducted 15 minutes later.

2.3.9. Experiment 4. Blood Alcohol Concentrations

This experiment was conducted to assess blood alcohol concentrations produced by ingesting alcohol that was delivered intermittently across a 44 to 47 minute session (as during Pavlovian conditioning or fluid exposure sessions) or via intraperitoneal injection (as during experiment 3).

Naïve rats (377 to 525 g at sampling) underwent Pavlovian conditioning as described above. Following their eighth, ninth, or tenth session, blood samples were collected from the tail vein immediately after the end of the session. In a group of non-naïve rats from experiment 3 (453 to 541 g at sampling), blood samples were collected from the tail vein 15 minutes after intraperitoneal injection of saline (1.0 ml) or varying doses of alcohol (0.4, 0.5, 1.0 g/kg). This time point was selected because of previous studies showing that blood alcohol concentrations following systemic injection peak after 15 minutes[117,118].

An NAD-ADH enzymatic assay was then conducted to determine blood alcohol concentrations[119]. Details for the tail vein blood extraction and NAD-ADH assay are located in Appendix S1.

2.3.10. Statistical Analyses

Initial and final sample sizes for each group in each experiment are reported in Table 1. In the home-cage alcohol exposure phase, we calculated the ingested dose of alcohol (g/kg; grams of alcohol consumed/body weight [kg]) for each rat in each session. Rats that ingested <1 g/kg averaged across the last 3 sessions were dropped from the experiment.

In Pavlovian conditioning, extinction, and test sessions, entries into the fluid port during the 20 second pre-CS, 20 second CS, 20 second post-CS, and variable intertrial intervals were recorded in each session. A normalized CS port entry (CS minus pre-CS) variable was calculated to account for individual differences in baseline port entry responding. In addition, the total duration of all CS-elicited port entries and the total latency to the first CS-elicited port entry were recorded. A maximum latency of 20 seconds was applied to CS trials in which a port

entry was not made. Rats that made <10 normalized CS-elicited port entries averaged across the last 3 Pavlovian conditioning sessions and also did not drink all the alcohol in the fluid port were excluded from the experiment (see Table 1).

Test data were compared to baseline extinction data, which was an average of the last 2 extinction sessions. In experiment 3, test 2 and 3 data were compared to the additional extinction session given in between test 1 and test 2. Data were analyzed using repeated measures analysis of variance (ANOVA), with the Huynh–Feldt correction applied when Mauchly's test of sphericity was significant. Post hoc analyses were conducted using the Bonferroni correction for all comparisons. Analyses were conducted with SPSS Statistics version 23 (IBM Corp., Armonk, NY) and evaluated using a statistical significance level of $p < 0.05$. Graphs were created with Prism 7 (La Jolla, CA).

Table 1. Sample-size trajectory across phase for each experiment

	Home-cage alcohol exposure		**Pavlovian Conditioning**		
Exp.	Initial	Dropped	Initial	Final	Dropped
1[a]	*n* = 26	*n* = 2	Alcohol, *n* = 12 Water, *n* = 12	Alcohol, *n* = 10 Water, *n* = 11	*n* = 3
2[b]	*n* = 26	*n* = 2	Alcohol, *n* = 12 Lemon, *n* = 12	Alcohol, *n* = 11 Lemon, *n* = 12	*n* = 1
3	*n* = 36	*n* = 0	0 g/kg, *n* = 12 0.5 g/kg, *n* = 12 1.0 g/kg, *n* = 12	0 g/kg, *n* = 11 0.5 g/kg, *n* = 11 1.0 g/kg, *n* = 11	*n* = 3
4[c]	*n* = 18	*n* = 0	8^{th} session, *n* = 4 9^{th} session, *n* = 4 10^{th} session, *n* = 4	8^{th} session, *n* = 4 9^{th} session, *n* = 4 10^{th} session, *n* = 3	*n* = 1

[a] After Test 1, all 24 rats were re-trained for 3 sessions. They all responded to criteria and were therefore included in Test 2 (Alcohol, *n* = 12; Water, *n* = 12).

[b] Following home-cage alcohol exposure, 1 rat was dropped because of low alcohol intake and 1 rat was dropped because of aggressive behaviour.

[c] 12 rats were used to examine blood alcohol levels after Pavlovian conditioning. One rat was dropped because of seizure-like behaviour. The remaining 6 rats were used to examine blood alcohol levels after intraperitoneal injection of saline (1.0 mL, *n* = 1) or alcohol (0.4 g/kg, *n* = 1; 0.5 g/kg, *n* = 2; 1.0 g/kg, *n* = 2).

2.4. Results

2.4.1. Acquisition and Extinction of Pavlovian Conditioning

During the home-cage alcohol exposure phase, ingested dose (g/kg) increased across sessions (Figure S2). Subsequently, rats learned to associate the CS with alcohol delivery during Pavlovian conditioning sessions (Figures S3 and S4; Table S2). Across Pavlovian conditioning and extinction training, port entries during the pre-CS remained low, whereas CS-elicited port entries increased across acquisition sessions and decreased across extinction sessions comparably in all groups.

2.4.2. Experiment 1. Reinstatement Triggered by Alcohol or Water (Oral)

Exposure to either alcohol or water in the fluid port (Figure 1A) reinstated CS-elicited port entries 24 hours later, with no difference at test between groups. This effect occurred following either 1 session of fluid exposure (test 1) or 3 sessions of fluid exposure (test 2). Normalized CS-elicited port entries (Figures 1B, E) were higher at test relative to extinction, Phase, test 1: $F_{(1, 19)} = 31.19$, $p < 0.001$; test 2: $F_{(1, 22)} = 43.99$, $p < 0.001$, in both groups, Group, test 1: $F_{(1, 19)} = 0.81$, $p = 0.380$; test 2: $F_{(1, 22)} = 0.034$, $p = 0.86$; Phase x Group, test 1: $F_{(1, 19)} = 2.01$, $p = 0.173$; test 2: $F_{(1, 22)} = 0.11$, $p = 0.749$. To determine whether the pattern of responding across test differed as a function of group, we examined port entries made during individual CS trials (Figures 1B, E, insets). In both tests, normalized CS-elicited port entries decreased across CS trials, Trials, test 1: $F_{(3.532, 67.108)} = 8.14$, $p < 0.001$; test 2: $F_{(3.625, 79.757)} = 22.79$, $p < 0.001$, comparably in both groups, Group, test 1: $F_{(1, 19)} = 1.32$, $p = 0.266$; test 2: $F_{(1, 22)} = 0.07$, $p = 0.796$; Trial x Group, test 1: $F_{(3.532, 67.108)} = 0.83$, $p = 0.500$; test 2: $F_{(3.625, 79.757)} = 1.12$, $p = 0.350$, due to within-session extinction.

The total latency to initiate a port entry following CS onset (Figures 1C, F) decreased at test relative to extinction, Phase, test 1: $F_{(1, 19)} = 18.31$, $p < 0.001$; test 2: $F_{(1, 22)} = 49.21$, $p < 0.001$, similarly in both groups, Group, test 1: $F_{(1, 19)} = 0.60$, $p = 0.450$; test 2: $F_{(1, 22)} = 0.07$, $p = 0.801$; Phase x Group, test 1: $F_{(1, 19)} = 1.22$, $p = 0.283$; test 2: $F_{(1, 22)} = 0.02$, $p = 0.887$.

Additionally, the total duration of CS-elicited port entries (Figures 1D, G) increased at test relative to extinction, Phase, test 1: $F_{(1, 19)} = 18.64$, $p < 0.001$; test 2: $F_{(1, 22)} = 37.77$, $p < 0.001$, in both groups, Group, test 1: $F_{(1, 19)} = 0.89$, $p = 0.357$; test 2: $F_{(1, 22)} = 0.46$, $p = 0.507$; Phase x Group, test 1: $F_{(1, 19)} = 1.60$, $p = 0.221$; test 2: $F_{(1, 22)} = 0.22$, $p = 0.642$.

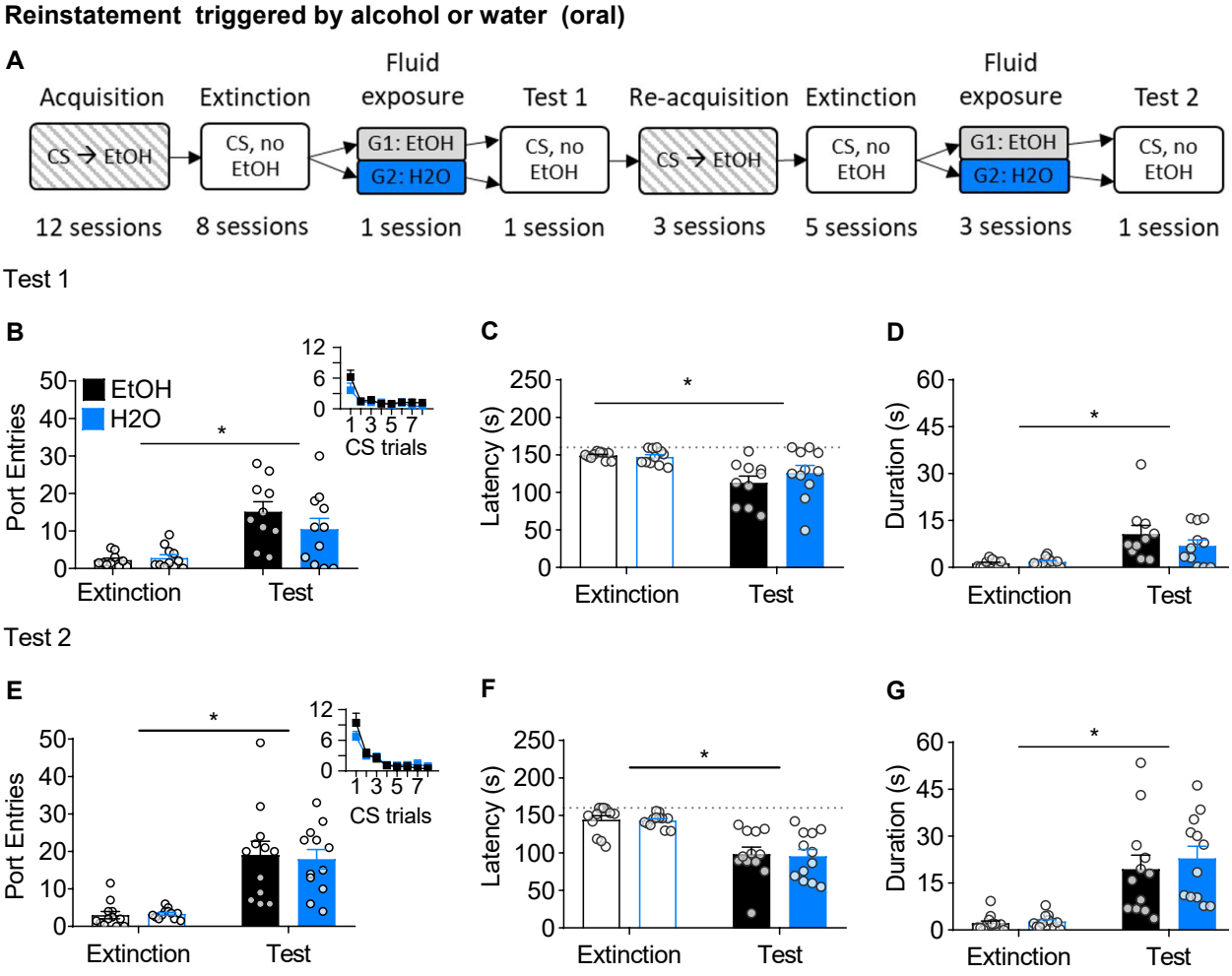


Figure 1. Exposure to orally ingested alcohol or water following extinction reinstated CS-elicited port entries at test 24 h later. **A** Schematic illustration of behavioural training. **B and E** Mean (± SEM) normalized CS-elicited port entries during extinction and test. Test 1 **(b)** occurred 24 h after 1 session of alcohol (EtOH; *n* = 10; black) or water (H2O; *n* = 11; blue) exposure, whereas Test 2 **(e)** occurred 24 h after 3 sessions of alcohol (*n* = 12) or water (*n* = 12) exposure. Insets depict mean (± SEM) normalized CS-elicited port entries across CS trials at Tests 1 and 2. **C and F** Mean (± SEM) total latency to initiate CS-elicited port entries during extinction, Test 1 **(c)** and Test 2 **(f)**. **D and G** Mean (± SEM) total duration of CS-elicited port entries during extinction, Test 1 **(d)** and Test 2 **(g)**. Here and in all subsequent figures, circles depict data from individual rats.

* $p < 0.05$, main effect of Phase (extinction vs. test).

2.4.3. Experiment 2. Reinstatement Triggered by Alcohol or Lemon-Flavored Liquid (Oral)

After extinction, exposure to either alcohol or the lemon-flavored liquid in the fluid port (Figure 2A) reinstated CS-elicited port entries 24 hours later; however, this effect was significantly greater following alcohol. Normalized CS-elicited port entries (Figure 2B) increased at test relative to extinction, Phase, $F_{(1, 21)} = 33.95$, $p < 0.001$, but this effect differed as a function of group, Group, $F_{(1, 21)} = 7.56$, $p = 0.012$; Phase x Group, $F_{(1, 21)} = 6.49$, $p = 0.019$. Although pairwise comparisons revealed increased normalized CS-elicited port entries at test relative to extinction following alcohol ($p < 0.001$) or lemon ($p = 0.027$) exposure, test responding was higher in rats that had previously received alcohol ($p = 0.010$).

At test, normalized CS-elicited port entries (Figure 2C) decreased across CS trials comparably in both groups, Trial, $F_{(6.017, 126.351)} = 3.83$, $p = 0.002$; Phase x Group, $F_{(6.017, 126.351)} = 1.09$, $p = 0.373$, due to within-session extinction; however, responding was higher overall in alcohol-exposed rats, Group, $F_{(1, 21)} = 8.05$, $p = 0.010$.

The total latency to initiate a CS-elicited port entry (Figure 2D) decreased at test, Phase, $F_{(1, 21)} = 24.61$, $p < 0.001$, and this effect differed across groups, Group, $F_{(1, 21)} = 6.35$, $p = 0.020$; Phase x Group, $F_{(1, 21)} = 4.91$, $p = 0.038$. Pairwise comparisons showed that relative to extinction, total latency to CS-elicited port entries at test was shorter after alcohol ($p < 0.001$), but not lemon-flavored liquid ($p = 0.061$) exposure. In addition, alcohol-exposed rats were faster to make CS-elicited port entries at test than rats that had received the lemon-flavored liquid ($p = 0.017$).

Last, the total duration of CS-elicited port entries (Figure 2E) was higher at test relative to extinction, Phase, $F_{(1, 21)} = 25.26$, $p < 0.001$, with a differential effect across groups, Group, $F_{(1, 21)} = 8.32$, $p = 0.009$; Phase x Group, $F_{(1, 21)} = 8.88$, $p = 0.007$. Pairwise comparisons showed that relative to extinction, total CS-elicited port entry duration was elevated in the alcohol ($p < 0.001$), but not the lemon-flavored liquid ($p = 0.154$) group. Also, total CS-elicited port entries at test were longer in duration in the alcohol-exposed group ($p = 0.008$).

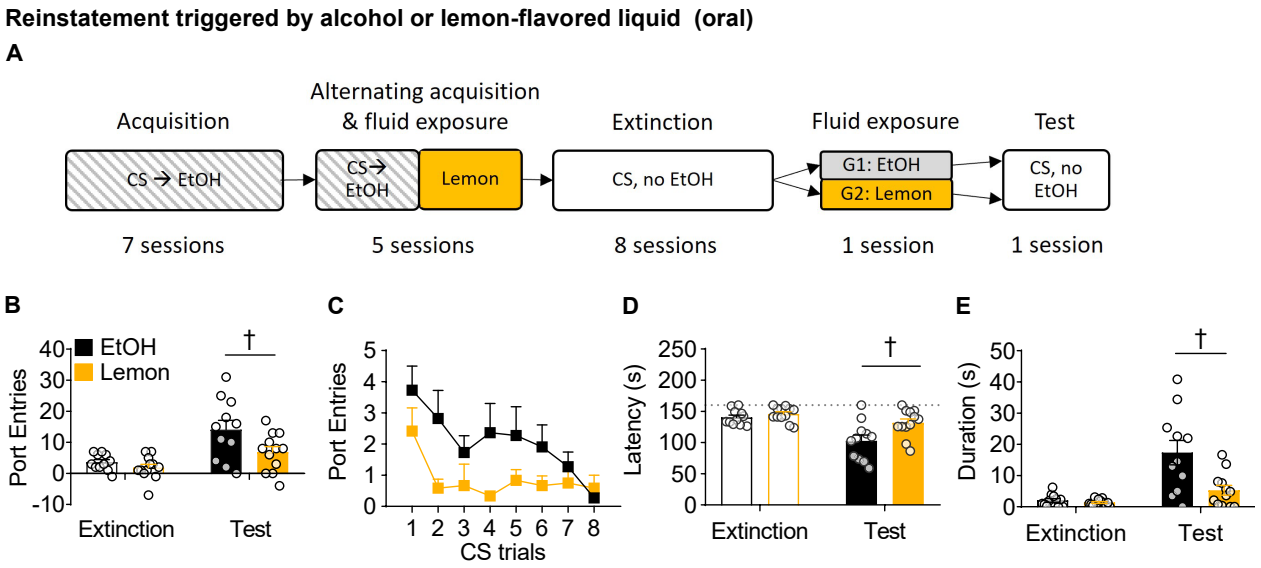


Figure 2. Exposure to orally ingested alcohol following extinction reinstated CS-elicited port entries at test 24 h later to a greater extent than did exposure to a lemon-flavored liquid. **A** Schematic illustration of behavioural training. **B** Mean (± SEM) number of normalized CS-elicited port entries during extinction and test for groups that received alcohol (EtOH; *n* = 11; black) or lemon-flavored liquid (Lemon; *n* = 12; yellow). **C** Mean (± SEM) normalized CS-elicited port entries across CS trials at test. **D** Mean (± SEM) total latency to initiate CS-elicited port entries during extinction and test. **E** Mean (± SEM) total duration of CS-elicited port entries during extinction and test.

† $p < 0.05$, Bonferroni-corrected pairwise comparison (alcohol vs. lemon).

2.4.4. Experiment 3. Reinstatement Triggered by Intraperitoneal Alcohol Injection

Twenty-Four Hours Before Test. Alcohol injected systemically 24 hours before test (Figure 3A) did not reinstate subsequent CS-elicited port entries. Relative to extinction, normalized CS-elicited port entries (Figure 3B) remained unchanged at test 1, Phase, $F_{(1, 30)} = 1.99$, $p = 0.168$, in both saline and alcohol groups, Group, $F_{(2, 30)} = 1.26$, $p = 0.298$; Phase x Group, $F_{(2, 30)} = 0.57$, $p = 0.572$.

Immediately Before Test. In rats that received a high dose of systemically injected alcohol immediately before test, there was an overall reduction in CS-elicited port entries (Figure 3C). Relative to extinction, normalized CS-elicited port entries decreased at test 2, Phase, $F_{(1, 30)} = 7.40$, $p = 0.011$, with a statistically significant main effect of group, Group, $F_{(2, 30)} = 4.82$, $p = 0.015$, but no statistically significant interaction, Phase x Group, $F_{(2, 30)} = 0.47$, $p = 0.632$. Pairwise comparisons revealed that, collapsed across extinction and test session, the 1 g/kg group made significantly fewer port entries than the saline group ($p = 0.024$) and near significantly fewer than the 0.5 g/kg group ($p = 0.053$).

To verify that this cohort of rats (399 to 502 g at test) was indeed capable of showing a reinstatement effect, they received an additional reinstatement test (test 3, Figure 3A) in which a 0.3 ml alcohol prime was delivered into the fluid port at the beginning of the 120 second delay that spanned program initiation and house light onset (Lacroix et al., 2016). Relative to an extinction session that occurred between tests 1 and 2, exposure to this drop of alcohol produced a significant reinstatement of normalized CS-elicited port entries (Figure 3D), Phase, $F_{(1, 32)} = 62.62$, $p < 0.001$.

Reinstatement triggered by intraperitoneal alcohol injection

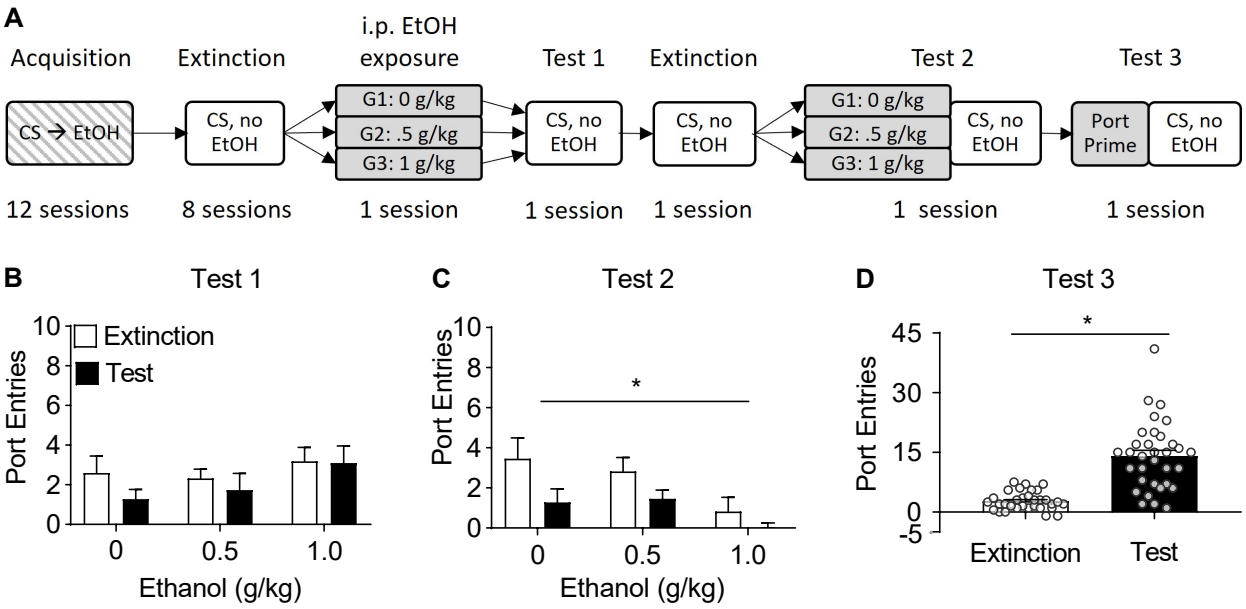


Figure 3. Intraperitoneal injection of alcohol did not reinstate CS-elicited port entries; however, an oral alcohol prime delivered into the fluid port immediately before test did. **A** Schematic illustration of behavioural training. **B-D** Mean (± SEM) normalized CS-elicited port entries during extinction (white bars) and test (black bars). Test 1 **(b)** occurred 24 h after an injection of saline (0 g/kg, *n* =11) or alcohol (0.5 g/kg, *n* = 11; 1.0 g/kg, *n* = 11), whereas Test 2 **(c)** occurred 15 min after an injection of saline (0 g/kg, *n* =11) or alcohol (0.5 g/kg, *n* = 11; 1.0 g/kg, *n* = 11). In Test 3 **(d)**, all rats (*n* = 33) received a 0.3 mL drop of alcohol in the fluid port at the beginning of the 120 s delay period before house light onset.

* $p < 0.05$, main effect of Phase (extinction vs. test).

2.4.5. Experiment 4. Blood Alcohol Concentrations

Blood sampled from rats immediately after a Pavlovian conditioning session revealed detectable levels of alcohol (Figure 4A). The mean SEM ingested alcohol dose was 0.55 ± 0.048 g/kg and mean SEM blood alcohol concentration was 23.06 ± 4.83 mg%. There was a significant, positive correlation between blood alcohol concentration and ingested alcohol dose ($r = 0.36$, $p = 0.05$).

Alcohol administered via systemic injection 15 minutes before blood sample collection produced detectable levels of alcohol in the blood (Figure 4B). Mean SEM blood alcohol concentrations were 12.97 ± 0 mg%, 41.19 ± 10.22 mg%, and 82.91 ± 2.31 mg% in rats injected with 0.4, 0.5, and 1.0 g/kg of alcohol, respectively. There was a significant, positive correlation between blood alcohol concentration and injected alcohol dose ($r = 0.97$, $p = 0.0018$).

Blood alcohol concentration

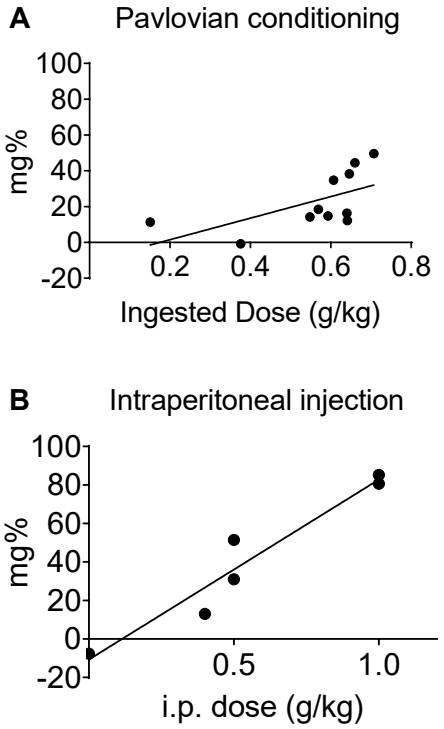


Figure 4. Detectable levels of alcohol in the blood were observed following oral alcohol intake in a Pavlovian conditioning session or after intraperitoneal injection of alcohol. Data represent the blood alcohol concentration (mg %) of individual rats as a function of **(A)** ingested dose of alcohol after a Pavlovian conditioning session (*n* = 11) or **(B)** following systemic injection of saline (*n* = 1) or alcohol (0.4 g/kg, *n* = 1; 0.5 g/kg, *n* = 2; 1.0 g/kg, *n* = 2).

2.5. Discussion

The objective of the present research was to develop new animal models of relapse to Pavlovian alcohol-seeking. We found that oral ingestion of alcohol, water, or a familiar lemon-flavored liquid following extinction reinstated CS-elicited port entries at test 24 hours later; however, reinstatement was significantly greater following alcohol compared to the lemon-flavored liquid. Systemically injected alcohol administered either 24 hours or 15 minutes before test did not reinstate CS-elicited alcohol-seeking. Importantly, both routes of alcohol administration (oral and intraperitoneal) produced detectable levels of alcohol in the blood that positively correlated with ingested or injected dose. These results were obtained in male rats and should be extended to females in future experiments.

The finding that oral alcohol ingestion after extinction reinstated CS-elicited port entries 24 hours later is consistent with results from aversive Pavlovian procedures in which re-exposure to an aversive US following extinction reinstated CS-evoked freezing 24 hours later[15,111]. This reinstatement effect has been attributed to an excitatory context–US association that is formed during the US exposure session and summates with the residual excitatory strength of the CS that survives extinction[50,112]. Support for a similar process underpinning reinstatement in our paradigm comes from the observation that the volume and pattern of alcohol ingestion that occurred during the fluid exposure session produced detectable blood alcohol concentrations that correlated positively with ingested dose (experiment 4; see also Cofresi and colleagues 2017[40]). A pharmacological effect of orally ingested alcohol may therefore have served as a US in an excitatory context–alcohol association during the fluid exposure session.

In aversive Pavlovian conditioning studies, control groups received either no US[111] or the same US as during Pavlovian conditioning, but at varying intensities[120]. In experiment 1 of the present research, we included a control group that received water, an ostensibly neutral stimulus, during the fluid exposure session. Surprisingly, exposure to water reinstated CS-elicited port entries 24 hours later. However, a parallel observation has been reported previously in an instrumental conditioning procedure, wherein exposure to water following extinction reinstated responding on an alcohol-associated lever in rats[121].

To explore this result further, we evaluated the possibility that water experienced in the fluid port might serve as a reinforcing, as opposed to neutral stimulus, even though our rats

were not food or water restricted. A subset of rats was retrained in the Pavlovian conditioning procedure for 3 sessions in which the CS was paired with alcohol or water in separate groups. There was an overall reduction in CS-elicited port entries in the group that received water (Figure S5), indicating that water was a less effective reinforcer than alcohol. We then considered the possibility that rats may have been unable to differentiate between the alcohol that they received during Pavlovian conditioning and the water that they received during the fluid exposure session. Indeed, both liquids were delivered into a fluid port in 0.3 ml aliquots across sessions of equal length, and water is the predominant component of the 10 and 15% ethanol solutions used in these studies. We reasoned that differentiating the alcohol and control liquid by altering the scent and taste of the control liquid would produce an alcohol-specific reinstatement effect. In experiment 2, exposure to either alcohol or a familiar, lemon-flavored liquid reinstated CS-elicited port entries 24 hours later; however, the reinstatement effect was significantly greater following alcohol. At test, CS-elicited port entries extinguished rapidly in rats that had been exposed to the lemon-flavored liquid but remained elevated across the session in the alcohol-exposed group. The latter group was also faster to make CS-elicited port entries and remained in the port for longer durations during the CS.

The results from experiments 1 and 2 suggest that ingesting a liquid from the fluid port is sufficient to reinstate conditioned responses to an alcohol-predictive CS 24 hours later. This nonspecific effect may be a function of the common consummatory behavior that rats used to ingest the 3 liquids that we tested (water, lemon-flavored liquid, alcohol). Performing the consummatory response, specifically in the fluid port, may have triggered a memory of the CS–alcohol association acquired during Pavlovian conditioning, which could have contributed to the reinstatement effect 24 hours later. Importantly, exposure to alcohol produced a significantly greater reinstatement effect, compared to a distinct, lemon-flavored liquid. This result may be attributable to less overall exposure to the lemon-flavored liquid than to alcohol, which could be addressed in future studies by delivering the control liquid during CS presentations in the extinction phase. Alternatively, the augmented reinstatement effect following alcohol may have been produced by the additive effects of an excitatory context–alcohol association summating with a residual CS–alcohol association that survived extinction.

Based on the hypothesis that a context–US association is needed for reinstatement in this paradigm, we predicted that an intraperitoneal injection of a pharmacologically effective

dose of alcohol would reinstate CS-elicited port entries 24 hours later. However, as seen in the results from experiment 3, this did not happen. One explanation is that this route of alcohol administration may have produced a conditioned place aversion, which would then have counteracted the psychological processes that induce reinstatement. Indeed, similar doses of alcohol have been shown to induce a conditioned place aversion in rats[122,123].

To examine this route of administration further, we gave the same rats a second test to determine whether an intraperitoneal injection of alcohol immediately before an extinction session would trigger reinstatement. This manipulation has been shown to produce a modest, dose-dependent reinstatement of instrumental responding on an alcohol-associated lever in rats[33], although our preliminary data failed to replicate this result (Figure S6). Interestingly, we did not observe a reinstatement effect. Instead, there was a reduction in CS-elicited port entries at test, relative to extinction, in all groups. Despite considerable habituation, the injection procedure may have been aversive or stressful, although in our experience stress produces a nonselective increase in port-entry behavior. Alternatively, if intraperitoneal injection of alcohol is not an effective stimulus for reinstating Pavlovian alcohol-seeking, then the reduced responding at test relative to extinction may simply reflect the natural progression of extinction that would have been observed even in the absence of injections. The test analysis also revealed a main effect of Group, attributable to an overall reduction in CS-elicited port entries on data averaged across extinction and test in the 1 g/kg group, relative to saline. This result could reflect a carryover effect of having previously received the same high dose of alcohol in test 1, as dose remained consistent within each group across tests.

The lack of reinstatement following systemic alcohol injections could also be due to differences in the interoceptive stimulus properties of voluntary, orally ingested alcohol (as experienced during Pavlovian conditioning) and involuntary, systemically injected alcohol (as experienced at test). These distinct routes of alcohol administration have unique kinetics, such as peak blood alcohol concentrations achieved and time to reach such blood alcohol concentrations[124,125], which could influence whether or not reinstatement is observed.

To rule out possible flaws in our injection procedure, we examined blood alcohol levels after intraperitoneal injections and found a significant, positive correlation between injected dose and blood alcohol concentration (experiment 4). Finally, we affirmed that this particular cohort of rats was capable of showing a reinstatement effect in response to a distinct trigger—

exposure to a 0.3 ml alcohol prime immediately before the test. Considering these results together, our data indicate that intraperitoneal injection of alcohol, either 24 hours or immediately before test, is not an effective trigger for relapse to Pavlovian alcohol-seeking.

In conclusion, we show that oral ingestion of alcohol reinstates CS-elicited alcohol-seeking 24 hours later. This effect is elevated when compared to a distinctive lemon-flavored liquid, but equivalent when compared to water. Thus, consummatory behavior involved in the ingestion of alcohol, particularly when performed in a context associated with alcohol availability and when paired with alcohol ingestion, may serve to augment the potential of alcohol-predictive cues to drive relapse in the future. This reinstatement paradigm offers the exciting possibility of investigating the long-term effects of a lapse in drinking on later cue-evoked alcohol-seeking. We also show that intraperitoneal administration of alcohol is not an effective trigger for relapse to Pavlovian alcohol-seeking. This new behavioral model of relapse to Pavlovian alcohol-seeking can be used in future research aimed at uncovering the neural underpinnings of relapse, as well as behavioral and pharmacological interventions against relapse.

Chapter 3: The Role of Context Conditioning in the Reinstatement of Responding to an Alcohol-Predictive Conditioned Stimulus

3.1. Abstract

Re-exposure to an unconditioned stimulus (US) can reinstate extinguished conditioned responding elicited by a conditioned stimulus (CS). We tested the hypothesis that the reinstatement of responding to an appetitive CS is driven by an excitatory association formed between the US and the context that the US was ingested in during US re-exposure. Male, Long-Evans rats were acclimated to drinking alcohol (15%, *v*/*v*) in the home-cage, then trained to associate an auditory CS with an alcohol-US that was delivered into a fluid port for oral intake. During subsequent extinction sessions, the CS was presented as before, but without alcohol. After extinction, rats were re-exposed to alcohol as in training, but without the CS (alcohol re-exposure). 24 h later at test, the CS was presented as in training, but without alcohol. First, we tested the effect of extinguishing the context-alcohol association, formed during alcohol re-exposure, on reinstatement. Conducting four context extinction sessions across four days (spaced extinction) after the alcohol re-exposure session did not impact reinstatement. However, four context extinction sessions conducted across two days (massed extinction) prevented reinstatement. Next, we conducted alcohol re-exposure in a context that either differed from, or was the same as, the test context. One alcohol re-exposure session in a different context did not affect reinstatement, however, three alcohol re-exposure sessions in a different context significantly reduced reinstatement during the first CS trial. These results partially support the view that a context-US association formed during US re-exposure drives the reinstatement of responding to an appetitive, alcohol-predictive CS.

3.2. Introduction

An important aspect of animal behaviour is the ability to associate a neutral, environmental stimulus with a salient, unconditioned stimulus (US). As a result of learning this predictive relationship, the environmental stimulus becomes a conditioned stimulus (CS) that can elicit conditioned responses. Conditioned responses allow animals to respond advantageously to environmental cues that predict appetitive stimuli (e.g., food, water) and aversive stimuli (e.g., predators, malaise). Importantly, in scenarios where the expected US stops occurring, animals learn to inhibit conditioned responding. This inhibition of responding, however, is not permanent and certain conditions can prompt a return of responding to the extinguished CS. For example, re-exposure to the US after extinction can reinstate conditioned responding to the CS[15,50,126]. Reinstatement is a fundamental phenomenon that is used to study learning and memory processes, including those related to extinction[111,127]. Reinstatement also has practical applications: it provides insight into how maladaptive reactions to environmental cues contribute to drug use and relapse[128,129] or post-traumatic stress disorder[130–132] in clinical populations. As such, it is critical to understand the psychological processes that underlie the reinstatement effect.

Reinstatement has been predominantly studied using an aversive Pavlovian conditioning procedure in which rats are trained to associate an auditory CS with a foot shock-US, then the shock-US is withheld to extinguish conditioned responding. Next, rats receive unsignalled re-exposure to the shock-US, which reinstates conditioned responding to the CS at test 24 h later. This reinstatement of conditioned responding to an aversive CS, however, is only observed under specific conditions. For example, US re-exposure must be conducted in the same context that the subsequent reinstatement test occurs in[50,56,111,120,133–135], or in the Pavlovian conditioning or extinction context (i.e., training contexts)[56], but not a novel context. When re-exposure to the shock-US occurs in a context that differs from the test or training contexts, reinstatement of the CS-evoked response does not occur. Additionally, if shock re-exposure is followed by sessions of repeated exposure to the context in which US re-exposure was conducted, then reinstatement is not observed[50]. Reinstatement of conditioned responding to appetitive stimuli also requires these specific conditions. Re-exposure to a food-pellet in a context that differs from the test context reduces reinstatement of a CS-evoked response[112] and of an operant lever pressing response[113]. Furthermore, repeated exposure to the context that re-exposure to a food-pellet occurred in also attenuates reinstatement of an operant lever-pressing response[113]. Based on these findings, it has

been suggested that US re-exposure produces an excitatory association between the context and US. This 'context-US association' is proposed to summate with the residual, predictive value of the CS that survives extinction to drive reinstatement[56,112,136]. This hypothesis is further supported by context preference tests conducted after US re-exposure. Rats avoid contexts associated with the shock-US delivered during US re-exposure, indicating that the context becomes associated with the shock-US[111,120].

The reinstatement of conditioned responding observed 24 h after US re-exposure is a reliable phenomenon observed in a variety of aversive and appetitive conditioning procedures. Re-exposure to an aversive US can reinstate various conditioned responses, such as freezing[56,137], suppression of an operant response[111,133,134,137–139], fear-potentiated startle[140,141], and taste aversion[142]. In appetitive conditioning, re-exposure to a food-pellet-US reinstated conditioned responding to an extinguished CS 24 h later[112,136]. In an operant task, re-exposure to a food-pellet reinstated an extinguished operant lever-pressing response 24 h later at test[113]. Conditioned responding for psychoactive substances can also be reinstated. After an established conditioned place preference for cocaine or methamphetamine was extinguished, re-exposure to the respective drug reinstated a preference for the drug-paired chamber 48 h later, relative to a chamber in which the drug was never delivered[143–145]. Therefore, reinstatement is a general phenomenon that occurs in a wide range of learning paradigms.

We recently extended this literature by demonstrating the reinstatement of responding to a CS that predicted an appetitive, psychoactive substance, alcohol[146]. In our task, following the acquisition and extinction of responding to an alcohol-predictive CS, re-exposure to an alcohol-US significantly reinstated responding to the CS 24 h later. Interestingly, a control group that received water instead of alcohol during US re-exposure showed similar reinstatement to that produced by alcohol. In a separate experiment where the control fluid was a familiar lemon-flavored water solution, making it distinct from alcohol, reinstatement was greater following re-exposure to the alcohol-US compared to the control fluid. In contrast, reinstatement was not observed when US re-exposure was delivered via systemic alcohol injection so that the pharmacological effects of alcohol were experienced without ingesting alcohol. These results provide new evidence of reinstatement to a CS that predicts a psychoactive substance, which occurs 24 h after exposure to the psychoactive substance. The present research was aimed at understanding the psychological processes underpinning this reinstatement effect.

As described above, research using aversive Pavlovian conditioning procedures suggests that a context-US association formed during US re-exposure summates with the residual, predictive value of the CS that survives extinction to drive reinstatement[56,112,136]. However, there are fundamental differences in how associations are formed with aversive and appetitive stimuli that may result in different processes underlying reinstatement to an alcohol-predictive CS. First, the rate at which conditioned responses are acquired differs. Although most studies conduct more than one conditioning trial, subjects can associate a CS and an aversive US after just one pairing[147–150]. Conversely, appetitive conditioning tasks typically require a greater number of CS-US pairings to form an association and evoke a conditioned response[151,152]. Aversive and appetitive tasks also differ in how subjects interact with the US. In many aversive tasks, the US (e.g., foot shock) is experimentally delivered and results in a passive, non-voluntary experience[50,142]. In appetitive tasks, the US is experimentally delivered (e.g., drops of alcohol, food pellets), but subjects must approach and ingest the US, which are voluntary behaviours[42,136,153]. Moreover, appetitive tasks often require subjects to engage in a consummatory response to ingest the US, unless the US is delivered via systemic injection[154], intragastric intubation[155] or intrajugular catheter[156,157]. In the latter cases, the route of US administration is non-voluntary, making these procedures more akin to passive aversive conditioning. Given these fundamental differences in appetitive and aversive conditioning, the extent to which a context-US association drives reinstatement to an alcohol-predictive CS is unknown.

We investigated the psychological processes underlying the reinstatement of responding to an alcohol-predictive CS using two distinct behavioural manipulations that assessed the role of a context-US association in reinstatement. In Experiment 1, we tested the effect of extinguishing the context-alcohol association formed during alcohol re-exposure on reinstatement. In Experiment 2, we tested the effect of conducting alcohol re-exposure in a context that differed from the subsequent test context on reinstatement.

3.3. Methodology

3.3.1. Subjects

Male, Long-Evans rats (Envigo, Indianapolis, IN; 220 – 240 g on arrival) were pair-housed upon arrival and left unhandled for three days. Next, rats were single housed and handled

for five days before experimental procedures began. Cages contained beta chip bedding (Aspen Sani chips, Envigo), a nylabone toy (Nylabones, Bio-Serv), a polycarbonate tunnel (Rat retreats, Bio-Serv), and shredded paper. Unrestricted access to water and rat chow (Purina Agribrands, Charles River) was provided throughout the experiment. Cages were held in a temperature (21° C) and humidity-controlled (40 50%) colony room that was on a 12 h light/dark cycle (lights on at 0700 h; all experimental procedures occurred during the light phase). All procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

3.3.2. Apparatus

Behavioural procedures were conducted in conditioning chambers (ENV-009A; Med Associates Inc., St-Albans, VT) that were enclosed in sound-attenuating, ventilated melamine cubicles (made in house). Chambers included a Plexiglass front door, back-wall and ceiling, stainless steel sidewalls, and a metal bar floor (ENV-009A-GF). A house light (ENV-215M; 75W, 100 mA) and a white-noise generator (ENV-225SM, 5 dB above background noise) were mounted on the upper left chamber wall. A dual-cup, fluid port (ENV-200R3AM) was located off-centred, on the lower right chamber wall. Alcohol was delivered to the fluid port via polyethylene tubing from a 20 mL syringe mounted on a syringe pump (PHM-100, 3.33 RPM) located outside the melamine cubicles. Port entries were measured via interruptions of an infrared beam across the entrance of the fluid port. Med PC IV software on a PC controlled stimulus delivery and recorded behavioural responses.

3.3.3. Solutions

Alcohol solutions (5%, 10%, 15% *v*/*v*) were prepared by diluting 95% ethanol in tap water. Odours for context configurations were prepared by diluting 10% *v*/*v* lemon oil (lemon odour; Sigma Aldrich, CAS# 8008-56-8) or 10% *v*/*v* benzaldehyde (almond odour; bought in house at Concordia University chemistry store) in tap water.

3.3.4. Behavioural Procedures

Intermittent Alcohol Access. Rats received 15 sessions of intermittent access to 15% (*v*/*v)* alcohol in the home-cage to induce high levels of alcohol intake[115,116,158]. During alcohol access

sessions (Monday, Wednesday, Friday), rats were first weighed, then pre-weighed 100 mL graduated cylinders containing alcohol and pre-weighed water bottles were inserted into home-cages via the cage lid. 24 h later, the alcohol cylinders were replaced with water cylinders (Tuesday, Thursday, Saturday, Sunday). Cylinder placement was randomized across the left and right sides of the cage lid to mitigate the impact of side preference on drinking. Cylinders and water bottles were weighed after each 24 h session to calculate fluid intake. Spillage was accounted for by subtracting the average amount of fluid lost from bottles placed on two empty control cages from the corresponding session data.

The unsweetened alcohol solution used in this procedure produces variable levels of alcohol intake[40,115,146]. For rats with low alcohol intake, we reduced the alcohol concentration to encourage drinking[40,159]. Rats that drank < 1 g/kg [g of alcohol consumed/kg of body weight] on three consecutive sessions subsequently received 5% (*v*/*v*) alcohol on session 5 (Experiment 1, *n* = 7) or on session 4 (Experiment 2, *n* = 2). Once 1 g/kg was obtained for two consecutive sessions, the alcohol concentration was increased to 10% (*v*/*v*). When 1 g/kg was obtained for two consecutive sessions the alcohol concentration was increased to 15% (*v*/*v*). Rats (*n* = 1) that drank < 1 g/kg for three consecutive sessions after session 6 subsequently received 10% (*v*/*v*) alcohol until 1 g/kg was obtained for two consecutive sessions and then the alcohol concentration was increased to 15% (*v*/*v*). The concentration of alcohol that rats received on the last intermittent alcohol access session was the alcohol concentration that they received during Pavlovian conditioning and alcohol re-exposure. Supplementary Table 1 depicts the number of rats that received 5% and 10% alcohol during subsequent training sessions.

Habituation. Two habituation sessions were conducted during the last week of intermittent alcohol access on days with only access to water. In session 1, rats were brought to the experimental room, weighed, and left in their home cages for 20 min. In session 2, rats were weighed and then placed into a designated conditioning chamber for 20 min, during which the house lights were illuminated, and the total number of port entries made was counted.

Pavlovian Conditioning. Pavlovian conditioning was conducted daily (Monday – Friday). Sessions began with a 2 min delay, after which the house lights were illuminated to signal the start of the session. In each session, eight trials of a 20 s continuous white-noise conditioned

stimulus (CS) were paired with 10 s activation of the fluid pump to deliver 0.3 mL of alcohol into the fluid port (2.4 mL per session). Pump activation began 10 s after CS onset and co-terminated with the CS. Trials occurred on a variable time 240 s schedule (intertrial intervals: 120, 200, 210, 280, 310, 320 s; not including 20 s preand post-CS intervals). Total session length was 42 45 min. Fluid ports were checked at the end of each session to verify that the alcohol was ingested.

Extinction. Extinction sessions were conducted daily (Sunday – Saturday). Session parameters were identical to Pavlovian conditioning except that the CS was paired with activation of empty syringe pumps (i.e., alcohol was not delivered).

Alcohol Re-exposure. During alcohol re-exposure, 2.4 mL of alcohol was delivered into the fluid port according to the same schedule as Pavlovian conditioning; however, the CS was not presented.

Reinstatement Test. During the reinstatement test, the CS was presented as during Pavlovian conditioning, except that CS trials were paired with activation of empty syringe pumps (i.e., alcohol was not delivered). The experimental procedures for all experiments are illustrated in Table 1.

3.3.5. Experiment 1A: The Effect of Spaced Context Extinction on Reinstatement

We tested the effect of extinguishing the context-alcohol association formed during the alcohol re-exposure session on reinstatement. After intermittent alcohol access and habituation, rats (n = 36) received 16 Pavlovian conditioning sessions, then seven extinction sessions. Four hours after each extinction session, all rats were habituated to a covered plastic bucket containing Sani-chip bedding for 20 min ('alternate context'). One alcohol re-exposure session was conducted 24 h after the last extinction session. Rats were then divided into three groups matched on body weight, ΔCS port entries, and total port entries across Pavlovian conditioning and extinction sessions.

Starting 24 h after alcohol re-exposure, each group received four context exposure sessions across four consecutive days, as this design has been shown to attenuate the reinstatement of operant responding for food-pellets[113]. Rats in the 'Context extinction' group (n

= 12) were placed into the conditioning chambers for daily context extinction sessions in which house light onset occurred after a 2 min delay, but no CS or alcohol were delivered. Rats in the 'No port' group (n = 12) received identical sessions; however, the fluid port was replaced with a metal panel. This control group was included to account for the possible effect on reinstatement of extinguishing consummatory port entry responses, rather than extinguishing the context-alcohol association. Rats in the 'No extinction' group (n = 12) were placed in the alternate context for the same duration as both other groups. At 24 h after the last context exposure session, all groups received a reinstatement test in the conditioning chambers.

Table 1. Experimental Designs.

Experiment 1A

Group	Conditioning	Extinction[a]	Alcohol re-exposure	Context exposure	Test
	16 sessions	7 sessions	1 session	4 sessions (1/day)	1 session
No extinction	CS+US	CS	US	Alternate context	CS
No port	CS+US	CS	US	Conditioning chamber (no port)	CS
Context extinction	CS+US	CS	US	Conditioning chamber	CS

Experiment 1B

Group	Conditioning	Extinction[a]	Alcohol re-exposure	Context exposure	Test
	2 sessions	5 sessions	1 session	4 sessions (2/day)	1 session
No extinction	CS+US	CS	US	Alternate context	CS
No US	CS+US	CS	No US	Conditioning chamber or alternate context	CS
Context extinction	CS+US	CS	US	Conditioning chamber	CS

Experiment 2A

Group	Conditioning	Extinction[b]	Alcohol re-exposure	Test
	16 sessions	7 sessions	1 session	1 session
Different	CS+US (Context A)	CS (Context A)	US (Context B)	CS (Context A)
Same	CS+US (Context A)	CS (Context A)	US (Context A)	CS (Context A)

Experiment 2B

Group	Conditioning	Extinction[c]	Alcohol re-exposure	Test
	2 sessions	5 sessions	3 sessions	1 session
Different	CS+US (Context A)	CS (Context A)	US (Context B)	CS (Context A)
Same	CS+US (Context A)	CS (Context A)	US (Context A)	CS (Context A)

[a] Experiment 1A and 1B: Habituation to the alternate context was conducted after every extinction session.

[b] Experiment 2A: Context B habituation sessions were conducted after extinction sessions 5 and 6.

[c] Experiment 2B: Context B habituation sessions were conducted after extinction session 3 and 4.

3.3.6. Experiment 1B: The Effect of Massed Context Extinction on Reinstatement

Because all groups in Experiment 1A showed reinstated conditioned responding to the CS, we tested a massed context extinction design which has been shown to attenuate the reinstatement of conditioned responding to a shock-predictive CS[50]. We reasoned that massed context extinction would be more effective than spaced context extinction at extinguishing the context-alcohol association, as massed extinction trials produce a more rapid reduction in conditioned responding[160], and more robust extinction that results in short-term reductions in conditioned responding[161]. However, spaced extinction trials better protect against a long-term return of conditioned responding relative to massed trials[160,162,163], which may be the result of a short intertrial interval in the latter producing a weaker extinction association[160].

Rats from Experiment 1A (n = 35) received two Pavlovian conditioning sessions, then five extinction sessions with habituation to the alternate context occurring four hours after every extinction session. Rats were then divided into the same three groups that they were previously assigned to in Experiment 1A. The 'Context extinction' (n = 12) and 'No extinction' (n = 11) groups received one alcohol re-exposure session followed by context extinction or exposure to the alternate context, respectively, as in Experiment 1A. Rats that were previously in the 'No port' group were included in a 'No US' control group (n = 12). This important control group was included to assess if an extinction-to-test delay or spontaneous recovery might contribute to increased responding at test. Rats in this group were placed in conditioning chambers with the house light illuminated but did not get alcohol during the alcohol re-exposure session. Next, half the rats received exposure to the conditioning chambers while the remainder received exposure to the alternate context. In this experiment, context exposure sessions were conducted across two days for all groups. The first session occurred at the time that previous phases of training had been conducted (1300 h) and the second session occurred at 1700 h. All groups received a test for reinstatement in the conditioning chambers 24 h after the last context exposure session.

3.3.7. Experiment 2A: The Effect of One Alcohol Re-Exposure Session in a Different Context on Reinstatement

We examined the effect of conducting alcohol re-exposure in a context that differed from the reinstatement test context on reinstatement. After intermittent alcohol access and habituation, rats (n = 28) received 16 Pavlovian conditioning sessions, then seven extinction sessions that

were all conducted in Context A. Four hours after the second-to-last and the third-to-last extinction sessions, rats were habituated to Context B for 20 min. Context A and B configurations were counterbalanced across Context Type 1 (grid floor, almond scent, transparent doors) and Context Type 2 (Perspex floor, lemon scent, black opaque doors). After extinction, rats were divided into two groups that were matched on body weight, ΔCS port entries, and total port entries, made across Pavlovian conditioning and extinction sessions. The 'Same' group (n = 14) received one alcohol re-exposure session in Context A, while the 'Different' group (n = 14) received one alcohol re-exposure session in Context B. At 24 h later, both groups were tested for reinstatement in Context A.

3.3.8. Experiment 2B: The Effect of Three Alcohol Re-Exposure Sessions in a Different Context on Reinstatement

Because in Experiment 2A both groups showed reinstatement, we tested the possibility that one alcohol re-exposure session was not sufficient for rats to discriminate between Contexts A and B. Consequently, we examined the effect of three alcohol re-exposure sessions in a different context on reinstatement. Rats from Experiment 2A (n = 28) received two Pavlovian conditioning sessions, then five extinction sessions in Context A. The third-to-last and second-to-last extinction sessions were followed by habituation to Context B, as described above. Rats were then divided into the same two groups that they were previously assigned to in Experiment 2A. The 'Same' group (n = 14) then received three alcohol re-exposure sessions in Context A, while the 'Different' group (n = 14) received three alcohol re-exposure sessions in Context B. At 24 h later, both groups were tested for reinstatement in Context A.

3.3.9. Data Management

Exclusion criteria. Rats did not transition from intermittent alcohol access to behavioural training if they drank <1 g/kg averaged across the last three sessions of intermittent alcohol access (Experiment 1; n = 1: Experiment 2; n = 0), received 10% alcohol on session 13 and onwards (Experiment 1; n = 1: Experiment 2; n = 0), or displayed aggressive behaviour (Experiment 1; n = 1: Experiment 2; n = *0*).

Following training, we used a behavioural criterion based on the probability of making a CS port entry [# of trials with ≥1 CS port entry / # of CS trials) * 100] to evaluate if rats had

learned to associate the CS with alcohol. Rats that responded on 70% or fewer trials averaged across the last two Pavlovian conditioning sessions were removed from statistical analyses as they were deemed to have not acquired the task (Experiment 1A, $n = 3$; Experiment 1B, $n = 4$; Experiment 2A, $n = 3$; Experiment 2B, $n = 3$). We also evaluated extinction and removed rats that were still responding to the CS on 60% or more of the trials averaged across the last two extinction sessions, as they were deemed to have not extinguished their conditioned responding (Experiment 1A, $n = 2$; Experiment 1B, $n = 3$; Experiment 2A, $n = 3$; Experiment 2B, $n = 2$). One rat from Experiment 1A became highly aggressive during training and was removed from the study. Table 2 depicts initial and final sample sizes for all experiments.

Variables. Our dependent variables were ΔCS port entries (CS port entries minus port entries during the 20 s pre-CS interval), total duration of CS port entries (length of time (s) spent in the fluid port summed across CS trials), and average latency to make a CS port entry (time (s) to initiate the first port entry during each CS trial averaged across CS trials). If a port entry was not made during a CS trial, a latency value of 20 s was used[146,164].

3.3.10. Statistical Analyses

Responding at test was compared to an extinction baseline obtained by averaging data across the last two extinction sessions. Data from the context extinction sessions in Experiment 1A and 1B were analyzed using a repeated measures analysis of variance (ANOVA). Data from the reinstatement test were analyzed using a mixed Phase x Group ANOVA and a mixed Trial x Group ANOVA. The Huynh-Feldt correction was applied when Mauchly's test of sphericity was violated. All post-hoc analyses were corrected for multiple comparisons with Scheffe's method. Statistical analyses were conducted with IBM SPSS (Version 23; IBM Corp., Armonk, NY) and evaluated using a statistical significance level of $p < 0.05$. Graphs were created with Graphpad Prism (Version 7; La Jolla, CA).

Table 2. Sample size across experimental phases.

	Intermittent alcohol access		Conditioning		Extinction	
Exp.	Initial	Dropped	Initial	Dropped	Dropped	**Final sample size**
1A	*n* = 39	*n* = 3[a]	Context extinction *n* = 12 No extinction *n* = 12 No port *n* = 12	*n* = 3	*n* = 3	Context extinction *n* = 10 No extinction *n* = 9[b] No port *n* = 11
1B	N/A	N/A	Context extinction *n* = 12 No extinction *n* = 11 No US *n* = 12	*n* = 4	*n* = 3	Context extinction *n* = 10 No extinction *n* = 9 No US *n* = 9
2A	*n* = 28	*n* = 0	Same *n* = 14 Different *n* = 14	*n* = 3	*n* = 3	Same *n* = 10 Different *n* = 12
2B	N/A	N/A	Same *n* = 14 Different *n* = 14	*n* = 3	*n* = 2	Same *n* = 12 Different *n* = 11

[a] Rats dropped because of <1 g/kg across last three intermittent access sessions (*n* = 1), received 10% alcohol on session 13 or onwards (*n* = 1), aggressive (*n* = 1).

[b] Rat dropped because aggressive (*n* = 1).

3.4. Results

3.4.1. Acquisition and Extinction of Conditioned Responding

Alcohol intake increased across intermittent alcohol access sessions in Experiment 1 and Experiment 2 (Supplementary Figure 1). Rats learned to associate the CS with alcohol, as ΔCS port entries significantly increased across Pavlovian conditioning sessions in Experiments 1 and 2 (Supplementary Figure 2). ΔCS port entries significantly decreased across extinction sessions in Experiment 1 and 2 (Supplementary Figure 2).

3.4.2. Experiment 1A: Spaced Context Extinction Did Not Affect Reinstatement

Following Pavlovian conditioning, extinction and alcohol re-exposure, rats received four daily sessions (i.e., spaced context extinction) of exposure to an alternate context ('No extinction'), the conditioning chambers ('Context extinction'), or the conditioning chambers without fluid ports ('No port'). Total port entries made by the 'Context extinction' group showed a significant reduction (Figure 1A) across context extinction sessions [$F_{(3, 27)} = 2.99$, $p = .048$], suggesting that context extinction had occurred.

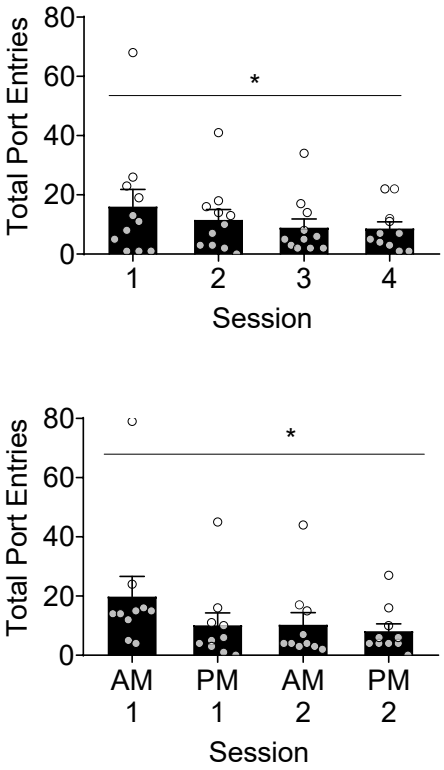


Figure 1. Conducting spaced or massed context extinction sessions significantly reduced total port entries across sessions. Data represent mean (± SEM) total port entries from rats in the 'Context extinction' group in **(A)** Experiment 1A (*n* = 10), and **(B)** Experiment 1B (*n* = 10). Open circles depict data of individual rats.

* $p < 0.05$, main effect of Session (1 – 4)

Following spaced context exposure sessions, all groups reinstated to a similar degree. Relative to extinction, ΔCS port entries (Figure 2A) significantly increased at test [Phase: $F_{(1, 27)} = 46.64$, $p < .001$] in all three groups [Group: $F_{(2, 27)} = 0.15$, $p = .865$; Phase x Group: $F_{(2, 27)} = 0.40$, $p = .673$]. Similar effects were found in the average latency to initiate the first CS port entry (Figure 2B) and the total duration of CS port entries (Figure 2C). At test, all groups showed a significant decrease in latency to CS port entries [Phase: $F_{(1, 27)} = 34.60$, $p < .001$; Group: $F_{(2, 27)} = 0.57$, $p = .574$; Phase x Group: $F_{(2, 27)} = 1.04$, $p = .367$], and a significant increase in the duration of CS port entries [Phase: $F_{(1, 27)} = 46.45$, $p < .001$; Group: $F_{(2, 27)} = 0.76$, $p = .480$; Phase x Group: $F_{(2, 27)} = 0.25$, $p = .785$], relative to extinction.

To determine if spaced context extinction affected the pattern of responding at test, we analyzed port entries as a function of CS trial. ΔCS port entries (Figure 2D) significantly decreased across CS trials due to within-session extinction [Trial: $F_{(5.097, 137.626)} = 10.82$, $p < .001$] with no differences between groups [Group: $F_{(2, 27)} = 0.20$, $p = .817$; Trial x Group: $F_{(10.195, 137.626)} = 0.38$, $p = .956$]. The average latency to initiate the first CS port entry (Figure 2E) significantly increased across CS trials [Trial: $F_{(7, 189)} = 7.69$, $p < .001$] in all groups [Group $F_{(2, 27)} = 0.89$, $p = .421$; Trial x Group: $F_{(14, 189)} = 0.97$, $p = .476$]. The total duration of CS port entries (Figure 2F) significantly decreased across CS trials [Trial: $F_{(3.923, 105.909)} = 12.14$, $p < .001$] in all groups [Group: $F_{(2, 27)} = 0.45$, $p = .641$; Trial x Group: $F_{(7.845, 105.909)} = 0.98$, $p = .454$]. These results indicate that spaced context extinction had no effect on reinstatement, despite producing a significant reduction in total port entries across context extinction sessions.

Spaced context extinction did not affect reinstatement

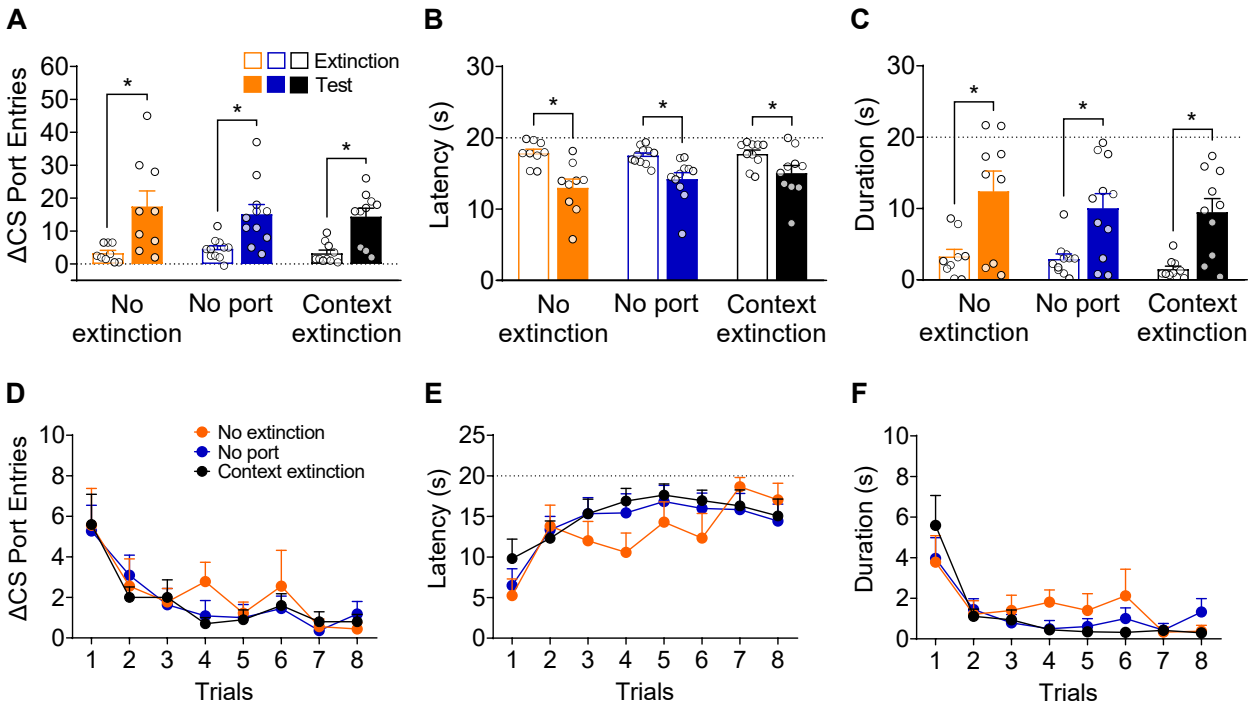


Figure 2. Conducting spaced context extinction after alcohol re-exposure did not reduce reinstatement. Data are from rats that received context exposure in an alternate context (No extinction; Orange; *n* = 9), the training context without fluid ports (No port; Blue; *n* = 11), or the training context (Context extinction; Black; *n* = 10). **A - C** Mean (± SEM) responding during extinction and test for **(a)** ΔCS port entries, **(b)** average latency to initiate the first CS port entry, and **(c)** total duration of CS port entries. **D - F** Mean (± SEM) responding across CS trials at test for **(d)** ΔCS port entries, **(e)** latency to initiate the first CS port entry, and **(f)** duration of CS port entries.

* $p < 0.05$, main effects of **A – C** Phase (Extinction vs. Test)

3.4.3. Experiment 1B: Massed Context Extinction Prevented Reinstatement

Following the reinstatement test, rats from Experiment 1A went through Pavlovian conditioning, extinction and alcohol re-exposure. Next, they received four sessions, conducted two times per day (i.e., massed context extinction), of exposure to an alternate context ('No extinction') or the conditioning chambers ('Context extinction'). An additional control group ('No US') did not receive alcohol re-exposure and was then exposed to either the alternate context or the conditioning chambers. As responding in these two subgroups was similar at test, their data were collapsed into one group (i.e., 'No US') for statistical analyses. Total port entries made by the 'Context extinction' group showed a significant reduction (Figure 1B) across context extinction sessions [$F_{(1.1721, 15.488)} = 5.15$, $p = .023$] suggesting that context extinction had occurred.

Conducting massed context extinction sessions after alcohol re-exposure prevented reinstatement (Figure 3A). Relative to extinction, ΔCS port entries significantly increased at test [Phase: $F_{(1, 25)} = 23.45$, $p < .001$]. There was no overall effect group [Group: $F_{(2, 25)} = 2.57$, $p = .097$]; however, reinstatement differed between groups as a function of phase [Phase x Group: $F_{(2, 25)} = 6.84$, $p = .004$]. Post-hoc analyses revealed that the 'No extinction' group made significantly more ΔCS port entries at test relative to extinction ($p < .001$), whereas the 'Context extinction' ($p = .093$) and 'No US' ($p = .509$) groups did not. The latency to initiate the first CS port entry (Figure 3B) significantly decreased at test [Phase: $F_{(1, 25)} = 20.94$, $p < .001$]. There was no overall effect of group [Group: $F_{(2, 25)} = 2.18$, $p = .134$]; however, reinstatement differed between groups as a function of phase [Phase x Group: $F_{(2, 25)} = 9.09$, $p = .001$]. Post-hoc analyses revealed that the 'No extinction' group was significantly quicker to initiate a CS port entry at test relative to extinction ($p < .001$), whereas the 'Context extinction' ($p = .245$) and 'No US' ($p = .797$) groups were not. The total duration of CS port entries (Figure 3C) significantly increased at test [Phase: $F_{(1, 25)} = 11.01$, $p = .003$] with no main effect of group or significant interaction [Group: $F_{(2, 25)} = 1.93$, $p = .166$; Phase x Group: $F_{(2, 25)} = 2.60$, $p = .094$].

In an analysis of responding as a function of CS trial at test, ΔCS port entries (Figure 3D) significantly decreased across CS trials [Trial: $F_{(2.875, 71.874)} = 14.13$, $p < .001$]. This effect differed as a function of group [Group: $F_{(2, 25)} = 4.83$, $p = .017$] with no significant interaction [Trial x Group: $F_{(5.750, 71.874)} = 0.88$, $p = .512$]. Post-hoc analyses revealed that the main effect of Group was driven by significantly more ΔCS port entries in the 'No extinction' group compared to the

'Context extinction' group ($p = .002$) or the 'No US' group ($p < .001$). The ΔCS port entries in the 'No US' and 'Context extinction' groups did not differ ($p = .809$). The average latency to initiate the first CS port entry (Figure 3E) significantly increased across CS trials [Trial: $F_{(6.690, 167.240)} = 1.98$, $p < .001$]; however, this effect differed as a function of group [Group: $F_{(2, 25)} = 5.40$, $p = .011$] with no significant interaction [Trial x Group: $F_{(12.379, 167.240)} = 1.13$, $p = .334$]. Post-hoc analyses revealed that the main effect of Group was driven by the 'No extinction' group making CS port entries more quickly than the 'Context extinction' group ($p < .001$) and the 'No US' group ($p < .001$). There was no significant difference between the 'Context extinction' and the 'No US' groups ($p = .694$). The total duration of CS port entries (Figure 3F) significantly decreased across CS trials [Trial: $F_{(4.134, 103.358)} = 5.07$, $p = .001$] in all three groups [Group: $F_{(2, 25)} = 2.83$, $p = .078$; Trial x Group: $F_{(8.269, 103.358)} = 0.54$, $p = .832$]. These results, obtained across multiple measures of conditioning, show that the massed context extinction procedure significantly attenuated reinstatement.

Massed context extinction prevented reinstatement

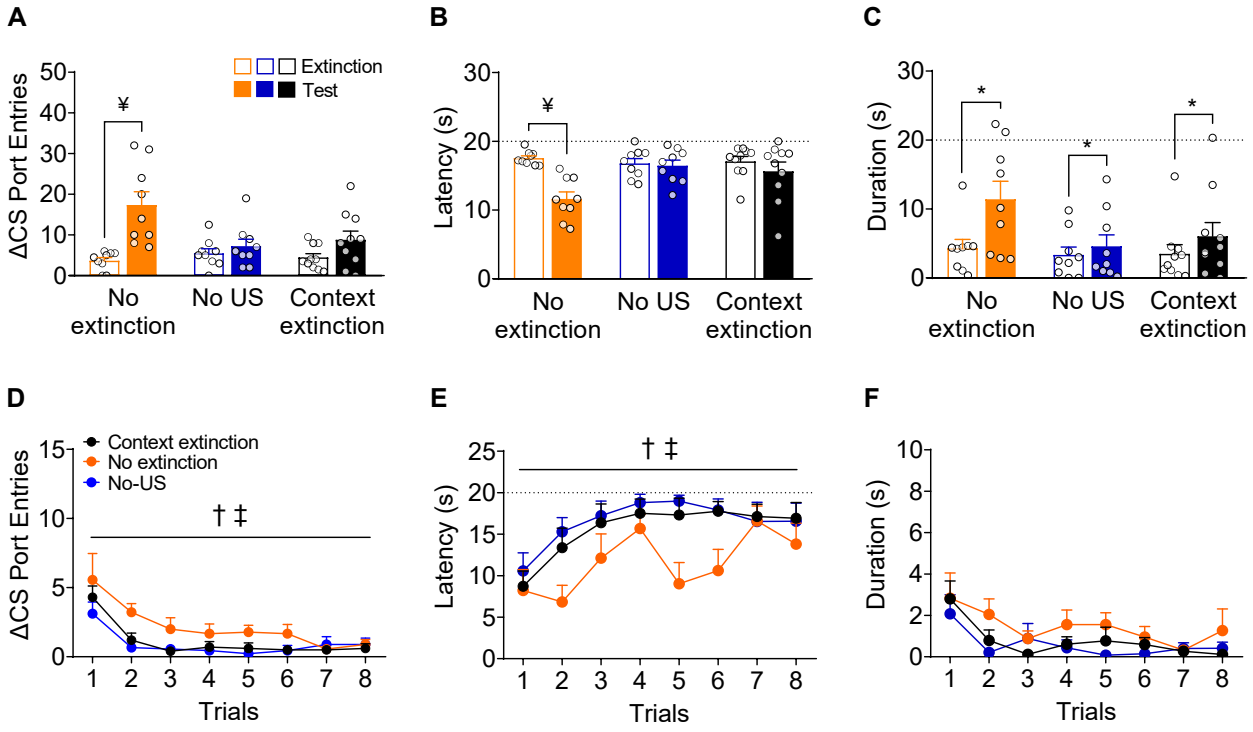


Figure 3. Conducting massed context extinction after alcohol re-exposure prevented reinstatement. Data are from rats that received context exposure in an alternate context (No extinction; Orange; *n* = 9), in the training context (Context extinction; Black; *n* = 10), or received no alcohol re-exposure then context exposure to the alternate or training context (No US; Blue; *n* = 9). **A – C** Mean (± SEM) responding during extinction and test for **(a)** ΔCS port entries, **(b)** average latency to initiate the first CS port entry, and **(c)** total duration of CS port entries. **D - F** Mean (± SEM) responding across CS trials at test for **(d)** ΔCS port entries, **(e)** latency to initiate the first CS port entry, and **(f)** duration of CS port entries.

¥ $p < 0.05$, Phase x Group interaction post-hoc (Extinction vs. Test)

* $p < 0.05$, main effects of **A – C** Phase (Extinction vs. Test)

‡ $p < 0.05$, main effect of Group post-hoc (No US vs. No extinction)

† $p < 0.05$, main effect of Group post-hoc (Context extinction vs. No extinction)

3.4.4. Experiment 2A: One Alcohol Re-Exposure Session in a Different Context Did Not Affect Reinstatement

Following Pavlovian conditioning and extinction in Context A, rats received one alcohol re-exposure session in either Context A ('Same' group) or Context B ('Different' group), followed by a reinstatement test in Context A.

Counter to our predictions, reinstatement was observed following alcohol re-exposure outside of the training context. Relative to extinction, ΔCS port entries (Figure 4A) significantly increased at test [Phase: $F_{(1, 20)} = 34.65$, $p < .001$] in both groups [Group: $F_{(1, 20)} = 0.04$, $p = .837$; Phase x Group: $F_{(1, 20)} = 0.01$, $p = .941$]. The average latency to initiate the first CS port entry (Figure 4B) significantly decreased at test [Phase: $F_{(1, 20)} = 32.83$, $p < .001$] in both groups [Group: $F_{(1, 20)} = 0.83$, $p = .374$; Phase x Group: $F_{(1, 20)} = 0.13$, $p = .723$]. The total duration of CS port entries (Figure 4C) significantly increased at test [Phase: $F_{(1, 20)} = 17.01$, $p < .001$] in both groups [Group: $F_{(1, 20)} = 0.22$, $p = .647$; Phase x Group: $F_{(1, 20)} = 2.19$, $p = .155$].

At test, ΔCS port entries (Figure 4D) significantly decreased across CS trials due to within session extinction [Trial $F_{(7, 140)} = 3.24$, $p = .003$] in both groups [Group: $F_{(1, 20)} = 0.03$, $p = .874$; Trial x Group: $F_{(7, 140)} = 0.60$, $p = .754$]. The average latency to initiate the first CS port entry (Figure 4E) significantly increased across CS trials [Trial: $F_{(7, 140)} = 2.83$, $p = .009$] in both groups [Group: $F_{(1, 20)} = 0.54$, $p = .470$; Trial x Group: $F_{(7, 140)} = 0.56$, $p = .786$]. The total duration of CS port entries (Figure 4F) also significantly decreased across CS trials [Trial: $F_{(5.249, 104.984)} = 3.07$, $p = .011$] in both groups [Group: $F_{(1, 20)} = 0.91$, $p = .351$; Trial x Group: $F_{(5.249, 104.984)} = 0.50$, $p = .786$]. These results show that conducting one alcohol re-exposure session in a different context did not affect reinstatement.

One alcohol re-exposure session in a different context did not prevent reinstatement

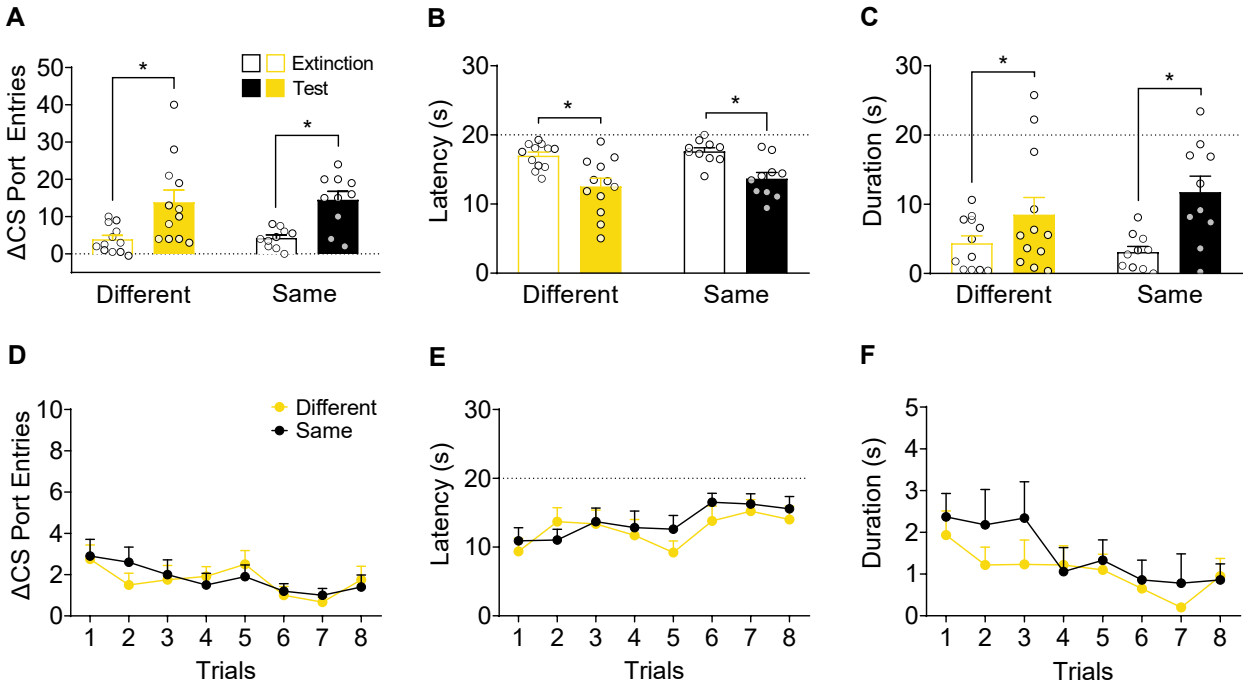


Figure 4. Conducting one alcohol re-exposure session in a different context did not reduce reinstatement. Data are from rats that received one alcohol re-exposure session in Context B (Different; Yellow; *n* = 12) or Context A (Same; Black; *n* = 10). All rats were tested in Context A. **A – C** Mean (± SEM) responding during extinction and test for **(a)** ΔCS port entries, **(b)** average latency to initiate the first CS port entry, and **(c)** total duration of CS port entries. **D – F** Mean (± SEM) responding across CS trials at test for **(d)** ΔCS port entries, **(e)** latency to initiate the first CS port entry, and **(f)** duration of CS port entries.

* $p < 0.05$, main effects of **A – C** Phase (Extinction vs. Test)

3.4.5. Experiment 2B: Three Alcohol Re-Exposure Sessions in a Different Context Reduced Reinstatement During the First CS Trial

Rats from Experiment 2A received additional Pavlovian conditioning and extinction in Context A, followed by three alcohol re-exposure sessions in either Context A ('Same' group) or Context B ('Different' group), then a reinstatement test in Context A.

Conducting three alcohol re-exposure sessions in a different context reduced reinstatement during the first CS trial. An analysis conducted on data averaged across the full test session showed that relative to extinction, ΔCS port entries (Figure 5A) significantly increased at test [Phase: $F_{(1, 21)} = 56.29$, $p < .001$] in both groups [Group: $F_{(1, 21)} = 0.02$, $p = .882$; Phase x Group: $F_{(1, 21)} = 0.29$, $p = .597$]. The average latency to initiate the first CS port entry (Figure 5B) significantly decreased at test [Phase: $F_{(1, 21)} = 91.16$, $p < .001$] in both groups [Group: $F_{(1, 21)} = 0.01$, $p = .929$; Phase x Group: $F_{(1, 21)} = 1.26$, $p = .274$]. The total duration of CS port entries (Figure 5C) also significantly increased at test [Phase: $F_{(1, 21)} = 27.01$, $p < .001$] in both groups [Group: $F_{(1, 21)} = 0.54$, $p = .472$; Phase x Group: $F_{(1, 21)} = 1.46$, $p = .240$].

At test, ΔCS port entries (Figure 5D) significantly decreased across CS trials [Trial: $F_{(7, 147)} = 3.26$, $p = .003$]. Although there was no effect of group [Group: $F_{(1, 21)} = 0.14$, $p = .717$], ΔCS port entries differed between groups as a function of CS trial [Trial x Group: $F_{(7, 147)} = 2.44$, $p = .022$]. Post-hoc analyses revealed that the 'Different' group made significantly fewer ΔCS port entries during the first CS trial, compared to the 'Same' group ($p = .010$). The average latency to initiate the first CS port entry and the total duration of CS port did not, however, follow this pattern of responding. The average latency to initiate the first CS port entry (Figure 5E) significantly increased across CS trials [Trial: $F_{(7, 147)} = 6.31$, $p < .001$] in both groups [Group: $F_{(1, 21)} = 0.42$, $p = .524$; Trial x Group: $F_{(7, 147)} = 1.57$, $p = .150$]. The total duration of CS port entries (Figure 5F) significantly decreased across CS trials [Trial: $F_{(6.269, 131.655)} = 2.81$, $p = .012$] in both groups [Group: $F_{(1, 21)} = 0.95$, $p = .340$; Trial x Group: $F_{(6.269, 131.655)} = 1.67$, $p = .130$]. Thus, conducting three alcohol re-exposure sessions in a different context modestly reduced reinstatement, as seen by a reduction in ΔCS port entries during the first CS trial.

Three alcohol re- exposure sessions in a different context reduced reinstatement in the first CS trial

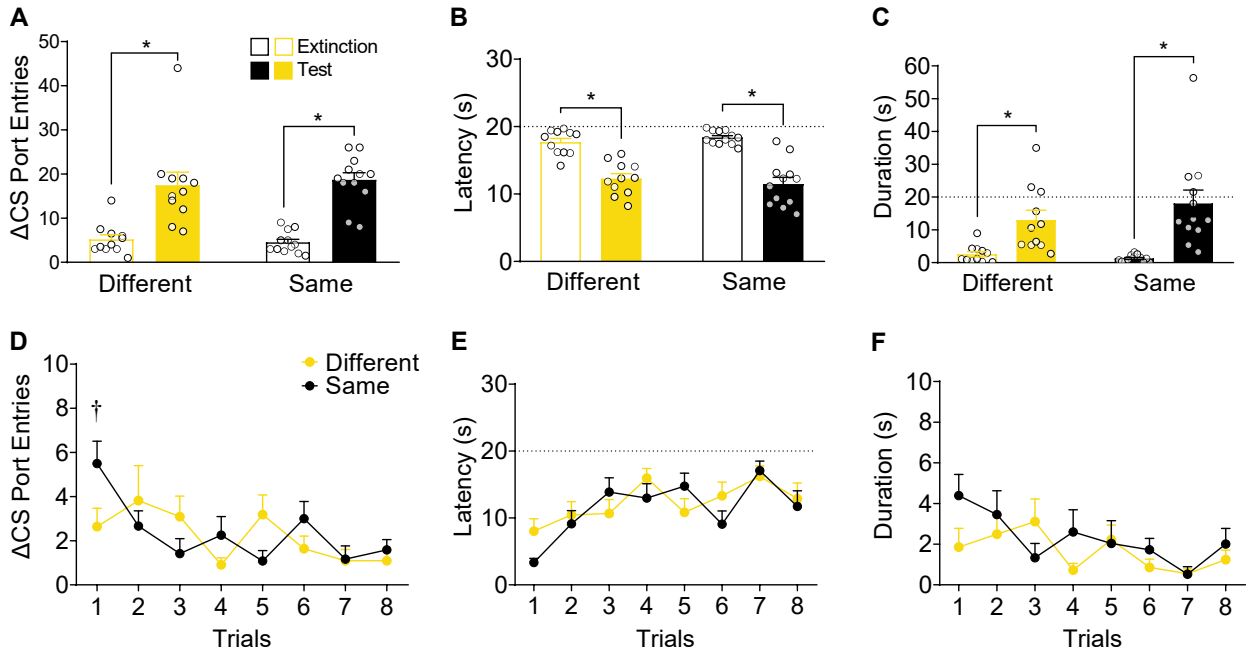


Figure 5. Conducting three alcohol re-exposure sessions in a different context reduced reinstatement during the first CS trial at test. Data are from rats that received three alcohol re-exposure sessions in Context B (Different; Yellow; *n* = 11) or Context A (Same; Black; *n* = 12). All rats were tested in Context A. **A - C** Mean (± SEM) responding during extinction and test for **(a)** ΔCS port entries, **(b)** average latency to initiate the first CS port entry, and **(c)** total duration of CS port entries. **D - F** Mean (± SEM) responding across CS trials at test for **(d)** ΔCS port entries, **(e)** latency to initiate the first CS port entry, and **(f)** duration of CS port entries.

* $p < 0.05$, main effects of **A – C** Phase (Extinction vs. Test)

† $p < 0.05$, Group x Trial interaction post-hoc (Different vs. Same on CS trial 1)

3.5. Discussion

This study examined the psychological processes involved in the reinstatement of responding to an appetitive, alcohol-predictive CS. We found that spaced context extinction did not affect reinstatement, whereas massed context extinction prevented reinstatement. Moreover, conducting one alcohol re-exposure session in a context that differed from the subsequent test context had no effect on reinstatement, whereas conducting three alcohol re-exposure sessions in a different context from the test context reduced the reinstatement of port entries during the first CS trial. Together, these findings partially support a view generated from aversive Pavlovian conditioning procedures, which posits that reinstatement is mediated by a context-US association that forms during US re-exposure.

In Experiment 1A, we extinguished the context-alcohol association formed during the alcohol re-exposure session by exposing rats to the conditioning chambers that alcohol re-exposure was conducted in across four daily sessions ('spaced context extinction'). Total port entries decreased across sessions in this group, suggesting that context extinction had occurred. Control groups were exposed either to the conditioning chambers without the fluid ports or to an alternate context for the same duration. Counter to our expectations, all three groups showed significant reinstatement as measured by ΔCS port entries, CS port entry duration, and CS port entry latency. These results differ from evidence that a comparable, spaced context extinction manipulation diminished the reinstatement of operant food-seeking[113]. Thus, a context-US association formed during US re-exposure may be involved in the reinstatement of operant responding for a food reinforcer, but not in the reinstatement of Pavlovian responding to an alcohol-predictive CS. Alternatively, our context extinction procedure may not have sufficiently extinguished the context-alcohol association, despite producing a decrease in total port entries across context extinction sessions.

To evaluate the latter possibility, we tested the effect of massed context extinction on reinstatement. Given that presenting CS trials in a temporally massed manner extinguishes conditioned responding to an aversive CS to a greater degree than temporally spaced CS trials[161], we hypothesized that conducting massed extinction sessions would deepen context extinction and reduce reinstatement. In Experiment 1B, rats were exposed to either the context that alcohol re-exposure occurred in or to an alternate context in four sessions delivered across two days. A third group that did not receive alcohol re-exposure and was then exposed to either the training or

alternate context served as a control for the potential spontaneous recovery of CS port entries after extinction. Interestingly, massed context extinction after alcohol re-exposure significantly reduced reinstatement, as indexed by ΔCS port entries and latency to make the first CS port entry, but not duration of CS port entries. Moreover, rats that did not receive alcohol re-exposure did not show changes in conditioned responding at test relative to extinction, indicating that spontaneous recovery did not contribute to reinstatement in our task. These results concur with previous work showing that context extinction conducted after US re-exposure reduced reinstatement to an aversive CS, and support the view that a context-US association formed during US re-exposure plays a role in reinstatement to an appetitive, alcohol-CS[50].

The findings of Experiment 1B suggest that context extinction sessions conducted after alcohol re-exposure prevented reinstatement; however, given that the same rats were used in Experiments 1A and 1B, this effect could be the cumulative result of repeated context extinction sessions across the two experiments. The robust reinstatement seen in the 'context extinction' group in Experiment 1A could be due to insufficient extinction of the context-US association, and additional spaced context extinction sessions may have prevented reinstatement as seen in Experiment 1B. Alternatively, there may be a difference in the efficacy of the spaced versus massed context extinction manipulations to extinguish the context-US association. Regardless of the precise mechanisms (i.e., a cumulative extinction effect or greater efficacy for the condensed extinction), our findings highlight the role of context conditioning in the reinstatement of responding to an appetitive CS. More research is needed to determine the nuanced effects of spaced versus massed context extinction sessions on reinstatement.

In Experiment 2A, we determined if conducting alcohol re-exposure in Context B, that differed from the subsequent test context (Context A), would impact reinstatement. Counter to our predictions, there was no effect of this manipulation on reinstatement as measured by ΔCS port entries, CS port entry duration, and CS port entry latency. Despite facilitating context discrimination with habituation to Context B, and our laboratory's long history of training rats to discriminate between the two identical context configurations used in the current study[42,53,54,115,153,165–167], reinstatement following alcohol re-exposure in Context B may be the result of context generalization. Context B and the test context and differed in terms of sensory stimuli (i.e., odour, tactile, visual); however, the innate features of the conditioning chambers were consistent across contexts (i.e., house light, speaker, and fluid port position). These

similarities could have resulted in a context-alcohol association formed in Context B generalizing to the test context.

To address this possibility that rats were unable to sufficiently discriminate between Context B and the test context, in Experiment 2B, we tested the effect of conducting three alcohol re-exposure sessions in Context B on subsequent reinstatement in Context A. This manipulation had no effect on reinstatement as assessed by data averaged across the full test session. However, it significantly reduced ΔCS port entries during the first CS trial at test. Although a seemingly modest effect, in aversive Pavlovian conditioning tasks learning is sometimes assessed after just one CS-US trial[168–170], and responding at test is sometimes assessed in one or two CS trials[50,170,171]. Arguably, conditioned responding elicited by the first CS trial at test may be the best indicator of an animal's expectation regarding whether or not the US will occur.

Our findings suggest that conducting US re-exposure in a context that differs from the test context may not robustly reduce reinstatement to an appetitive CS, which differs from previous reports using aversive conditioning procedures[50,111,133,134]. One consideration with the experimental design of Experiment 2, however, is that the same rats were used in Experiments 2A and 2B. Therefore, the reduction in reinstatement observed during CS trial 1 in the 'Different' group may have been the result of repeated testing under the same conditions. It is possible that under these conditions, even one alcohol re-exposure session could have evoked a difference in reinstatement between groups. Alternatively, it is possible that, due to the potential generalization between Context B and the test context, additional sessions of alcohol re-exposure were needed to unmask an effect on reinstatement. Future studies could assess the extent to which rats discriminate between Context B and the test context by conducting context preference tests after alcohol re-exposure. If rats that are re-exposed to alcohol in Context B show a similar preference for Context B and the test context, this would suggest that the context-US association has generalized across the two distinct contexts.

Several published studies conducted using aversive conditioning procedures have shown that conducting US re-exposure in a context that differs from the test context prevents reinstatement[50,111,133,134]. One interpretation of these data is that reinstatement does not occur because the context-US association formed during US re-exposure is not present at test to summate with the residual predictive value of the CS that survived extinction to drive reinstatement. An alternate hypothesis regarding the reinstatement effect is that reinstatement

may be due to the US re-exposure session reactivating the CS representation, as the context can become associated with the CS during previous Pavlovian conditioning and extinction training. This CS representation would be experienced in tandem with the US delivery during the US re-exposure session, which could result in a strengthened CS-US association and drive reinstatement 24 h later[56,172]. If the US re-exposure session were conducted in a different context, which was never associated with the CS through extinction training, the CS representation would not be activated. Therefore, the CS representation would not become associated with the US and would not produce subsequent reinstatement. According to either hypothesis, we should not have observed reinstatement when alcohol re-exposure was conducted in an alternate context. Interestingly, prior data has shown that re-exposure to a food-US in either the test context or a different context reinstated conditioned responding to a food-predictive CS; however, reinstatement was more robust when food re-exposure occurred in the same context as the subsequent reinstatement test[112]. Therefore, it is not surprising that we found some reinstatement of CS port entries at test following one or three alcohol re-exposure sessions in Context B.

A unique aspect of appetitive conditioning tasks is that a context-US, or strengthened CS-US, association may not be the sole mechanisms contributing to reinstatement. A consummatory response is required to voluntarily ingest an appetitive US like alcohol and this consummatory response may contribute to reinstatement. This possibility is supported by our previous work showing that re-exposure to water as a control condition reinstated responding to an alcohol-predictive CS to the same degree as re-exposure to alcohol, whereas when alcohol re-exposure occurred via systemic injection reinstatement was not observed[146]. This additional factor of a consummatory response does not occur in aversive conditioning tasks; therefore, this difference could account for discrepancies in our findings and previous findings using aversive conditioning procedures. Future studies could assess the impact of consummatory behaviour on the reinstatement of responding to an alcohol-predictive CS by delivering alcohol during the alcohol re-exposure session in a manner that produces a different consummatory response from that used during Pavlovian conditioning (e.g., via a sipper tube instead of in the fluid port).

Finally, an important consideration in the present research is that we only used male rats. Given the generality of the reinstatement effect and its importance in evaluating the role of cues in people with substance use disorders or post-traumatic stress disorders, it is critical for preclinical research to be conducted using both male and female subjects[173–176]. Our recent,

unpublished data show that female rats reinstate responding to a sucrose-predictive CS to a greater degree relative to male rats[177], and we are currently using male and female rats in ongoing experiments to study the role of μ-opioid receptors in reinstatement[177].

In conclusion, our findings show that a context-US association plays a role in the reinstatement of responding to an alcohol-predictive CS, thereby extending the literature on the psychological processes underlying reinstatement to an appetitive stimulus. However, more research is needed to elucidate the role that consummatory behaviours may play in this reinstatement effect. Thus, these findings provide the basis for future studies aimed at investigating processes that may uniquely underlie appetitive reinstatement.

Chapter 4: Blocking μ-Opioid Receptors Attenuates Reinstatement of Responding to an Alcohol-Predictive Conditioned Stimulus Through Actions in the Ventral Hippocampus

4.1. Abstract

The μ-opioid system is important for the reinstatement of responding that is immediately evoked by alcohol-predictive cues. The extent of this involvement in a novel model of reinstatement that evaluates the delayed effects of re-exposure to alcohol, however, is unclear. Therefore, we investigated the role of μ-opioid receptors (MORs) in the delayed reinstatement of an extinguished, Pavlovian conditioned response that was evoked 24 h after re-exposure to alcohol. We further investigated the necessity of MORs in the ventral hippocampus in this reinstatement effect. Female and male Long-Evans rats received Pavlovian conditioning in which a conditioned stimulus (CS; 20 s white-noise) was paired with the delivery of an appetitive unconditioned stimulus (US; Experiments 1, 2, and 4: 15% *v*/*v* alcohol; Experiment 3: 10% *w*/*v* sucrose) that was delivered into a fluid port for oral intake (0.3 ml/CS; 2.4 mL/session). During subsequent extinction sessions, the CS was presented as before but without the US. Next, the US was delivered as during Pavlovian conditioning, but without the CS. A reinstatement test was conducted 24 h later, during which the CS was presented in the absence of the US. Silencing MORs via pretest administration of systemic naltrexone (0.3 or 1.0 mg/kg; subcutaneous) attenuated reinstatement of port entries elicited by an alcohol-CS, without significantly affecting reinforced conditioned responding. Naltrexone did not, however, affect reinstatement of port entries elicited by a sucrose-CS. Finally, silencing MORs in the ventral hippocampus via bilateral microinfusion of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP; 2.5 or 5.0 μg/hemisphere) prevented reinstatement of port entries elicited by an alcohol-CS. Together, these data show that MORs are involved in the delayed reinstatement of a Pavlovian conditioned response in an alcohol-specific manner. Importantly, these data illustrate, for the first time, that MORs located in the ventral hippocampus are necessary for reinstatement of responding to an alcohol-predictive cue.

4.2. Introduction

An established theory in the research on alcohol use disorders is that environmental stimuli that accompany alcohol intake can become cues that predict alcohol availability. Consequently, exposure to alcohol-predictive cues can significantly influence human behaviour, such as evoking craving for alcohol and precipitating relapse[4,129,178]. Similarly in preclinical studies, exposure to various types of alcohol-predictive cues such as contexts[108,179], discrete cues[30,40], discriminative stimuli[36], and a small 'priming' dose of alcohol[33,35], have been shown to prompt the reinstatement of extinguished, operant and Pavlovian conditioned responding for alcohol. These reinstatement models are valuable tools that provide insight into how maladaptive behaviours in response to environmental cues contribute to alcohol use disorders and relapse in humans[129]. Therefore, it is essential to understand the neural mechanisms that drive reinstatement of responding to alcohol-predictive cues.

There is considerable evidence supporting the involvement of the endogenous opioid system in conditioned responding evoked by alcohol-predictive cues. One of the few pharmacotherapies approved for treating alcohol use disorders is the μ-opioid receptor (MOR) antagonist naltrexone, which reduces both alcohol intake and the likelihood of relapse[180,181]. It has been posited that naltrexone's efficacy is in part due to the treatment reducing cue-evoked craving for alcohol[3,182]. Similarly, in animal models, systemic administration of MOR antagonists like naltrexone and CTOP attenuate reinstatement of operant alcohol-seeking evoked by an alcohol-predictive context[82,95,183], discrete cue[83], discriminative stimulus[94,184,185], and alcohol-prime delivered via systemic injection[34]. Furthermore, the robust reinstatement of alcohol-seeking observed after the presentation of an alcohol-predictive discrete cue and an ingested alcohol-prime combination is reduced by naltrexone[186,187]. Blocking MORs does not affect other motivated behaviours such as reinstatement of alcohol-seeking evoked by stressful stimuli[34,83] or reinstatement of sucrose-seeking[188], thereby demonstrating that the capacity for MOR antagonists to reduce reinstatement is specific to alcohol-predictive cues.

While systemic administration of MOR antagonists consistently attenuates reinstatement of alcohol-seeking, the neural loci of this effect are less understood. Currently, the strongest evidence implicates brain regions that are substrates of the reward neurocircuitry, specifically the nucleus accumbens (NAc), basolateral amygdala (BLA), and ventral pallidum[189]. Administering the MOR antagonist CTAP into the NAc prevents context-induced reinstatement of alcohol-

seeking[190]; inversely, intra-NAc administration of the agonist DAMGO enhances cue-induced reinstatement[191]. The BLA is another promising neural locus, as neuronal activity in the BLA during context-induced reinstatement of alcohol-seeking was diminished with systemic naltrexone[95]. Furthermore, localized administration of the MOR antagonist naloxone into the BLA attenuates context-induced reinstatement of alcohol-seeking[96]. Finally, the reduction of context-induced reinstatement of alcohol-seeking following intra-ventral pallidum administration of CTAP provides evidence for the recruitment of MORs in the ventral pallidum in this behaviour[192]. Together, these findings implicate only a handful of brain regions as neural loci for MORs involved in reinstatement of responding to alcohol-predictive cues. The extent to which MORs in other brain regions contribute to reinstatement is unknown, despite there being various promising possibilities.

MORs in the hippocampus may also regulate responding to alcohol-predictive cues. The dorsal subregion of the hippocampus contributes to various forms of reinstatement of conditioned responding. For example, inactivation of the dorsal hippocampus (dHipp) abolishes context-induced reinstatement of a Pavlovian fear response[193], operant cocaine-seeking[92], and operant alcohol-seeking[93]. Moreover, context- or discriminative stimuli-induced reinstatement of alcohol-seeking is associated with neuronal activity in the dHipp, and this effect is reversed with systemic naltrexone[94,95]. Blocking MORs specifically in the dHipp with naloxone, however, does not affect context-induced reinstatement of alcohol-seeking[96]. MORs in the dHipp are therefore unlikely involved in mediating responding to alcohol-predictive cues. Alternatively, the ventral subregion of the hippocampus may be a more promising target, given that it is an integral substrate of the reward neurocircuitry[189] and has a rich expression of MORs[89,194]. Inactivating the ventral hippocampus (vHipp) attenuates context-induced reinstatement of cocaine-seeking[97], heroin-seeking[98], and alcohol-seeking[195], as well as prime- and cue-induced reinstatement of cocaine-seeking[99,100]. Despite this wealth of evidence, to date, the role of ventral hippocampal MORs in responding to alcohol-predictive cues has not been investigated.

The involvement of MORs in responding to alcohol-predictive cues has, overwhelmingly, been investigated using established operant reinstatement models. While the established reinstatement procedures used in the current literature are valuable tools to evaluate the immediate impact of exposure to alcohol-predictive cues on behaviour, they do not assess the delayed impact of exposure to such cues. A novel model of reinstatement addresses this issue. In

this model, following the acquisition and extinction of conditioned responding to an alcohol-predictive conditioned stimulus (CS), rats are re-exposed to alcohol. When tested 24 h later, responding to the CS in the absence of alcohol significantly reinstates[146,196]. This delayed reinstatement effect provides a unique insight into how exposure to alcohol affects Pavlovian conditioned responding to an extinguished alcohol-predictive cue at a future timepoint. Given the research on the role of MORs in responding to alcohol-predictive cues being primarily studied in established reinstatement models using operant tasks, the extent to which MORs are involved in the delayed reinstatement of Pavlovian conditioned responding to an alcohol-cue is uncertain.

We investigated the role of MORs in Pavlovian conditioned responding to an alcohol-predictive CS using the delayed reinstatement model. First, the effects of systemic naltrexone on the reinstatement of responding to an alcohol-CS were tested in male (Experiment 1) and female rats (Experiment 2). A separate control experiment examined the effect of systemic naltrexone on the reinstatement of responding to a sucrose-predictive CS (Experiment 3). Given that naltrexone attenuated reinstatement of responding to an alcohol-CS, the second aim of the present study was to examine the effects of administering the MOR antagonist, CTAP, into the vHipp on the reinstatement of responding to an alcohol-CS (Experiment 4).

4.3. Methodology

4.3.1. Subjects

Female and male Long-Evans rats (Envigo, Indianapolis, IN; 8 weeks on arrival) were same-sex pair-housed upon arrival, then single-housed three days later (Experiment 1: n = 15 males; Experiment 2: n = 18 females, n = 18 males; Experiment 3: n = 12 females, n = 12 males; Experiment 4: n = 13 females, n = 13 males). Rats were then handled for five days before experimental procedures began. Cages contained beta chip bedding (Aspen Sani chips, Envigo), a nylabone toy (Nylabones, Bio-Serv), a plastic tunnel (Rat retreats, Bio-Serv), and shredded paper. Unrestricted access to water and rat chow (Purina Agribrands, Charles River) was provided throughout all experiments. Cages were held in a temperature- (21.0° C) and humidity-controlled (40-50%) colony room that was on a 12 h light/dark cycle (lights on at 0700 h; all experiments were conducted during the light phase). All procedures were conducted following the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

4.3.2. Apparatus

Behavioural procedures were conducted in 12 conditioning chambers (ENV-009A; Med Associates Inc., St-Albans, VT) that were enclosed in sound-attenuating, ventilated melamine cubicles (made in house). Chambers were comprised of a Perspex front door and back-wall, stainless steel sidewalls, and a metal bar floor (ENV-009A-GF). A white-noise generator (ENV-225SM, 5 dB above background noise) and a white house light (ENV-215M; 75W, 100 mA) were mounted on the upper left chamber wall. A dual-cup, fluid port (ENV-200R3AM) was located off-centre on the right chamber wall. Alcohol (Experiments 1, 2, and 4) or sucrose (Experiment 3) was delivered to the fluid port via polyethylene tubing from a 20 ml syringe mounted on a syringe pump (PHM-100, 3.33 RPM) located outside the melamine cubicles. Port entries were measured via interruptions of an infrared beam that crossed the entrance of the fluid port. Med PC IV software on a PC controlled stimulus delivery and recorded behavioural responses.

4.3.3. Drugs and Solutions

Alcohol solutions (5%, 10%, 15%; *v*/*v*) were prepared by diluting 95% ethanol in tap water. A 10% (*w*/*v*) sucrose solution was prepared by dissolving sucrose (500070, Bioshop) in tap water. Naltrexone solutions were prepared the day of use by dissolving naltrexone hydrochloride (Sigma Aldrich; N3136) in sterile, physiological saline (0.9%) to obtain a 0.3 mg/ml or 1.0 mg/ml dose and was administered at a volume of 1 ml/kg. D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP; Tocris CAT# 1560) was dissolved in sterile, physiological saline (0.9%) to obtain a 2.5 µg/0.3 µl or 5.0 µg/0.3 µl dose and was administered at a volume of 0.3 µl/hemisphere. Aliquots of each dose were premade and stored at -20°C until use.

4.3.4. Surgery

After 12 sessions of intermittent alcohol access (described below), rats underwent stereotaxic surgery using standard procedures[197] to bilaterally implant stainless steel cannulae (26 gauge; Plastics One C235G-1.2-SPC) into the ventral hippocampus. Coordinates used for the ventral hippocampus were -5.5 mm anterior-posterior (AP), ±5.4 mm medial-lateral (ML), and -3.0 ventral from the skull surface. During subsequent intracranial drug infusions, the injector tip (Plastics One C235I-SPC) protruded 3.0 mm below the cannula base, resulting in a final ventral

coordinate of -6.0 mm. Ventral hippocampus coordinates were based on previous studies[198,199] and the 2007 Paxinos and Watson rat brain atlas[200]. After surgery, guide cannulae were occluded with 7.5 mm dummy canulae and dust cap. Postsurgical pain was managed with buprenorphine (Buprenex; 0.1 mg/kg, subcutaneous), and rats were monitored daily to ensure recovery and regular weight gain. Three additional intermittent alcohol access sessions were conducted 1 week after surgery.

4.3.5. Intracranial Drug Microinfusions

Bilateral microinfusions of saline or CTAP were conducted using standard procedures[49,197]. Microinfusions were administered with a 26 gauge injector attached to polyethylene tubing (PE20, VWR, CA-63 018-645) that was connected to a 10 µL Hamilton syringe (Hamilton, 1701N). Microinfusions were delivered by a syringe pump (Pump 11 Elite, Harvard Apparatus, 704 501) at a volume of 0.3 µl/hemisphere at a rate of 0.3 µl/min. Injectors remained in place for 2 min after microinfusion completion to ensure proper drug diffusion.

4.3.6. Behavioural Procedures

Intermittent Alcohol Access. Fifteen sessions of intermittent access to 15% (*v*/*v)* alcohol were conducted in the home-cage to familiarize rats with the taste and pharmacological effects of alcohol[116,158], as detailed in previous studies[146].

Intermittent access to unsweetened alcohol produces varying levels of alcohol intake[40,196]. To encourage drinking in rats with low alcohol intake, we lowered the alcohol concentration. Starting on session 3, rats that drank <0.9 g/kg [g/kg; g of alcohol consumed/ kg of body weight] for 2 consecutive sessions subsequently received 5% (*v*/*v*) alcohol during access sessions (Experiment 1 $n = 4$; Experiment 2 $n = 4$; Experiment 4 $n = 5$). One rat in Experiment 2 met this criterion, however, was not given 5% alcohol due to experimenter error. When 1 g/kg was obtained for three consecutive sessions, the alcohol concentration was increased to 10% (*v*/*v*). Once 1 g/kg was obtained for two consecutive sessions, the alcohol concentration was then increased to 15% (*v*/*v*). Starting on Session 10 rats that drank <0.9 g/kg for 2 consecutive sessions subsequently received 10% (*v*/*v*) alcohol during access sessions (Experiment 2 $n = 2$). When 1 g/kg was obtained for three consecutive sessions, the alcohol concentration was increased to 15% (*v*/*v*).

The alcohol concentration that rats received during the last intermittent alcohol access session was the same concentration that they received during Pavlovian conditioning and alcohol re-exposure sessions. In Experiment 2, one rat remained on 5% alcohol. In Experiment 4, one rat remained on 5% and one rat remained on 10% alcohol. All rats obtained 1.0 g/kg averaged across the last three intermittent alcohol access sessions, except one rat in Experiment 4. All rats were retained for behavioural training.

Sucrose Habituation. Habituation to 10% (*w*/*v*) sucrose was conducted in the home-cage to familiarize rats with sucrose. Pre-weighed, 100 ml graduated cylinders containing sucrose and pre-weighed water bottles were inserted into home-cages via lids for two consecutive days.

Chamber Habituation. Two habituation sessions were conducted during the last week of intermittent alcohol access on days with only access to water (Experiments 1, 2, and 4), and after the last day of sucrose habituation (Experiment 3). In session 1, rats were brought to the experimental room and left in their home-cages for 20 min. In session 2, rats were placed into a designated conditioning chamber for 20 min, during which the house lights were illuminated, and total port entries were recorded.

Pavlovian Conditioning. Pavlovian conditioning sessions were conducted daily (42 - 45 min sessions). Program onset was followed by a 2 min delay, after which house lights were illuminated to signal the start of the session. Eight trials of a 20 s continuous white-noise conditioned stimulus (CS) were paired with a 10 s activation of the fluid pump which delivered 0.3 ml of the unconditioned stimulus (US) into the fluid port (Experiments 1, 2, and 4: alcohol; Experiment 3: sucrose). Pump activation began 10 s after CS onset and co-terminated with the CS. Trials were presented on a variable time 240 s schedule (inter-trial intervals: 120, 200, 210, 280, 310, 320 s), not including 20 s pre- and post-CS intervals. Fluid ports were checked at the end of each session to verify that the US was ingested.

Extinction. Extinction sessions were conducted daily (42 - 45 min sessions). Session parameters were identical to Pavlovian conditioning except that CS presentations were paired with the activation of empty syringe pumps (i.e., the US was not delivered).

US Re-exposure. US re-exposure sessions occurred after the last extinction session (42 - 45 min sessions). During the US re-exposure session, 2.4 ml of the US was delivered to the fluid port according to the same schedule of delivery as Pavlovian conditioning; however, the CS was not presented. Fluid ports were checked at the end of each session to verify that the US was ingested.

Reinstatement Test. A reinstatement test was conducted 24 h after the US re-exposure session (42 - 45 min sessions). During the reinstatement test, the CS was presented as during Pavlovian conditioning, except that CS presentations were paired with activation of empty syringe pumps (i.e., the US was not delivered).

4.3.7. Experiment 1A: The Effect of Systemic Naltrexone on Reinstatement of Responding to an Alcohol-CS

We tested the effect of systemic administration of naltrexone on the reinstatement of responding to an alcohol-CS, using a within-subjects design. After intermittent alcohol access and habituation, naïve rats (n = 15 males) received Pavlovian conditioning with an alcohol-US, extinction, alcohol-US re-exposure, then a test for reinstatement 24 h later. Naltrexone (0, 0.3, 1.0 mg/kg; counterbalanced across tests) was subcutaneously injected 10 – 15 min before the reinstatement test (doses based on previous studies[82,83,94]). Rats were habituated to saline injections before the second last extinction session and the alcohol-US re-exposure session.

Test 1 was conducted after 12 Pavlovian conditioning sessions and eight extinction sessions (i.e., training cycle 1). Test 2 was conducted after two Pavlovian conditioning retraining sessions and six extinction sessions (i.e., training cycle 2). Test 3 occurred after two Pavlovian conditioning retraining sessions and five (n = 11) or six (n = 4) extinction sessions (i.e., training cycle 3). The experimental procedure is illustrated in Figure 3A.

4.3.8. Experiment 1B: The Effect of Systemic Naltrexone on Responding to a CS Paired with Alcohol

To assess if naltrexone affected the ability to make reinforced port entries, we tested the impact of systemic naltrexone on conditioned responding to a CS that was paired with alcohol delivery. After the last reinstatement test in Experiment 1A, rats (n = 15 males) received five

Pavlovian conditioning retraining sessions. Naltrexone (0, 0.3, 1.0 mg/kg;) was subcutaneously injected 10 – 15 min before the third, fourth, and fifth Pavlovian conditioning sessions, using a counterbalanced, within-subjects design.

4.3.9. Experiment 2: The Effect of Systemic Naltrexone on Reinstatement of Responding to an Alcohol-CS in Female and Male Rats

We next examined the potential sex differences in the capacity of naltrexone to reduce reinstatement of responding to an alcohol-CS, using a within-subjects design in a new group of rats. After intermittent alcohol access and habituation, female (n = 18) and male (n = 18) rats received Pavlovian conditioning with an alcohol-US, extinction, alcohol-US re-exposure, then a test for reinstatement 24 h later. Naltrexone (0, 0.3, 1.0 mg/kg; counterbalanced across tests) was subcutaneously injected 10 – 15 min before the reinstatement test. Rats were habituated to saline injections before the second last extinction session and the alcohol-US re-exposure session.

Test 1 was conducted after 16 Pavlovian conditioning sessions and seven extinction sessions (i.e., training cycle 1). Test 2 was conducted after two Pavlovian conditioning sessions and six extinction sessions (i.e., training cycle 2). Test 3 occurred after two Pavlovian conditioning sessions and five (n = 35) extinction sessions (i.e., training cycle 3). One rat received eight in order to reach extinction criteria.

4.3.10. Experiment 3: The Effect of Systemic Naltrexone on Reinstatement of Responding to a Sucrose-CS

We assessed the ability of naltrexone to reduce reinstatement in an alcohol-specific manner by testing the effect of naltrexone on reinstatement of responding to a sucrose-CS, using a within-subjects design in a new group of rats. After sucrose habituation, rats (n = 12 females, n = 12 males) received Pavlovian conditioning with a sucrose-US, extinction, sucrose-US re-exposure, then a test for reinstatement 24 h later. Naltrexone (0, 0.3, 1.0 mg/kg; counterbalanced across tests) was subcutaneously injected 10 – 15 min before the reinstatement test. Rats were habituated to saline injections before the second last extinction session and the sucrose-US re-exposure session.

Test 1 occurred after nine Pavlovian conditioning sessions and nine extinction sessions (i.e., training cycles 1). Test 2 occurred after two Pavlovian conditioning sessions and seven

extinction sessions (i.e., training cycles 1). Test 3 occurred after two Pavlovian conditioning sessions and six extinction sessions (i.e., training cycles 1). The experimental procedure is illustrated in Figure 5A.

4.3.11. Experiment 4: The Effect of Intra-Ventral Hippocampal CTAP on Reinstatement of Responding to an Alcohol-CS

We examined the effect of CTAP microinfused into the ventral hippocampus on reinstatement of responding to an alcohol-CS. CTAP was used in order to specifically target MORs as naltrexone binds, with lower affinity, to delta and kappa opioid receptors[201]. After intermittent alcohol access and habituation, a new group of rats (n = 13 males, n = 13 females) received Pavlovian conditioning with an alcohol-US, extinction, alcohol-US re-exposure, then a test for reinstatement 24 h later. CTAP (0, 2.5, 5.0 µg/hemisphere; counterbalanced across tests) was bilaterally microinfused into the ventral hippocampus 5 min before the reinstatement test (doses based on previous studies[190,192,202,203]. Rats were habituated to intracranial microinfusions of saline (0.3 ul/hemisphere) during the first training cycle, before the second last extinction session and the US re-exposure session.

Test 1 occurred after 17 Pavlovian conditioning sessions and eight extinction sessions (i.e., training cycle 1). Test 2 occurred after three Pavlovian conditioning sessions and seven extinction sessions (i.e., training cycle 2). Test 3 occurred after three Pavlovian conditioning sessions and seven extinction sessions (i.e., training cycle 3). The experimental procedure is illustrated in Figure 6A.

4.3.12. Histology

After Experiment 4 was completed, coronal sections (40 µm) were collected from paraformaldehyde-fixed brains using a cryostat (−20°C) for Nissl staining using a standard protocol[197]. Placements of ventral injector tips were identified using standard light microscopy and the 2007 Paxinos and Watson rat brain atlas[200].

4.3.13. Data Management

Exclusion criteria. We used a criterion based on the probability of making a CS port entry [# of trials with ≥1 CS port entry / # of CS trials) * 100] to evaluate if rats learned to associate the

CS with the US[196]. Rats that responded on <70 % of trials averaged on the last two (Experiments 1, 2, and 3) or three (Experiment 4) Pavlovian conditioning sessions were removed from statistical analyses as they were deemed to have not acquired the task. Rats with a probability score of > 60% averaged across the last two extinction sessions, were removed from statistical as they were deemed to have not extinguished conditioned responding. Rats were also excluded if they had a difference score of ≤ 0 ΔCS port entries (last extinction session subtracted from reinstatement test) under saline treatment as these rats were deemed to not reinstate under the control condition. Lastly, rats were excluded from the study due to a detached headcap, obstructed cannulae, or serious injury. Initial and final sample sizes are depicted in Table 1.

Variables. The dependent variables were ΔCS port entries (CS port entries minus 20 s pre-CS port entries), and intertrial interval port entries (port entries made outside of the 20 s CS, pre-CS, and post-CS intervals). Responding at reinstatement test was compared to an extinction baseline, which was the average responding during the last two extinction sessions.

Table 1. Sample size across experimental phases.

	Intermittent alcohol access	Pavlovian conditioning		Extinction		
Exp.	Initial	Initial	Dropped	Initial	Dropped	Final
1A	*n* = 15	*n* = 15	*n* = 0	*n* = 15	*n* = 4	*n* = 11
1B	N/A	*n* = 15	*n* = 0	N/A	N/A	*n* = 15
2	*n* = 36 ♀ *n* = 18 ♂ *n* = 18	*n* = 36 ♀ *n* = 18 ♂ *n* = 18	*n* = 7 ♀ *n* = 2 ♂ *n* = 5	*n* = 29 ♀ *n* = 16 ♂ *n* = 13	*n* = 1 ♀ *n* = 1 ♂ *n* = 0	*n* = 24[c] ♀ *n* = 12 ♂ *n* = 12
3	N/A	*n* = 24 ♀ *n* = 12 ♂ *n* = 12	*n* = 0	*n* = 24 ♀ *n* = 12 ♂ *n* = 12	*n* = 4 ♀ n = 2 ♂ n = 2	*n* = 19[c] ♀ n = 9 ♂ n = 10
4	*n* = 26[a] ♀ *n* = 13 ♂ *n* = 13	*n* = 24 ♀ *n* = 11 ♂ *n* = 13	*n* = 10[b] ♀ *n* = 5 ♂ *n* = 5	*n* = 14 ♀ *n* = 6 ♂ *n* = 8	*n* = 1 ♀ *n* = 1 ♂ *n* = 0	*n* = 10[c] ♀ *n* = 3 ♂ *n* = 7

[a] *n* = 2 females dropped during intermittent alcohol access due to serious injury.

[b] *n* = 2 females and 2 males rats dropped due to inability to reach Pavlovian conditioning criteria, *n* = 1 female dropped due to serious injury, *n* = 1 female and *n* = 1 male due to obstructed cannulae, *n* = 1 female and *n* = 2 males due to lost headcaps.

[c] Rats excluded due to a difference score of ≤ 0 ΔCS port entries (last extinction session subtracted from reinstatement test) under saline treatment; Experiment 2: *n* = 3 females, *n* = 1 male; Experiment 3: *n* = 1 female; Experiment 4: *n* = 2 females, *n* = 1 male.

4.3.14. Statistical Analyses

Potential test order effects were controlled for as Dose was counterbalanced across test sessions in all experiments; therefore, statistical analyses reported herein do not include an Order factor. Data were analyzed using analysis of variance (ANOVA). The Huynh-Feldt correction was applied when Mauchly's test of sphericity was violated. All post-hoc analyses were corrected for multiple comparisons with Scheffe's method. Statistical analyses were conducted with RStudio (Version 2021.9.0.351, R Foundation for Statistical Computing) and evaluated using a statistical significance level of $p < 0.05$. Graphs were created with Graphpad Prism (Version 8; La Jolla, CA).

4.4. Results

4.4.1. Intermittent Alcohol Access

Alcohol intake (Figure 1A) increased across intermittent alcohol access sessions in Experiment 1 [Session: $F_{(14, 140)} = 5.18$, $p < .001$]. In Experiment 2, alcohol intake (Figure 1B) remained stably elevated across sessions [Session: $F_{(6.126, 134.763)} = 1.973$, $p = .072$], similarly between sexes [Sex: $F_{(1, 22)} = 4.046$, $p = .057$; Session x Sex: $F_{(6.126, 134.778)} = 1.910$, $p = .082$]. In Experiment 4, alcohol intake (Figure 1C) increased across sessions [Session: $F_{(14, 112)} = 2.258$, $p = .010$], similarly between sexes [Sex: $F_{(1, 8)} = 0.092$, $p = .769$; Session x Sex: $F_{(14, 112)} = 0.687$, $p = .783$].

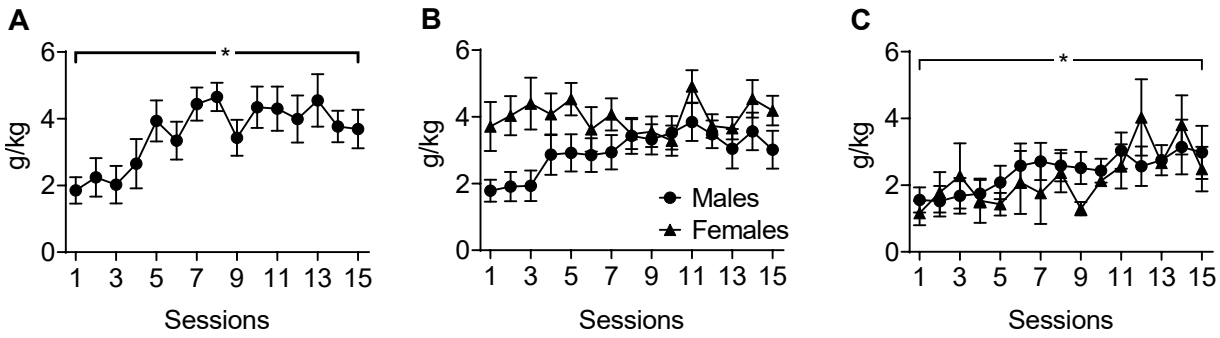


Figure 1. Alcohol intake (g/kg) increased, or remained stably elevated, across intermittent alcohol access sessions. Data are mean (± SEM) g/kg obtained across sessions for male (circles) and female (triangles) rats during **(A)** Experiment 1, **(B)** Experiment 2, and **(C)** Experiment 4.

* $p < 0.05$, main effect of Session (1 – 15)

4.4.2. Acquisition and Extinction of Conditioned Responding

Experiment 1. Rats learned to associate the CS with alcohol delivery across Pavlovian conditioning sessions. In training cycle 1, ΔCS port entries (Figure 2A) significantly increased across Pavlovian conditioning sessions [**Cycle 1** Session: $F_{(15, 150)} = 24.394$, $p < .001$], and remained stably elevated in training cycles 2 and 3 [**Cycle 2** Session: $F_{(1, 10)} = 0.53$, $p = .823$; **Cycle 3** Session: $F_{(1, 10)} = 4.423$, $p = .062$].

In all training cycles, ΔCS port entries (Figure 2A) significantly decreased from the first to the last extinction session [**Cycle 1** Session: $F_{(1, 10)} = 41.365$, $p < .001$; **Cycle 2** Session $F_{(1, 10)} = 39.625$, $p = < .001$; **Cycle 3** Session $F_{(1, 10)} = 30.262$, $p = < .001$].

Experiment 2. In all training cycles, ΔCS port entries (Figure 2B) significantly increased across Pavlovian conditioning sessions [**Cycle 1** Session: $F_{(5.572, 122.589)} = 27.781$, $p < .001$; **Cycle 2** Session: $F_{(1, 22)} = 11.856$, $p = .002$; **Cycle 3** Session: $F_{(1, 22)} = 5.417$, $p = .030$], similarly between sexes [**Cycle 1** Sex: $F_{(1, 22)} = 0.349$, $p = .561$; Session x Sex: $F_{(5.572, 122.589)} = 0.443$, $p = .836$; **Cycle 2** Sex: $F_{(1, 22)} = 1.041$, $p = .319$; Session x Sex: $F_{(1, 22)} = 0.004$, $p = .947$; **Cycle 3** Sex: $F_{(1, 22)} = 1.267$, $p = .273$; Session x Sex: $F_{(1, 22)} = 3.980$, $p = .059$].

In all training cycles, ΔCS port entries (Figure 2B) significantly decreased from the first to the last extinction session [**Cycle 1** Session: $F_{(1,22)} = 91.515$, $p < .001$; **Cycle 2** Session: $F_{(1,22)} = 86.919$, $p < .001$; **Cycle 3** Session: $F_{(1,22)} = 100.108$, $p < .001$], similarly between sexes [**Cycle 1** Sex: $F_{(1,22)} = 0.008$, $p = .931$; Session x Sex: $F_{(1,22)} = 0.002$, $p = .965$; **Cycle 2** Sex: $F_{(1,22)} = 0.433$, $p = .518$; Session x Sex: $F_{(1,22)} = 0.019$, $p = .891$; **Cycle 3** Sex: $F_{(1,22)} = 0.029$, $p = .867$; Session x Sex: $F_{(1,22)} = 0.000$, $p = .984$].

Experiment 3. ΔCS port entries (Figure 2C) significantly increased across Pavlovian conditioning sessions during training cycle 1 [**Cycle 1** Session: $F_{(3.850, 65.442)} = 31.839$, $p < .001$] and remained stably elevated during training cycles 2 and 3 [**Cycle 2** Session: $F_{(1, 17)} = 0.165$, $p = .690$; **Cycle 3** Session: $F_{(1, 17)} = 0.345$, $p = .565$]. This pattern of responding occurred similarly between sexes [**Cycle 1** Sex $F_{(1, 17)} = 3.110$, $p = .096$; Session x Sex: $F_{(3.850, 65.442)} = 1.308$, $p = .277$; **Cycle 2** Sex: $F_{(1, 17)} = 0.020$, $p = .889$; Session x Sex: $F_{(1, 17)} = 0.407$, $p = .532$; **Cycle 3** Sex: $F_{(1, 17)} = .002$, $p = .961$; Session x Sex: $F_{(1, 17)} = 1.519$, $p = .235$].

Across all training cycles, ΔCS port entries (Figure 2C) significantly decreased from the

first to the last extinction session [**Cycle 1** Session: $F_{(1, 17)} = 131.541$, $p < .001$; **Cycle 2** Session: $F_{(1, 17)} = 100.461$, $p < .001$; **Cycle 3** Session: $F_{(1, 17)} = 124.317$, $p < .001$], similarly between sexes [**Cycle 1** Sex: $F_{(1, 17)} = 3.119$, $p = .095$; Session x Sex: $F_{(1, 17)} = .493$, $p = .492$; **Cycle 2** Sex: $F_{(1, 17)} = 2.041$, $p = .171$; Session x Sex: $F_{(1, 17)} = .751$, $p = .398$; **Cycle 3** Sex: $F_{(1, 17)} = 1.185$, $p = .292$; Session x Sex: $F_{(1, 17)} = .826$, $p = .376$].

Experiment 4. ΔCS port entries (Figure 2D) significantly increased across Pavlovian conditioning sessions in training cycle 1 [**Cycle 1** Session: $F_{(16, 128)} = 11.531$, $p < .001$] and remained stably elevated across training cycles 2 and 3 [**Cycle 2** Session: $F_{(1,239, 9.914)} = 1.554$, $p = .248$; **Cycle 3** Session: $F_{(2, 16)} = .425$, $p = .661$]. This pattern of responding occurred similarly between sexes [**Cycle 1** Sex: $F_{(1, 8)} = .071$, $p = .796$; Session x Sex: $F_{(16, 128)} = .985$, $p = .477$; **Cycle 2** Sex: $F_{(1, 8)} = 1.711$, $p = .227$; Session x Sex: $F_{(1,239, 9.914)} = 1.530$, $p = .252$; **Cycle 3** Sex: $F_{(1, 8)} = .2.564$, $p = .148$; Session x Sex: $F_{(2, 6)} = .230$, $p = .797$].

Across training cycles, ΔCS port entries (Figure 2D) significantly decreased from the first to the last extinction session [**Cycle 1** Session: $F_{(1, 8)} = 18.179$, $p = .003$; **Cycle 2** Session: $F_{(1, 8)} = 25.253$, $p = .001$; **Cycle 3** Session: $F_{(1, 8)} = 37.758$, $p < .001$], similarly between sexes [**Cycle 1** Sex: $F_{(1, 8)} = 0.330$, $p = .582$; Session x Sex: $F_{(1, 8)} = 0.341$, $p = .576$; **Cycle 2** Sex: $F_{(1, 8)} = 0.542$, $p = .283$; Session x Sex: $F_{(1, 8)} = 2.715$, $p = .138$; **Cycle 3** Sex: $F_{(1, 8)} = 0.066$, $p = .803$; Session x Sex: $F_{(1, 8)} = 2.159$, $p = .180$].

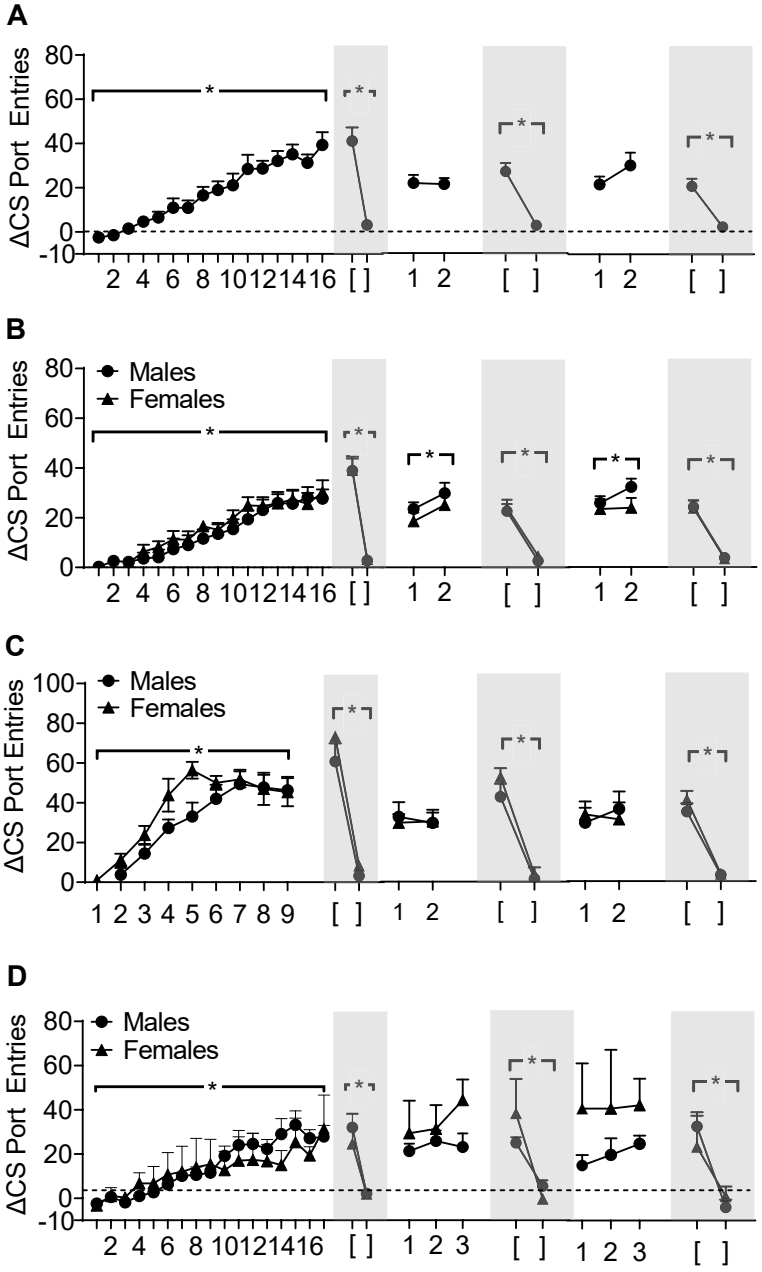


Figure 2. Rats learned to associate the CS with the US. ΔCS port entries increased or remained stably elevated, across Pavlovian conditioning sessions. This conditioned response was subsequently extinguished, as ΔCS port entries decreased from the first ([ ) to the last ( ]) extinction session. Data are mean (± SEM) ΔCS port entries made by male (circles) and female (triangles) rats during **(A)** Experiment 1, **(B)** Experiment 2, **(C)** Experiment 3, and **(D)** Experiment 4.

* $p < 0.05$, main effect of Session

4.4.3. Experiment 1A. Systemic Naltrexone Reduced Reinstatement of Responding to an Alcohol-CS

Systemic injection of naltrexone reduced reinstatement of responding to the alcohol-CS. Relative to extinction, ΔCS port entries (Figure 3B) significantly increased at test [Phase: $F_{(1,10)}$ = 49.249, $p < .001$]; however, this increase differed by naltrexone dose [Phase x Dose: $F_{(2,20)}$ = 10.959, $p < .001$; Dose: $F_{(2,20)} = 3.673$, $p = 0.044$]. Post-hoc analyses revealed that reinstatement of ΔCS port entries occurred following injections of saline ($p < .001$) and 0.3 mg/kg of naltrexone ($p = .002$), whereas the reinstatement was prevented by 1.0 mg/kg of naltrexone (p = .166). Moreover, relative to saline, reinstatement of ΔCS port entries was reduced by 0.3 mg/kg ($p = .005$) and 1.0 mg/kg ($p = .002$) of naltrexone.

To examine the effects of naltrexone on the pattern of responding at test, CS port entry as a function of trial was analyzed. At test, ΔCS port entries (Figure 3C) significantly decreased across CS trials due to within-session extinction [Trial: $F_{(7, 70)} = 16.757$, $p < .001$]; however, this responding differed by naltrexone dose [Trial x Dose: $F_{(14, 140)} = 4.887$, $p < .001$; Dose: $F_{(2,20)}$ = 7.546, $p = .004$]. Post-hoc analyses revealed that, relative to saline, 0.3 mg/kg ($p < .001$) and 1.0 mg/kg ($p < .001$) of naltrexone reduced ΔCS port entries during the first CS trial.

Naltrexone did not affect intertrial interval (ITI) port entries during reinstatement tests. Relative to extinction, ITI port entries (Figure 3D) did not increase at test [Phase: $F_{(1,10)} = 0.597$, $p = 0.458$], and did not differ by naltrexone dose [Phase x Dose: $F_{(2,20)} = 0.220$, $p = 0.804$; Dose: $F_{(2,20)} = 2.046$, $p = 0.155$].

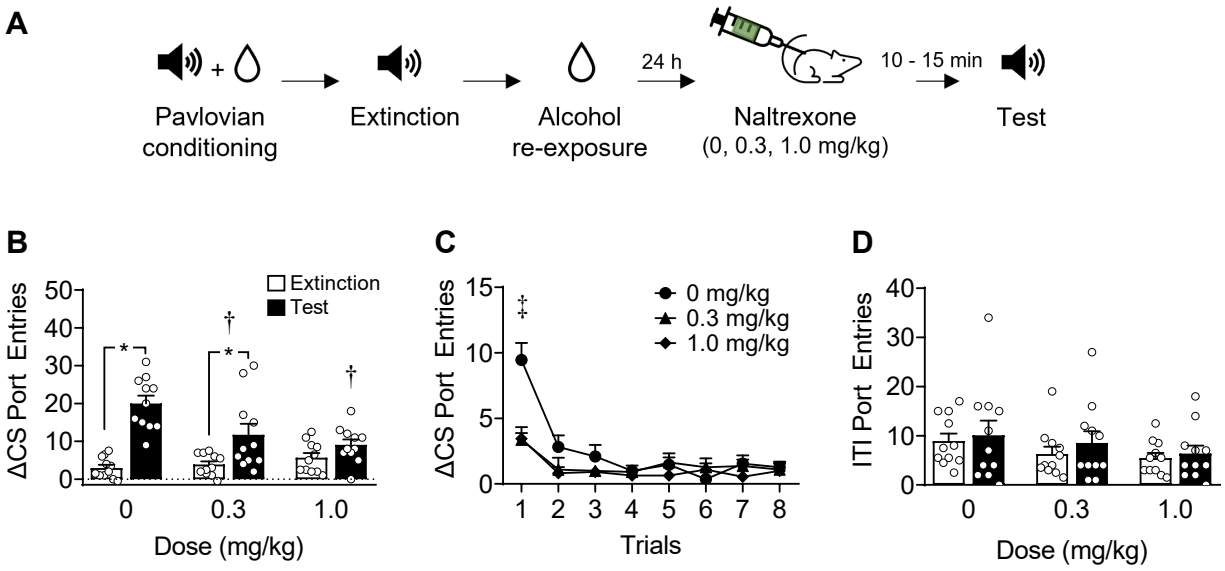


Figure 3. Systemic naltrexone attenuated reinstatement of responding to an alcohol-CS. **A** Schematic representation of the behavioural design. Data are from rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone before reinstatement tests. **B** Mean (± SEM) ΔCS port entries made during extinction and test. **C** Mean (± SEM) ΔCS port entries across CS trials during test. **D** Mean (± SEM) intertrial interval port entries made during extinction and test. Herein, open circles depict individual data of male rats.

* $p < 0.05$, main effect of Phase (Extinction < Test)

† $p < 0.05$, Phase x Dose interaction post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg at Test)

‡ $p < 0.05$, Trial x Dose interaction post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg on CS trial 1)

4.4.4. Experiment 1B. Systemic Naltrexone Minimally Affected Conditioned Responding to a Reinforced Alcohol-CS

Systemic injection of naltrexone did not impact responding to a CS that was paired with alcohol delivery. A repeated measures ANOVA showed that ΔCS port entries (Figure 4) made during Pavlovian conditioning sessions significantly differed across naltrexone dose [$F_{(2, 28)}$ = 4.582, p = 0.019]. ΔCS port entries appear to decrease from saline (M = 33.80) to 0.3 mg/kg (M = 26.87) to 1.0 mg/kg (M = 19.33) of naltrexone. However, post-hoc analyses revealed that, relative to saline, ΔCS port entries were not significantly affected by 0.3 mg/kg (p = .520) or 1.0 mg/kg (p = .066) of naltrexone, nor by 1.0 mg/kg relative to 0.3 mg/kg (p = 0.463) of naltrexone.

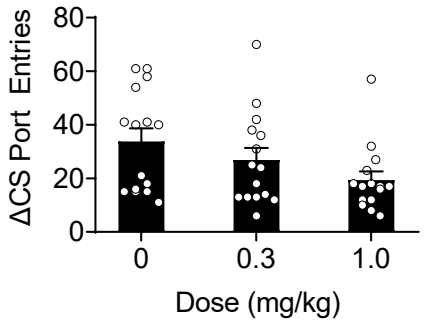


Figure 4. Systemic naltrexone did not affect responding to an alcohol reinforced CS. Data are mean (± SEM) ΔCS port entries made during Pavlovian conditioning sessions in rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone.

4.4.5. Experiment 2. Systemic Naltrexone Reduced Reinstatement of Responding to an Alcohol-CS in Female and Male Rats

Systemic injection of naltrexone reduced reinstatement of responding to an alcohol-CS in both female and male rats. Relative to extinction, ΔCS port entries (Figure 5A) significantly increased at test [Phase: $F_{(1,22)} = 65.994$, $p < .001$]; however, this differed by naltrexone dose [Phase x Dose: $F_{(2,44)} = 7.552$, $p = .002$; Dose: $F_{(2,44)} = 12.887$, $p < .001$]. Post-hoc analyses revealed that reinstatement of ΔCS port entries occurred following injections of saline ($p < .001$), 0.3 mg/kg ($p < .001$) and 1.0 mg/kg ($p = .001$) of naltrexone. However, relative to saline, reinstatement of ΔCS port entries was reduced by 0.3 mg/kg ($p = .016$) and 1.0 mg/kg ($p < .001$) of naltrexone. This effect did not differ between sexes [Sex: $F_{(1, 22)} = 0.253$, $p = .620$; Sex x Phase $F_{(1, 22)} = 0.207$, $p = .654$; Sex x Dose: $F_{(2,44)} = 0.672$, $p = .516$; Sex x Dose x Phase: $F_{(2,44)} = 2.161$, $p = .127$].

In an analysis of responding across CS trials at test, ΔCS port entries (Figure 5B) significantly decreased across CS trials [Trial: $F_{(5.717, 125.770)} = 12.141$, $p < .001$]; however, this differed by naltrexone dose [Trial x Dose: $F_{(8.991, 197.809)} = 3.543$, $p < .001$; Dose: $F_{(2, 20)} = 12.838$, $p < .001$]. Post-hoc analyses revealed that, relative to saline, 0.3 mg/kg ($p < .001$) and 1.0 mg/kg ($p < .001$) of naltrexone reduced ΔCS port entries during the first CS trial. This effect did not differ by sex [Sex: $F_{(1, 22)} = 0.621$, $p = .439$; Sex x Trial $F_{(7, 154)} = 0.272$, $p = .964$; Sex x Dose: $F_{(2,44)} = 2.562$, $p = .089$; Sex x Dose x Trial: $F_{(14, 308)} = 1.085$, $p = .370$].

Naltrexone did not affect the reinstatement of ITI port entries in female or male rats. Relative to extinction, ITI port entries (Figure 5C) increased at test [Phase: $F_{(1,22)} = 12.748$, $p = .002$]. This effect did not differ by dose [Phase x Dose: $F_{(2,44)} = 1.375$, $p = 0.264$; Dose: $F_{(1.453, 31.963)} = 1.752$, $p = 0.195$], nor by sex [Sex: $F_{(1,22)} = 0.012$, $p = 0.914$; Sex x Dose: $F_{(2, 44)} = 0.349$, $p = 0.708$; Sex x Phase: $F_{(1,22)} = 0.543$, $p = 0.469$; Sex x Dose x Phase: $F_{(2, 44)} = 0.352$, $p = 0.705$].

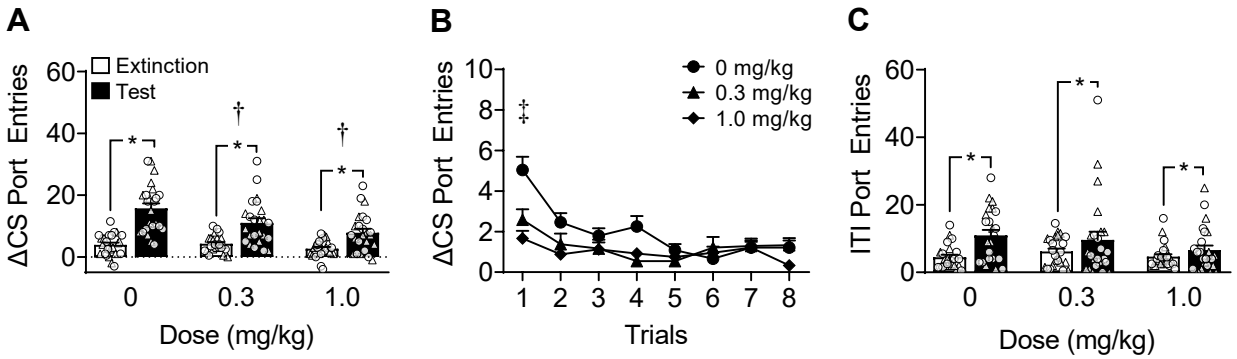


*Figure 5. Systemic naltrexone attenuated reinstatement of responding to an alcohol-CS in both female and male rats.*Data are from rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone before reinstatement tests. **A** Mean (± SEM) ΔCS port entries made during extinction and test. **B** Mean (± SEM) ΔCS port entries across CS trials at test. **C** Mean (± SEM) intertrial interval port entries made during extinction and test. Herein, open triangles depict individual data of female rats, and open circles depict individual data of male rats.

* $p < 0.05$, main effect of Phase (Extinction < Test)

† $p < 0.05$, Phase x Dose interaction post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg at Test)

‡ $p < 0.05$, Trial x Dose interaction post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg on CS trial 1)

4.4.6. Experiment 3. Systemic Naltrexone Did Not Affect Reinstatement of Responding to a Sucrose-CS

Systemic injection of naltrexone did not affect reinstatement of responding to a sucrose-CS. Relative to extinction, ΔCS port entries (Figure 6B) significantly increased at test [Phase: $F_{(1,17)}$ = 143.912, $p < .001$] similarly across naltrexone doses [Dose: $F_{(2, 34)} = 0.922$, $p = .407$; Phase x Dose: $F_{(2, 34)} = 0.813$, $p = 0.452$]. Reinstatement did, however, significantly differ between sexes [Phase x Sex: $F_{(1,17)} = 6.518$, $p = 0.021$; Sex: $F_{(1,17)} = 11.771$, $p = 0.003$], regardless of dose [Sex x Dose: $F_{(2, 34)} =1.624$, $p = .212$; Sex x Dose x Phase: $F_{(2, 34)} =1.826$, $p = .177$]. Post-hoc analyses revealed that both female ($p < .001$) and males ($p < .001$) reinstated; however, ΔCS port entries were higher in females compared to males during test ($p < .001$).

In an analysis of responding across CS trials at test, ΔCS port entries (Figure 6C) decreased across CS trials [Trial: $F_{(7, 119)} = 20.050$, $p < .001$], similarly across doses [Dose: $F_{(2,34)} = 0.899$, $p = .417$; Trial x Dose: $F_{(24, 238)} = 238\ 0.724$, $p = .749$]. Again, ΔCS port entries across trials significantly differed between the sexes [Sex: $F_{(1,17)} = 9.453$, $p = 0.007$], regardless of CS trial [Sex x Trial: $F_{(7, 119)} = 0.776$, $p = .608$] or dose [Sex x Dose: $F_{(2, 34)} =1.794$, $p = .182$; Sex x Dose x Trial: $F_{(14, 238)} =0.611$, $p = .855$]. Post-hoc analyses revealed that females made significantly more ΔCS port compared to males across all CS trials ($p < .001$).

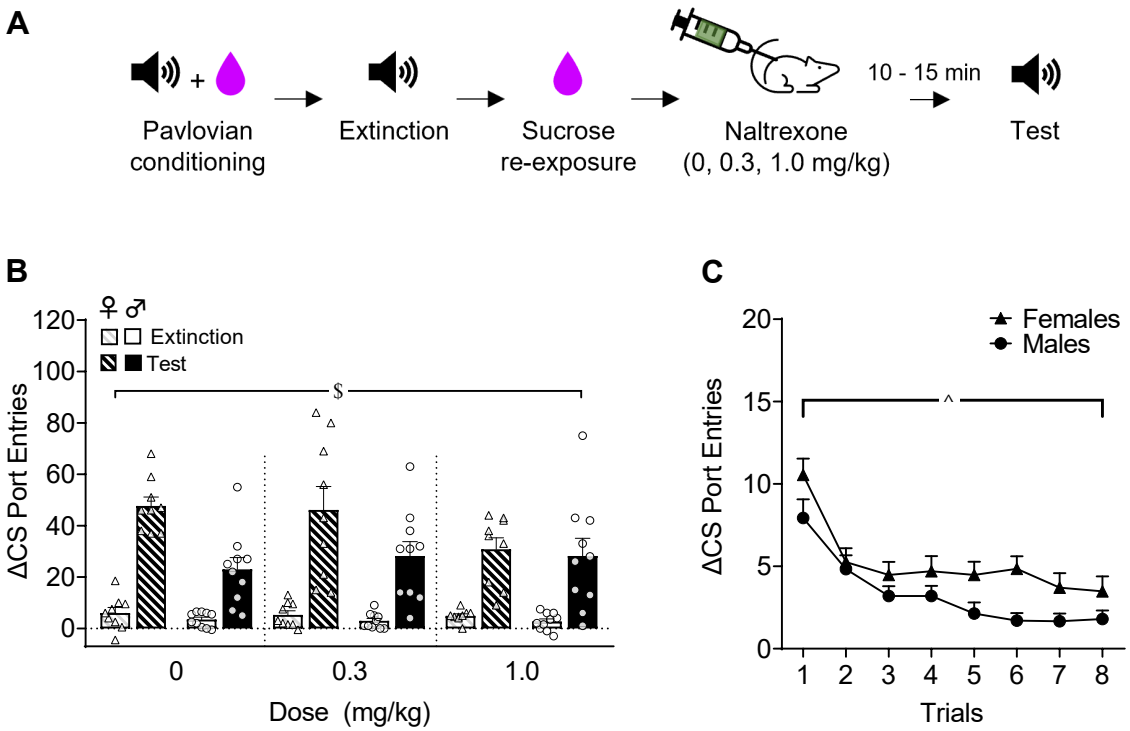


Figure 6. Systemic naltrexone did not attenuate reinstatement of responding to a sucrose-CS. Data are from rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone before reinstatement tests. **A** Schematic representation of the behavioural design. **B** Mean (± SEM) ΔCS port entries made during extinction and test for female (hatched bars) and male (filled bars) rats. **C** Mean (± SEM) ΔCS port entries across CS trials at test.

$ $p < 0.05$, Phase x Sex interaction post-hoc (Female > Male at Test)

^ $p < 0.05$, main effect of Sex (Female > Male)

4.4.7. Experiment 4. Intra-Ventral Hippocampal CTAP Administration Prevented Reinstatement of Responding to an Alcohol-CS

Given the lack of sex differences in the effects of naltrexone on the reinstatement of responding to an alcohol-CS (Experiment 2), and due to the small sample size, data from this experiment were not analyzed with a between-subjects factor of Sex.

CTAP microinfusions into the ventral hippocampus prevented reinstatement of responding to an alcohol-CS. Relative to extinction, ΔCS port entries (Figure 7B) significantly increased at test [Phase: $F_{(1,9)} = 5.668$, $p = .041$]; however, this differed across CTAP doses [Phase x Dose: $F_{(2, 18)} = 4.455$, $p = .027$; Dose: $F_{(2, 18)} = 1.462$, $p = .258$]. Post-hoc analyses revealed that reinstatement of ΔCS port entries occurred following microinfusions of saline ($p = .002$), whereas reinstatement was prevented by 2.5 µg ($p = .970$) and 5.0 µg ($p = .970$) of CTAP. Moreover, relative to saline, reinstatement was reduced by 2.5 µg ($p = .037$) and 5.0 µg ($p = .016$) of CTAP.

In an analysis of responding across CS trials at test, ΔCS port entries (Figure 7C) decreased across CS trials at test [Trial: $F_{(7, 63)} = 5.402$, $p < .001$], however, this differed across CTAP doses [Trial x Dose: $F_{(14, 126)} = 2.430$, $p = .005$; Dose: $F_{(2, 18)} = 2.979$ $p = .076$]. Post-hoc analyses revealed that, relative to saline, 2.5 µg ($p < .001$) and 5.0 µg ($p < .001$) of CTAP reduced ΔCS port entries during the first CS trial.

Intra-ventral hippocampal administration of CTAP did not affect reinstatement of ITI port entries. ITI port entries (Figure 7D) significantly reinstated at test [Phase: $F_{(1,9)} = 11.824$, $p = .007$], similarly across doses [Dose: $F_{(2, 18)} = .662$, $p = .528$; Phase x Dose: $F_{(2, 18)} = .885$, $p = .430$].

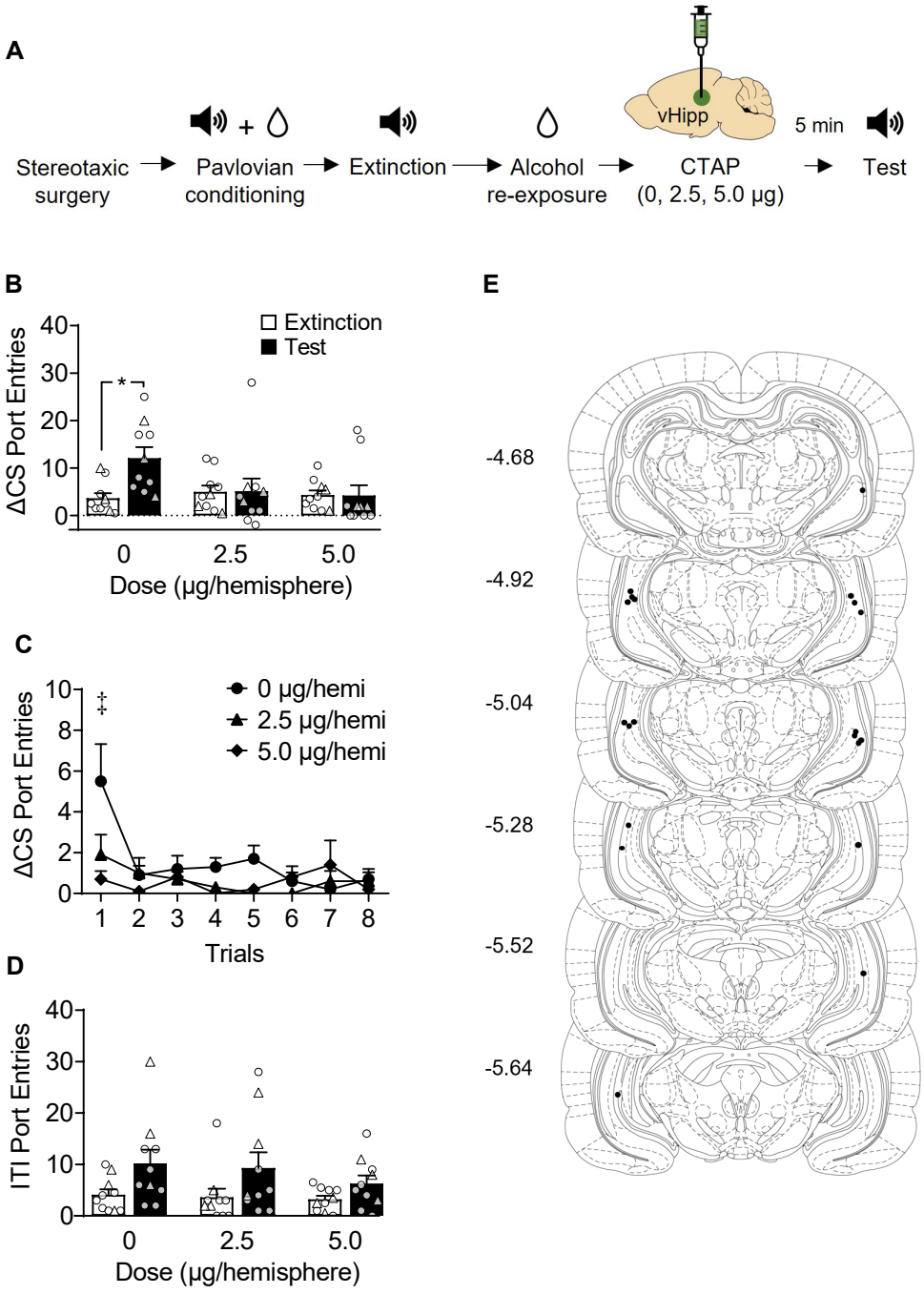


Figure 7. CTAP bilaterally microinfused into the ventral hippocampus prevented reinstatement of responding to an alcohol-CS. Data are from rats that received 0 µg (Black), 2.5 µg (Light green), or 5.0 µg (Dark green) of CTAP before reinstatement tests. **A** Schematic representation of the behavioural design. **B** Mean (± SEM) ΔCS port entries made during extinction and test. **C** Mean (± SEM) ΔCS port entries across CS trials at test. **D** Mean (± SEM) intertrial interval port entries made during extinction and test. **E** Representation of injector tip placements in the ventral hippocampus. Numbers indicate AP coordinates from bregma.

* $p < 0.05$, main effect of Phase (Extinction < Test)

‡ $p < 0.05$, Trial x Dose interaction post-hoc (2.5 µg and 5.0 µg < 0 µg on CS trial 1)

4.5. Discussion

Our data show that blocking μ-opioid receptors (MORs) with the antagonist, naltrexone, attenuates reinstatement of responding to an alcohol-CS in both female and male rats, while minimally affecting conditioned responding to a CS that was paired with alcohol. Naltrexone did not, however, affect reinstatement of responding to a sucrose-CS. Finally, intra-ventral hippocampal administration of CTAP prevented reinstatement of responding to an alcohol-CS at both doses tested. These findings illustrate that MORs are involved in the reinstatement of Pavlovian conditioned responding to a CS in an alcohol-specific manner, which complements studies showing the recruitment of these receptors in established reinstatement models using operant alcohol-seeking tasks. Importantly, our findings show for the first time that MORs located in the ventral hippocampus (vHipp) are necessary for the reinstatement of responding to an alcohol-CS.

In the present study, systemic naltrexone attenuated reinstatement of port entries evoked by an alcohol-CS, even at the low 0.3 mg/kg dose. These findings are consistent with studies that show similar doses of naltrexone reduced reinstatement of operant alcohol-seeking evoked by an alcohol-predictive context[82,95], discriminative stimuli[83,94,184], or a priming dose of alcohol[34]. Our findings do, however, contrast evidence that a different MOR antagonist, CTOP, did not affect cue-induced reinstatement of operant alcohol-seeking[183]. This difference could be attributable to the distinct reinstatement models used. Discrete cues have been found to evoke less vigorous reinstatement of operant alcohol-seeking[179,183], whereas the model used in the present study consistently produces robust reinstatement of responding to an alcohol-CS[146,196]. Therefore, the reinstatement model used in the present study may have been more sensitive to the behavioural changes produced by naltrexone. Together, our results suggest that MORs are part of the neural mechanism that mediates reinstatement of a Pavlovian conditioned response to a discrete alcohol-CS, specifically in this novel reinstatement model.

An important aspect of the present study is the inclusion of both female and male rats. The current literature examining responding to alcohol-predictive cues has often overlooked sex differences, and the findings that do exist are inconsistent. Some studies have shown a lack of sex differences in reinstatement of cue-induced operant alcohol-seeking[204] and Pavlovian responding to a CS evoked by an alcohol-prime[41], whereas others have reported that males have greater reinstatement of operant alcohol-seeking evoked by a cue and alcohol-prime combination relative

to females[205,206]. Still, others have shown greater cue- and context-induced reinstatement of alcohol-seeking in females relative to males[207,208]. We show that, under saline conditions, reinstatement of port entries made during an alcohol-CS occurred similarly in female and male rats. The systematic comparison of the effects of naltrexone on the reinstatement of responding to an alcohol-CS in female and male rats also shows that naltrexone attenuates reinstatement similarly in both sexes, which is consistent with the finding that naltrexone reduces home-cage alcohol intake similarly in female and male rodents[209]. Interestingly, 1.0 mg/kg of naltrexone prevented reinstatement in Experiment 1, however, the same dose only reduced reinstatement in Experiment 2. Although not statistically significant, the persistent reinstatement in Experiment 2 may be driven by the greater responding at test relative to extinction in females (Extinction M = 2.29, Test M = 9.08) compared to males (Extinction M = 3.00, Test M = 6.58). This hypothesis follows the pattern of responding observed in Experiment 3, in which females showed greater reinstatement of the sucrose-CS relative to males. Future research should investigate this potential nuanced sex difference, as the current experimental design may not have been sensitive enough to statistically detect sex differences. Overall, our findings provide novel evidence that suggests the recruitment of MORs in the reinstatement of Pavlovian responding to an alcohol-CS occurs independent of sex, and contributes to the burgeoning body of literature investigating potential sex differences in conditioned responding to alcohol-cues.

An important consideration when interpreting our data is that the reduction in reinstatement could be attributable to naltrexone producing non-specific behavioural effects that impact the ability to make a port entry. To address this concern, port entries made during the intertrial interval (ITI) were analyzed, which showed that naltrexone did not affect ITI port entries at test. We did observe reinstatement of ITI port entries in Experiments 2 and 4, but not Experiment 1. This effect is inconsistent; it has been observed in our unpublished data that is associated with published research[196], but not observed in other datasets[146]. ITI port entries can be interpreted as responding elicited by only the context, as no other stimuli are presented during this interval. Given that the delayed reinstatement effect is mediated by a context-alcohol association[196], the unreliable reinstatement of ITI port entries could be due to the context-alcohol association influencing behaviour outside of the CS presentation. This may be a small effect, and thus is not consistently observed.

A secondary assessment examined the effect of naltrexone on responding to a CS that was

paired with alcohol. Naltrexone minimally impacted responding to the reinforced alcohol-CS, as post-hoc analyses did not support the significant main effect of naltrexone dose identified by a mixed ANOVA. Together, these findings provide evidence that the doses of naltrexone used in the present study did not cause non-specific behavioural effects, like depressing locomotor activity, as demonstrated by others[94,95,210]. The latter finding that naltrexone may have minimally reduced responding to a reinforced alcohol-CS is not surprising. Similar doses of naltrexone reduce operant alcohol self-administration[34,187], as well as alcohol intake using an intermittent access procedure[211]. Therefore, MORs appear to be important for Pavlovian conditioned responding driven by the memory of an alcohol-CS association, and less so for the maintenance of a Pavlovian conditioned response for alcohol.

Systemic naltrexone did not impact reinstatement of port entries evoked by a sucrose-CS in either female or male rats. This finding complements prior work showing similar doses of naltrexone do not affect cue-induced reinstatement of operant sucrose-seeking[188]. Thus, naltrexone selectively attenuates reinstatement of responding to an alcohol-CS, but not a CS associated with a natural reward like sucrose, supporting the specificity of naltrexone to reduce responding to alcohol-predictive cues. This finding also strengthens our claim that naltrexone did not reduce reinstatement of responding to an alcohol-CS through non-specific effects on behaviour.

Interestingly, female rats showed greater reinstatement of responding to a sucrose-CS relative to males, regardless of naltrexone dose. The literature concerning sex differences in the reinstatement to sucrose-predictive cues is similarly as variable as those in responding to alcohol-predictive cues. In contrast to our findings, there is evidence that context-induced reinstatement of sucrose-seeking is greater in male compared to female rats[212], however, others have also reported no sex differences in this behaviour[204,213,214]. This inconsistency of findings across studies again highlights the necessity for continuing the investigation of sex differences in conditioned responding to appetitive cues.

The role of MORs in the neurocircuitry mediating reinstatement of responding to alcohol-cues is poorly understood, despite the extensive literature examining the systemic effects of altering these receptors. Our pharmacology experiment shows that localized administration of the MOR antagonist CTAP into the vHipp prevented reinstatement of port entries evoked by an alcohol-CS, at both examined doses. Administration of CTAP did not affect ITI port entries,

suggesting that the lack of reinstatement was likely not due to depressant effects of CTAP on behaviour. This experiment demonstrates, for the first time, that MORs in the vHipp are required for the delayed reinstatement of responding to an alcohol-CS. These findings are consistent with previous studies that have demonstrated that the vHipp is critically involved in reinstatement, as reversible inactivation of the ventral structures of the hippocampus attenuates reinstatement of responding evoked by a variety of drugs- and alcohol-cues[97–100,195]. Our findings extend this work by demonstrating that MORs, specifically in this region, are necessary for reinstatement to an alcohol-cue.

The dorsal subregion of the hippocampus has traditionally been associated with reinstatement of conditioned responding[84]; however, localized administration of a MOR antagonist into the dorsal hippocampus does not affect reinstatement of operant alcohol-seeking evoked by an alcohol-context[96]. Therefore, MORs in the ventral, but not the dorsal, hippocampus may mediate reinstatement of responding to alcohol-cues. This idea is supported by the growing evidence that the ventral and dorsal subregions of the hippocampus are functionally separate structures, with distinct projections, and involved in unique behaviours[215]. For example, reversible inactivation of the vHipp, but not the dorsal hippocampus impedes responding to a distinct cue paired with sucrose during a Pavlovian discrimination task, as well as the memory of this association[216]. Therefore, we posit that MORs in the dorsal versus vHipp have segregate roles in the reinstatement of responding to alcohol-predictive cues; however, future studies should conduct a systematic comparison to confirm these separable roles.

In conclusion, we show that silencing MORs attenuated the delayed reinstatement of Pavlovian conditioned responding to a CS in an alcohol-specific manner, and this effect is independent of sex. Moreover, for the first time, we provide evidence that MORs in the vHipp are necessary for the reinstatement of responding to an alcohol-CS. Our findings complement the research on the role of MORs in the responding to alcohol-cues that has predominantly been studied using established reinstatement models with operant alcohol-seeking tasks. These findings also provide the basis for future studies to further investigate the role of hippocampal MORs and their afferent projections in the reinstatement of responding to alcohol-cues.

Chapter 5: General Discussion

Animal models of relapse are valuable tools that have allowed researchers to model different aspects of alcohol use disorder (AUD), and to parse out the learning processes that are involved in AUD[27]. Models such as cue-induced reinstatement, priming-induced reinstatement, and renewal (i.e., context-induced reinstatement) of responding for alcohol have been particularly valuable, as they rigorously examine how alcohol-predictive cues can influence relapse-like behaviour[28,44]. The main focus of these reinstatement models has been to examine the immediate impact that re-exposure to these reinstating stimuli have on alcohol-seeking behaviour; however, they do not address the delayed impact of re-exposure to such stimuli on behaviour. The current thesis extends the established body of research on reinstatement by developing a novel model that assesses the delayed impact of re-exposure to alcohol on the reinstatement of responding to an alcohol-predictive cue (Chapter 2). Further, the role of a context-alcohol association (Chapter 3), and μ-opioid receptors (MORs) (Chapter 4), as psychological and neural mechanisms underlying the delayed reinstatement of responding to an alcohol-cue were examined to gain a comprehensive understanding of the new model.

5.1. The Capacity for Alcohol Re-Exposure to Reinstate Responding to an Alcohol-CS at a Future Timepoint

One of the main reasons for developing animal models of relapse is to capture diverse aspects of human addiction, which is crucial for understanding the complex mechanisms that contribute to relapse to drug use. The preclinical reinstatement model is frequently used to examine how alcohol-predictive cues can precipitate the relapse-like return – or 'reinstatement' – of extinguished, conditioned responding for a drug[28,44]. For example, re-exposure to a discrete alcohol-cue[30,31,40] or a priming dose of alcohol[33,34,108,165] can reinstate conditioned responding for alcohol. Similarly in a renewal task, which is also known as context-induced reinstatement, responding for alcohol can return when subjects are re-exposed to an alcohol-predictive context, following extinction of responding in a different context[54,108,217]. Typically, these reinstating stimuli are presented directly before, or during a non-reinforced test in which responding in the absence of alcohol is assessed. This sequence of events provides great insight into the immediate impact of re-exposure to alcohol-cues or alcohol-primes on responding for alcohol, but it does not assess the delayed impact that these stimuli may have on this behaviour.

Investigating the delayed impact of re-exposure to alcohol-cues and -primes on extinguished responding for alcohol is an equally important focus. This effect captures a unique aspect of human addiction that the established reinstatement models do not, such as how drinking one alcoholic beverage could affect the likelihood of an alcohol-abstinent person fully relapsing days later. Moreover, investigating this relationship can reveal unique psychological and neural mechanisms that contribute to relapse which the established reinstatement models cannot detect[27]. In the current thesis, a new delayed reinstatement model was developed in order to examine this, arguably, understudied impact of re-exposure to alcohol on extinguished responding for alcohol at a future timepoint.

The new delayed reinstatement model consists of acquisition and extinction of responding to a conditioned stimulus (CS) paired with alcohol delivery, followed by re-exposure to alcohol delivered according to the same schedule of access as Pavlovian conditioning, then a test for reinstatement 24 h later. Using this model, we demonstrate that a Pavlovian conditioned response to an alcohol-CS is reinstated 24 h after re-exposure to alcohol, relative to responding during extinction. This delayed reinstatement is a robust effect. It was reliably produced across multiple measures of conditioned responding during CS presentations, including the number of port entries made, the total duration of port entries, and the latency to initiate the first port entry. Moreover, rats that were implanted with cannulae targeting the ventral hippocampus also showed delayed reinstatement of responding to the alcohol-CS, thus replicating the effect even under invasive surgical conditions. Finally, delayed reinstatement was similarly evoked in both female and male rats. Our findings, therefore, validate the new model and demonstrate that it is a reliable model to examine how re-exposure to alcohol can impact responding to an alcohol-CS at a future timepoint.

The delayed reinstatement model uniquely demonstrates how a lapse in drinking can produce the relapse-like return of responding to an alcohol-predictive cue at a future timepoint. This finding is particularly relevant for individuals receiving cue exposure therapy (CET) as a treatment for AUD[218,219]. In CET, discrete cues that are associated with alcohol, like a picture of one's favourite alcoholic beverage, are repeatedly presented in the absence of alcohol. This treatment extinguishes cue-evoked craving and consequentially should protect against relapse[220,221]. These beneficial effects of CET, however, are not permanent and patients can still relapse to heavy alcohol use[22]. The findings presented in this thesis suggest that despite

extinguishing an alcohol-cue through CET, a small lapse in drinking alcohol could facilitate such relapse when encountering alcohol-cues days after the lapse.

We believe that the findings from the new delayed reinstatement model also contribute to a larger discussion about harm reduction strategies as a treatment approach for AUD[222]. Within the framework of addiction, harm reduction refers to the recognition that drug use in society will never be entirely eliminated, and therefore does not focus on preventing drug use *per se* but rather minimizes the negative consequences that result from drug use[222,223]. The findings in the current thesis highlight a potential obstacle when implementing harm reduction strategies. When generalized to a clinical population, our findings suggest that individuals maintaining a level of alcohol use may be at greater risk for relapse to heavy levels of alcohol use when encountering alcohol-predictive cues, even days later. As such, the efficacy of harm reduction strategies should be bolstered by providing adjunct therapies to help individuals cope with cue-evoked craving[223]. Ultimately, the effects of a lapse in drinking on future alcohol-cue reactivity are an important factor in the precipitation of relapse and should be considered in clinical research investigating AUD.

In sum, Chapter 2 presents a new delayed reinstatement model in which re-exposure to alcohol reinstated responding to an alcohol-CS 24 h later. These unique findings extend the information gained from the established reinstatement models which typically examine the immediate effect of re-exposure to discrete alcohol-cues[30], alcohol-primes[33,35], and alcohol-predictive contexts[108] on behaviour. Importantly, the delayed reinstatement model offers the possibility of investigating the prolonged impact of a lapse in drinking alcohol on cue-evoked responding for alcohol at a future timepoint, which can further elucidate the processes that contribute to relapse to alcohol use.

5.2. The Role of Context in Reinstatement of Responding to an Alcohol-CS

The capacity for physical contexts to facilitate responding to discrete alcohol-cues has been demonstrated in various animal models, such as the Pavlovian conditioning with context alternation[42,49] and renewal of alcohol-seeking[52,54] tasks, in which alcohol-predictive contexts can facilitate responding to the discrete-alcohol-cue. Further, contexts have been implicated in reinstatement of responding to a discrete cue associated with either an aversive-US (e.g., foot shock) or appetitive-US (e.g., food pellet), which is evoked using a model that is commonly used

to investigate fundamental memory and learning processes[50,111,112]. In this unique model, which is similar to the one used in this thesis, a context-US association is necessary to produce the reinstatement of responding to the aversive or appetitive discrete cue[50,112]. The current thesis used a behavioural approach to test if a similar psychological mechanism, involving an association between context and alcohol, also facilitates the delayed reinstatement of responding to an alcohol-predictive CS.

In Chapter 3, the role of a context-alcohol association in delayed reinstatement of responding to an alcohol-CS was investigated through two separate procedures that manipulated the context-alcohol association that was formed during alcohol re-exposure. First, the context that alcohol re-exposure had occurred in was extinguished by repeatedly exposing rats to the context without presentation of other stimuli, until responding significantly decreased. This was done in order to extinguish the context-alcohol association such that it would not be present during the subsequent reinstatement test. As predicted, this manipulation prevented reinstatement of responding to the alcohol-CS 24 h later, indicating that the presence of an association between alcohol and the test context is necessary for the expression of delayed reinstatement. This finding is consistent with studies demonstrating that extinguishing the context-US association after re-exposure to an aversive- or appetitive-US reduced reinstatement of Pavlovian conditioned responding to an aversive-CS[50] and operant responding for food-pellets[113], respectively.

In the second behavioural procedure, alcohol re-exposure was delivered in a context that differed in terms of multimodal sensory stimuli from the context used during Pavlovian conditioning, extinction, and reinstatement test (i.e., training context). This was done in order to establish an association between alcohol and the distinct re-exposure context, which would not be present when the rats were subsequently tested in the training context. Accordingly, this manipulation reduced reinstatement to the alcohol-CS 24 h later, which parallels previous findings in which re-exposure to a US in a different context different from the test context reduced reinstatement to an aversive- or appetitive-CS[50,112]. This finding further supports the notion that a context-alcohol association that is formed during alcohol re-exposure mediates delayed reinstatement.

An important theoretical consideration when interpreting Chapter 3 findings is whether a context-alcohol association was formed during alcohol re-exposure since these experiments aimed to manipulate the context-alcohol association and determine if it mediated reinstatement.

The formation of a context-US association is often assessed with context preference tasks[111]. In these tasks, rats choose to spend time in one side of a chamber that has a distinct contextual configuration and where the US was previously delivered, or in the other side of the chamber which has a unique context and where the US was never delivered. When less time is spent in the context associated with an aversive-US, and when more time is spent in the context associated with an appetitive-US, it is believed that a context-US association was formed[111]. The current thesis did not conduct such context preference tests after an alcohol re-exposure session, however, the experimental procedure used did provide a setting that was expected to establish a context-alcohol association. During alcohol re-exposure, rats could associate the taste and the smell of alcohol with the chamber context as they drank the alcohol. Moreover, the dose of alcohol consumed during the re-exposure session produced significant levels of alcohol in the blood that would produce intoxication[40,158,224]. This pharmacological effect of alcohol would therefore be salient, and likely to become associated with the chamber context.

Responding during the intertrial interval (ITI) during the delayed reinstatement tests also provides support for the formation of a context-alcohol association during alcohol re-exposure. The ITI is an interval during which no stimuli are presented other than the context, therefore, responding during this interval can be interpreted as being driven primarily by the context. ITI port entries were found to reinstate at test (see Figure 1), relative to extinction, in some experiments in Chapters 3 and 4 (i.e., Chapter 3 Experiments 2A and B; Chapter 4 Experiments 2, 3, and 4), and there was a trend towards statistically significant reinstatement of ITI port entries in Chapter 2 (i.e., Experiments 1 and 2; but see also related discussion in Chapter 4). The increase in ITI port entries at test may illustrate the context-alcohol association evoking responding for alcohol. Together, these methodological considerations support the claim that the alcohol re-exposure session conducted in this delayed reinstatement model likely established a context-alcohol association.

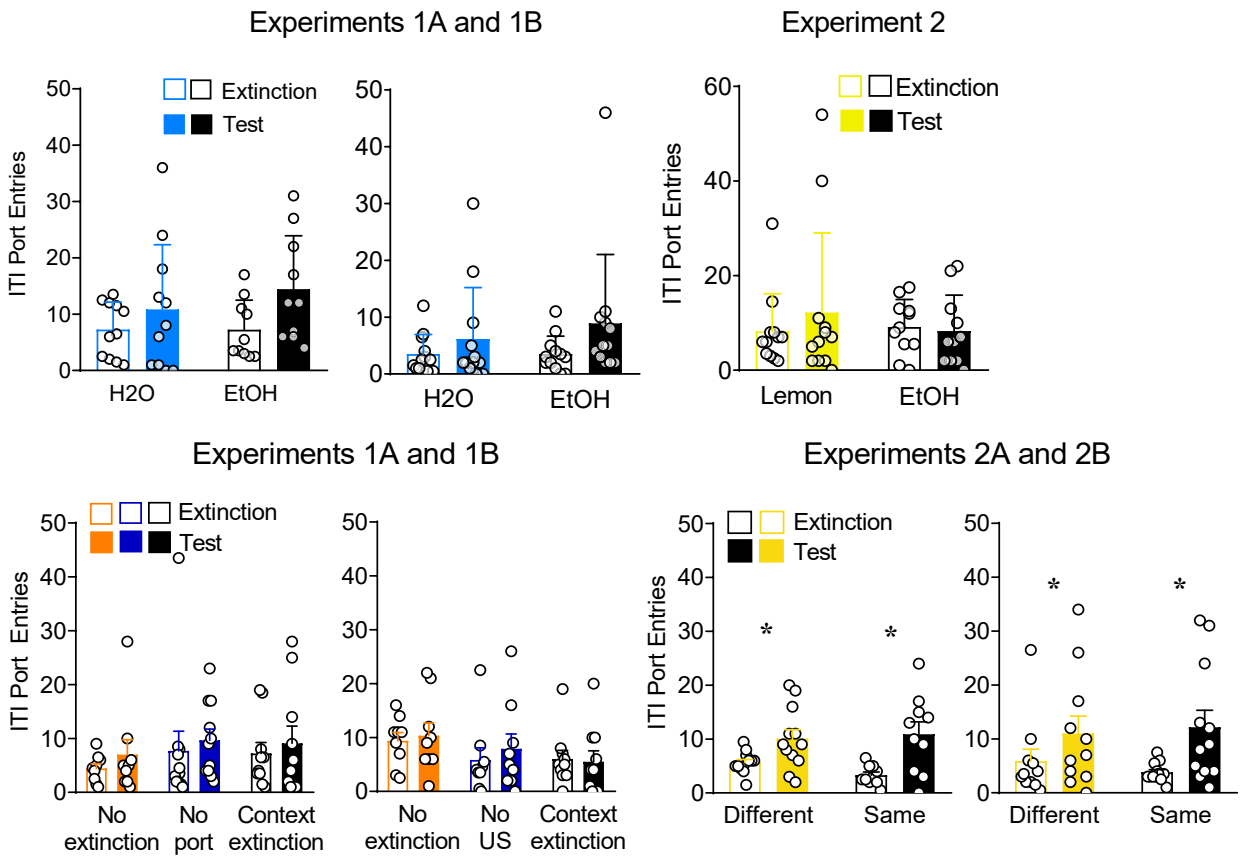


Figure 1. Intertrial interval (ITI) port entries during extinction and delayed reinstatement tests across experiments. Data are mean (±SEM) ITI port entries during extinction and test in Chapters 2 and 3. ITI port entries in Chapter 4 experiments are provided in the Results section of Chapter 4.

* $p < 0.05$ main effect of Phase (Extinction vs. Test)

Understanding exactly *how* the context-alcohol association mediates delayed reinstatement is an important matter, and two main explanations exist in the literature[57,58]. One hypothesis is that the context-US association returns the subject to the background condition that was present during the initial acquisition of the CS-US association, which drives reinstatement[50,57,58]. The context-US association is a part of the background that is present when the CS-US association is acquired. Therefore, a return to this US-associated context after US re-exposure could be returning the animal to the background condition under which the CS-US association was formed, resulting in activation of the CS-US memory and ultimately facilitating reinstatement[50,112]. This background condition could be the physical location of the context that acquisition was conducted in, as illustrated by reinstatement being evoked after US re-exposure and test were conducted in the acquisition context[50,56,112]. Importantly, the background condition could also be the response (e.g., freezing) or emotional state (e.g., fear) that resulted from the US which occurred during initial acquisition, as illustrated by reinstatement evoked after US re-exposure and test were both conducted in a context that differed from the acquisition context[56]. A similar process may drive delayed reinstatement of responding to an alcohol-CS, such that returning to the context that alcohol re-exposure occurred in returns the subject to the background condition experienced during initial CS-alcohol acquisition.

A second manner through which the context-US association may evoke reinstatement is through context-mediated reconditioning of the CS to the US[56,57]. According to this hypothesis, during extinction, a CS can become associated with the context that it is presented in because the CS is presented together with the context in the absence of the US. When returned to this extinction context during US re-exposure, the context could activate a cognitive representation of the CS. Consequently, the CS representation can become reconditioned with the US during the re-exposure session via the context-CS association. This reconditioning of the CS to the US would facilitate reinstatement of responding to the CS when tested 24 h later, regardless of the context that it was delivered in, as demonstrated by Westbrook and colleagues[56]. The methodology used in this thesis does not allow for the assessment of whether context-mediated conditioning contributes to the delayed reinstatement of responding to an alcohol-predictive CS, as the reinstatement test was not conducted in a novel context. This potential context-mediated process in the delayed reinstatement model should, therefore, be delineated. Future research can conduct alcohol re-exposure sessions in the context that extinction had occurred in, then test for

reinstatement of responding to the CS in a novel context, and in the extinction context. If context-mediated conditioning of the CS to alcohol does occur during the alcohol re-exposure session, then reinstatement should be observed regardless of the context that it is tested in 24 h later. The suggested experiments could reveal an exciting new learning process that contributes to delayed reinstatement to an alcohol-CS and ultimately would provide greater insight into learning processes involved in relapse to alcohol use.

In sum, Chapter 3 provides evidence that an association between alcohol and the context that alcohol re-exposure occurred in contributes to the delayed reinstatement of responding to an alcohol-CS, and thus establishes a psychological process that contributes to this delayed reinstatement effect. We also recommend experimental approaches for future research to gain a greater understanding of *how* this context-alcohol association facilitates reinstatement.

5.3. The Role of MORs in the Reinstatement of Responding to an Alcohol-CS

There is a rich history regarding the role of MORs in AUD. This relationship has been largely demonstrated by evidence that blocking these receptors with antagonists reduces alcohol intake and delays relapse to alcohol use in humans[79,180]. Moreover, MOR antagonists reduce alcohol-cue evoked reactivity in humans[3,81], and in rodents as repeatedly shown with established reinstatement models[34,83,94] and renewal models[95]. The current thesis used a pharmacological approach to examine the extent to which MORs are involved in the new delayed reinstatement model. Further, the role of MORs specifically in the ventral hippocampus (vHipp) in the delayed reinstatement effect was also assessed.

The robust delayed reinstatement of responding to an alcohol-CS was attenuated by the MOR antagonist naltrexone, which replicates previous findings[34,36,95,179,210]. This effect was replicated across two experiments, and in both female and male rats, thus indicating that there is a role of MORs in delayed reinstatement in both sexes. This reduction in reinstatement was CS-specific, as naltrexone did not affect ITI port entries nor reinstatement of responding to a sucrose-CS. These results again replicate previous findings that MORs have a more specific role in responding to a drug-cue versus other appetitive cues[188,210].

An important methodological consideration regarding the set of experiments included in Chapter 4 must be discussed: the differing roles of opioid receptor subtypes. Naltrexone is a non-specific opioid receptor antagonist and thus has binding affinity to δ-opioid receptors (DORs) and

κ-opioid receptors (KORs) in addition to MORs. Consequently, the reduction in delayed reinstatement observed after systemic naltrexone could be attributable, to some degree, to the blockade of DORs and/or KORs. Indeed, DOR antagonists have been shown to attenuate reinstatement of alcohol-seeking evoked by context or discriminative stimuli[183,184]. Conversely, studies using KOR antagonists are less definitive, as they have been shown to either not affect cue-induced reinstatement[225], or to reduce it[226]. We reason that the reduction in delayed reinstatement is likely greatly driven by blocking MORs, as naltrexone has substantially higher binding affinity and potency to this receptor over DORs and KORs[201,227,228]. Further support for this reasoning stems from research showing that selective blockade of MORs with the antagonist naloxonazine attenuates reinstatement evoked by discriminative alcohol stimuli[184].

Experiments in Chapter 4 also demonstrate, for the first time, that selectively blocking ventral hippocampal MORs prevented delayed reinstatement of responding to an alcohol-CS. This finding provides evidence that MORs in the vHipp are necessary for delayed reinstatement and thus identify a new neural mechanism that underlies this model. Moreover, this finding further supports the notion that drug actions on MORs, and not other opioid receptors, are responsible for attenuating delayed reinstatement.

The vHipp was targeted as a region of interest because of its role in associative learning[215,229,230]. In terms of drug-seeking, the dorsal hippocampus primarily plays a role in context-induced drug-seeking behaviour like renewal[92], whereas the vHipp appears to play a more diverse role. Stimulating the ventral subiculum, which is a structure in the vHipp, reinstates cocaine-seeking behaviour[231], and inactivation of the vHipp attenuates both cue- and priming-induced cocaine-seeking[99,100]. Moreover, the vHipp is involved in context-evoked drug-seeking, such as acquisition of context-cocaine associations[232], and renewal of cocaine-seeking[97]. Given this body of literature, in combination with the dense MOR population located in the vHipp[89,194], it was deemed a promising brain region to target in the investigation of the neural locus for the MORs involved in delayed reinstatement of responding to an alcohol-CS.

Research examining the involvement of MORs in responding to alcohol-cues is limited and, as such, the neural mechanisms through which blocking MORs in the hippocampus reduces reinstatement are unclear. One such mechanism may be through disinhibiting GABAergic neurons. Within the hippocampus, MORs are predominantly localized on inhibitory GABAergic interneurons, which are vital for gating excitatory and inhibitory signalling in the brain[233,234].

Accordingly, activating inhibitory MORs in the hippocampus with a MOR agonist inhibits interneurons, and reduces inhibitory GABAergic neurotransmission. Electrophysiological studies have shown that activating hippocampal MORs inhibits both spontaneous GABAergic inhibitory postsynaptic currents (IPSCs) and GABAergic IPSCs evoked by action potentials; moreover, the reduction in synaptic inhibition results in an increase in excitatory activity throughout the hippocampus[235,236]. It is possible that intra-ventral hippocampal administration of CTAP blocks MORs on GABAergic interneurons, which removes the inhibitory influence of and facilitates GABA transmission. Consequently, this increase in inhibitory activity in the vHipp could lead to a reduction in hippocampal activity and consequently the attenuated delayed reinstatement observed in Chapter 4 of this thesis. This hypothesis is consistent with pharmacological inactivation studies, in which inactivating ventral hippocampal structures with GABA agonists attenuates reinstatement of drug-seeking evoked by discrete and contextual cues[97–99,195]. As discussed above, the ventral hippocampus is not only implicated in drug-seeking driven by contexts but also discrete cues[97,99]. Therefore, the inhibition of MORs via antagonist administration, and potentially the consequential disinhibition of GABA neurons, may reduce delayed reinstatement by impacting cue processing that occurs in the hippocampus.

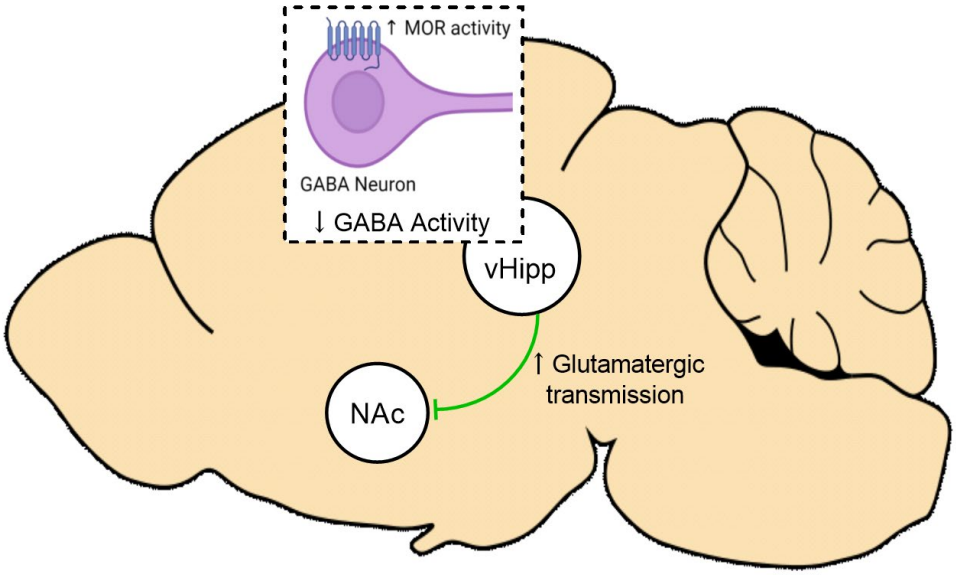
Although delayed reinstatement of responding to an alcohol-CS was prevented following intra-ventral hippocampal administration of CTAP, the potential caveat of drug diffusion into other brain structures must be addressed. It is well established that MORs mediate reward-related behaviours through actions in conventional reward-processing brain regions, such as the nucleus accumbens (NAc), ventral pallidum (VP), and basolateral amygdala (BLA)[237]. Therefore, it could be speculated that delayed reinstatement was prevented by CTAP diffusing to these other brain regions, rather than through actions in the vHipp. These regions, however, are quite anteriorly and medially distant from the ventral hippocampal coordinates used in the current experiment[42,96,190,192,200], and thus, diffusion of CTAP into these regions is unlikely. A second possibility is that CTAP diffused from the vHipp into the lateral ventricle, given that the histological assessment determined the microinjector tip placements were located directly medial to the lateral ventricle[200]. Diffusion of CTAP into the lateral ventricle would result in a systemic effect, and not a hippocampal-dependent effect, which would have prevented delayed reinstatement. However, support against this possibility is provided by research that administered CTAP into the caudate putamen of rats, using anatomical coordinates with similar proximity to

the lateral ventricle, which did not report issues concerning drug diffusion in a CPP established with cocaine[238]. Moreover, CTAP reduced expression of CPP when administered into the NAc shell, but not when administered in the core, despite being adjacent structures[238]. Thus, the prevented delayed reinstatement of responding to an alcohol-CS following administration of CTAP into the vHipp is unlikely to be due to diffusion across different brain structures, but rather to actions within the vHipp.

The vHipp is a central region in the medial temporal lobe that gives rise to afferent projections to multiple brain regions[239,240]. Of these connections, the projection from the vHipp to the NAc may be of particular interest for its potential involvement in the delayed reinstatement of responding to an alcohol-CS. The vHipp sends glutamatergic projections to the NAc, a brain region that is responsible for various reward- and goal-directed behaviours[241], including responding to alcohol-cues[42,242]. Activation of the vHipp-to-NAc pathway supports the development of conditioned place preference and operant self-administration[241]. Moreover, this projection has been implicated in responding to drug-predictive cues[243]. Disconnecting projections from the ventral subiculum to the NAc shell using an inhibitory chemogenetic approach attenuated renewal of alcohol-seeking[195]. It is plausible that a similar vHipp-to-NAc pathway, which is influenced by MOR activity in the vHipp, is involved in delayed reinstatement of responding to an alcohol-CS. Consequently, blocking this MOR activity may encourage GABAergic activity in the vHipp which could then inhibit excitatory, glutamatergic projections to the NAc (see Figure 2).

A possible caveat to this hypothesis, however, is that the existing literature supporting the role of the vHipp-to-NAc circuit in responding to drug cues has been assessed with a renewal model of drug-seeking[195,243]. Therefore, the proposed vHipp-to-NAc pathway may be uniquely involved in the renewal of drug-seeking, but not in the delayed reinstatement. Future research should first investigate the role of the vHipp-to-NAc projection in delayed reinstatement, and subsequently, identify the specific role of MORs in this projection's influence on delayed reinstatement.

A Delayed reinstatement of responding to an alcohol cue



B Attenuated delayed reinstatement of responding to an alcohol cue

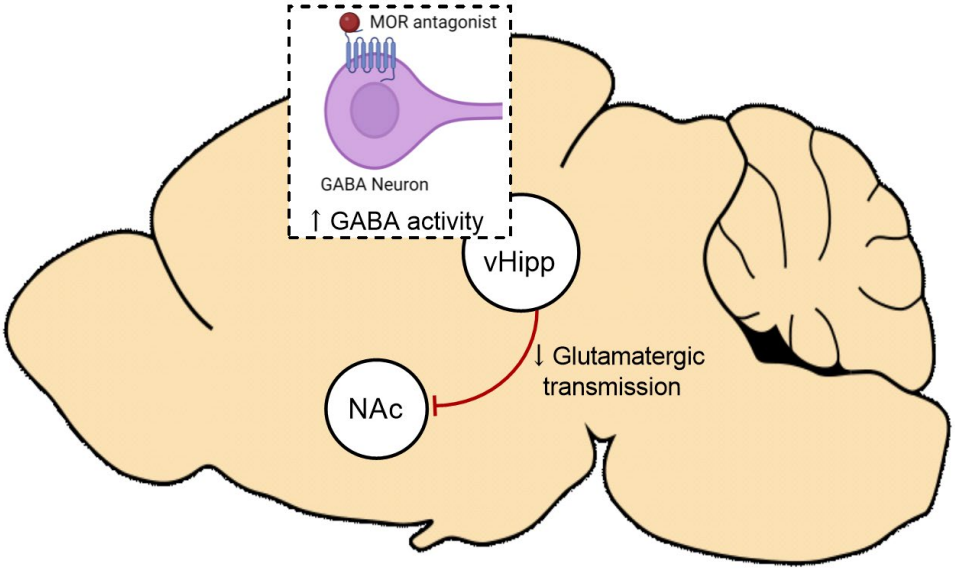


Figure 2. The proposed neurocircuitry involved in the delayed reinstatement of responding to an alcohol-CS. **(A)** During reinstatement, MORs located on GABAergic neurons in the vHipp may be activated, resulting in inhibition of GABAergic transmission and increased activity of glutamatergic neurons that send excitatory inputs to the NAc, thus driving reinstatement. **(B)** The inverse may occur when a MOR antagonist is administered into the vHipp, such that blocking MORs may disinhibit GABAergic neurons in the vHipp, resulting in reduced glutamatergic transmission to the NAc, and thus attenuating reinstatement.

The role of MORs in the neurocircuitry mediating conditioned responding, particularly to alcohol-cues, remains underexplored. While the present thesis adds to this body of research by demonstrating that MORs in the vHipp are also necessary for responding to an alcohol-CS using the delayed reinstatement model, there remain considerable gaps in the literature. Foremost, MORs in different brain regions appear to be selectively involved in different reinstatement models. Blocking MORs in the NAc shell reduces renewal but not priming-induced reinstatement of alcohol-seeking[190] while activating MORs in this region enhances cue-induced reinstatement[191]. Conversely, blocking MORs in the VP attenuates both renewal and prime-induced reinstatement[192]. Given the selective involvement of MORs in these brain regions in responding to different types of cues, it is unknown if ventral hippocampal MORs mediate responding to alcohol-cues in other reinstatement procedures. Furthermore, the understanding of the role of MORs within other hippocampal structures in responding to alcohol-cues is also incomplete. Dorsal hippocampal MORs are not involved in renewal of alcohol-seeking[96], however, this has not been tested with other reinstatement models, including the new delayed reinstatement model. These gaps in the literature offer new, exciting avenues of research to continue investigating the neural loci of MORs in responding to alcohol-cues, which will provide a more comprehensive understanding of how of MORs mediate responding to alcohol-cues.

In sum, Chapter 4 of this thesis provides evidence for a role of MORs in the delayed reinstatement model. For the first time, the vHipp was identified as a neural locus for MORs involved in any reinstatement of responding to an alcohol-CS, and a potential mechanism of action through which this effect occurs was proposed. These findings contribute to the larger, burgeoning literature that aims to delineate the brain regions that express the MORs responsible for the reinstatement of responding to alcohol-cues.

5.4. General Methodological Considerations

5.4.1. The Role of the Consummatory Response in the Delayed Reinstatement Model

The potential involvement of the consummatory response of drinking alcohol in the delayed reinstatement model must be considered. The act of using a drug like alcohol can be separated into two behaviours: 1) the seeking behaviour that is required to obtain access to the drug (e.g., pressing a lever to obtain alcohol delivery), and 2) a consummatory behaviour that is required to contact and consume the drug (e.g., licking alcohol from a fluid port). This distinction

between seeking and consummatory behaviour in animal models of addiction is important, as seeking behaviour can model the human condition of craving, whereas the consummatory response can model the loss of control or regulation during drug-taking events[5,244–246].

In Chapter 2, preferential delayed reinstatement of responding to the alcohol-CS was observed 24 h after re-exposure to alcohol, compared to re-exposure to a distinct lemon-flavoured control liquid. There was, however, still a small degree of reinstatement following re-exposure to the lemon-flavoured control liquid. This finding suggests that an additional factor, other than just re-exposure to alcohol, may have contributed to reinstatement. The consummatory response of entering the fluid port and drinking a liquid is a common feature between consuming alcohol and the lemon-flavoured control liquid. Therefore, we reason that this common consummatory response may have contributed to the enduring, lower level of reinstatement observed after re-exposure to the lemon control.

A similar persistent delayed reinstatement effect was observed in Chapter 3 of this thesis. When alcohol re-exposure was conducted in the non-training context ('B'), a small degree of reinstatement still occurred when tested in the training context ('A'). This was unexpected. If a context-alcohol association drives delayed reinstatement, then conducting alcohol re-exposure in the non-training context ('B') should not facilitate reinstatement in the training context ('A'). This is because the association established between alcohol and the non-training context ('B') would not be present when tested in the training context ('A')[112]. This unexpected effect has been demonstrated with other appetitive conditioning tasks which require an active consummatory response to ingest the US[112,113]; however, it has not been found in aversive conditioning tasks in which the shock-US is passively experienced[50]. Taken together, these findings again suggest that the consummatory response required to ingest an appetitive-US may play a role in contributing to delayed reinstatement. It is possible that performing the consummatory response of ingesting the US, specifically in the receptacle that the US is delivered, may trigger the memory of the CS-US association that was initially acquired during Pavlovian conditioning. This activated CS-US association may contribute to the reinstated responding to the CS observed 24 h later. In Chapter 3, the consummatory response of licking alcohol from a well in the fluid port was a common feature between the two different context configurations. Thus, the low level of reinstatement observed following alcohol re-exposure in the non-training context ('B') may be attributable to an activated CS-US association. The augmented reinstatement observed following alcohol re-

exposure in the training context ('A') may result from the additive effects of the activated CS-US association and the context-alcohol association.

The potential role of a consummatory response in contributing to the delayed reinstatement of responding to an alcohol-CS was addressed in Chapter 2. Re-exposure to alcohol was delivered in a manner that did not require the same consummatory response as that for the conditioned response (i.e., licking alcohol from a well in the fluid port). Alcohol was administered by systemic injection, after which rats were placed in the conditioning chambers, which did not produce reinstatement of responding to the alcohol-CS 24 h later. This finding could indicate that the consummatory response largely contributes to the delayed reinstatement effect. Alternatively, the level of intoxication resulting from a systemic alcohol injection compared to ingested alcohol is very different. An identical dose of alcohol is absorbed significantly quicker into the bloodstream when delivered by systemic injections compared to ingested alcohol[124]. The difference in blood alcohol concentrations likely produced different levels of intoxication, such that systemic injections may have produced an internal state that was vastly different from the one experienced during other phases of training, under which reinstatement could not be expressed. Therefore, the extent to which the consummatory response is involved in delayed reinstatement of responding to an alcohol-CS cannot be fully explained by the results obtained from delivering alcohol re-exposure via systemic injection.

The potential role of a consummatory response in delayed reinstatement is an important factor, as it may be a unique process that underlies reinstatement to drug cues. Two behavioural approaches can be used in future research to elucidate this potential role. First, alcohol re-exposure could be conducted in a manner that requires a consummatory response that differs from the response required during other phases of training, but still produces similar levels of intoxication. If re-exposure to alcohol by a method other than delivery to a well in the fluid port, like a sipper tube[40,159], does not evoke reinstatement, then reinstatement may be largely driven by a consummatory response that is similar to that required to initially consume alcohol. Second, a Pavlovian discrimination task would assess the capacity for alcohol re-exposure to reinstate responding to an alcohol-CS over a CS that predicts a different stimulus such as sucrose[36,210]. Here, a distinct CS (CS1) would be paired with alcohol and a separate CS (CS2) would be paired with sucrose. After extinction of both CSs, alcohol re-exposure would be conducted. Then, 24 h later, reinstatement to either CS1 or CS2 would be tested. If reinstatement to CS2 occurs, the CS

that was previously paired with sucrose, then reinstatement is likely driven by the common consummatory response that occurs when consuming either alcohol or sucrose. These experiments could further elucidate the unique role of a consummatory response in the delayed reinstatement of responding to an alcohol-CS.

Importantly, the potential role of the consummatory response does not minimize the validity of the delayed reinstatement model, as we have shown that preferential delayed reinstatement is produced by re-exposure to alcohol, relative to a distinct control liquid. Rather the involvement of the consummatory response in reinstatement implies that the act of ingesting alcohol is an additional feature that may contribute to relapse to alcohol use, which should also be considered in animal models of addiction[5,244–246].

5.4.2. Sex Differences in the Delayed Reinstatement of Responding to an Alcohol-CS

The necessity of including female subjects in preclinical research is undeniable[174]. Many differences have been reported in women and men living with AUD. Relative to men, women are more sensitive to the pharmacological effects of alcohol, progress from recreational use to dependence quicker, and are more susceptible to stress-induced relapse[247], indicating that various aspects of alcohol use disorder differ between genders. In order to capture these differences observed in clinical populations, and to produce comprehensive research, female subjects must be included in preclinical models of relapse[175]. Therefore, although the focus of the current thesis was not to determine sex differences in the delayed reinstatement model, female rats were included in this thesis.

Delayed reinstatement of responding to an alcohol-CS occurred similarly in both female and male rats. Moreover, blocking MORs with naltrexone attenuated delayed reinstatement in females and males, indicating that MORs are recruited during delayed reinstatement in both sexes. A caveat to the current thesis, however, is that the role of a context-alcohol association in delayed reinstatement was only examined in male rats. A context-alcohol association may mediate delayed reinstatement in female rats, as previous studies show that a context-US association is necessary for the reinstatement to an aversive-CS in samples of only female rats[50,112]. However, the manner that alcohol-predictive contexts govern responding to discrete alcohol-cues are disparate in female and male rats. Male rats tailor their responding to discrete alcohol-cues based on the context, such that responding is higher in alcohol contexts and lower in

neutral contexts. Females, however, respond to discrete alcohol-cues to a similar degree regardless of the context[165]. Given that females appear to use discrete cues more than contexts to guide conditioned responding, delayed reinstatement in female rats may be mediated by a mechanism other than a context-alcohol association. If this is the case, then delayed reinstatement of responding to the alcohol-CS in females may persist after extinguishing the context-alcohol association or delivering alcohol re-exposure in a context that differs from the test context. Given this ambiguity, future research should investigate the role of a context-alcohol association in mediating delayed reinstatement in both female and male rats. Importantly, this work would further elucidate potential sex differences in the psychological mechanisms that underly reinstatement.

5.5. General Conclusions

The current established reinstatement models illustrate how re-exposure to alcohol and alcohol-predictive cues can be potent triggers for relapse[44,248]. However, these models have primarily examined the immediate impact of reinstating stimuli on behaviour[30,33,52]. Chapter 2 of this thesis expands on this research by demonstrating that re-exposure to alcohol also reinstates responding to an alcohol-predictive cue at a later timepoint. The separate sets of experiments described in Chapters 3 and 4 provide insight into the psychological and neural mechanisms that contribute to this delayed reinstatement of responding to an alcohol-predictive cue, such that contexts associated with alcohol, and MORs in the vHipp, mediate delayed reinstatement. Furthermore, various questions that arose from these findings were discussed, and future research avenues to continue investigating the underlying mechanisms of delayed reinstatement were suggested. The new delayed reinstatement model helps establish a more comprehensive understanding of how alcohol and alcohol-predictive cues can influence relapse. Moreover, a detailed understanding of the psychological and neural mechanisms underlying the delayed reinstatement effect can further inform the development of new behavioural and pharmacological treatment interventions against relapse.

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Appendix A: Chapter 2 Supplementary Material

Table A.1. Alcohol concentration used during Pavlovian conditioning for each experiment. During Pavlovian conditioning, CS trials were paired with 15% ethanol unless rats were receiving 10% ethanol at the end of the home-cage alcohol exposure phase, in which case 10% ethanol was used during Pavlovian conditioning.

Exp.	Number of rats on 10% EtOH	Number of rats on 15% EtOH
1*	*n* = 5	*n* = 19
2	*n* = 5	*n* = 19
3	*n* = 7	*n* = 29
4	*n* = 0	*n* = 12

* In Experiment 1, *n* = 5 rats started receiving 10% ethanol in session 6 due to low normalized-CS port entries in the prior sessions. They were maintained on this concentration for the remainder of the study.

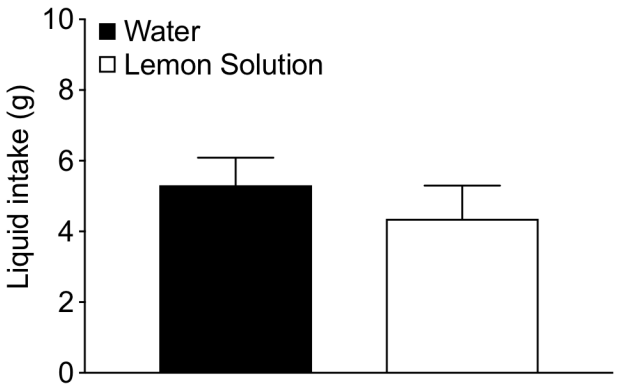

Figure A.1. Rats in Experiment 2 drank equivalent amounts of water and lemon-flavored liquid in the home cage. Data represent mean (± SEM) intake of water averaged across the last two sessions in which rats had access only to water (black bar), and across the last two sessions in which rats had access to either lemon-flavored liquid or water in separate bottles (white bar). There was no significant difference in the intake of these two liquids [Paired t-test: t (23) = 1.14, p = 0.265].

Supplementary methods for tail vein blood extraction and NAD-ADH assay.

To extract blood from the lateral tail vein, rats were anesthetized with isoflurane. Illumination of an infrared lamp over the tail for 5 min was used to dilate the lateral tail vein and increase blood flow. The tail was cleaned with sterile saline and a horizontal incision was made between the scales on the right side of the tail. Blood was collected from the incision into heparinized capillary tubes that were emptied into centrifuge vials which were kept on ice. The incision was cleaned with an alcohol swab and once bleeding ceased, a mixture of xylocaine and polysporin was topically applied.

An NAD-ADH enzymatic assay was then conducted to determine blood alcohol concentrations (Carnicella et al., 2009). This technique uses the enzymatic reduction of the alcohol dehydrogenase co-enzyme NAD+, to NADH, so that blood alcohol levels can be interpolated by measuring spectrophotometric absorbance of NADH at 340 nm. Standard samples of varying millimolar alcohol (EtOH) concentrations, ranging from 0 mM to 50 mM (99% EtOH diluted in distilled water) were used to create the standard curve, which was used to estimate the alcohol content in blood samples. Ten µL of the standard sample supernatants and blood plasma, collected from whole blood samples, were pipetted into a centrifuge tube, to which 40 µL of 3.4% perchloric acid (≥70% perchloric acid diluted in distilled water) was added. Supernatants and blood plasma samples were then centrifuged for six min at 2000 RPM and at 4 °C. The assay was then prepared by pipetting triplicates of 7 µL of the supernatants and blood plasma samples into a transparent flat-bottomed 96-well plate. Next, 343 µL of a tris-buffer solution (6.057 g trizma Base, Sigma-Aldrich, T1503; 100 mL distilled water) was added. The tris-buffer solution pH was adjusted to 8.8 using hydrochloric acid and included ADH from saccharomyces cerevisiae (0.275 mg; Sigma-Aldrich, A7011) and β-NAD lithium salt (50 mg; Sigma-Aldrich, N7132). The assay was left to incubate for 40 min, then was run through a spectrophotometer at 340 nm. A standard curve was created from the absorbance values of the standard samples, and from this blood alcohol content in mg% (mg/dL) was estimated.

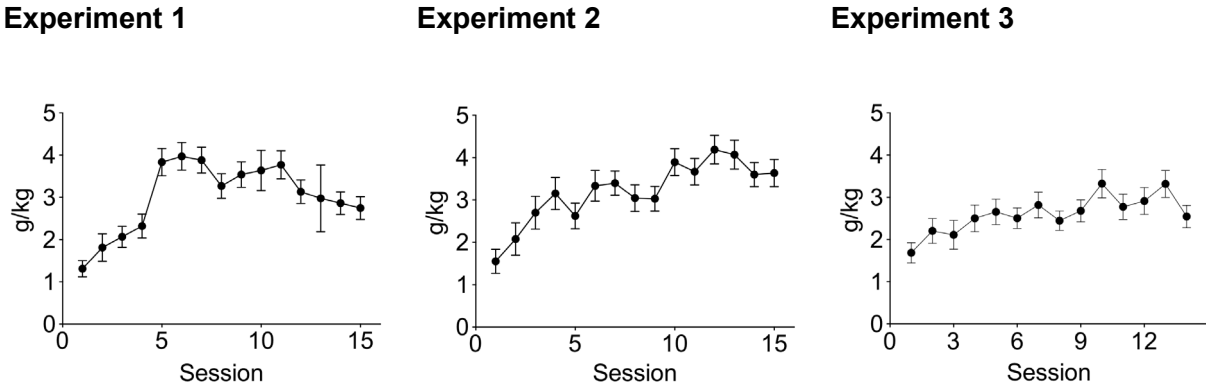

Figure A.2. The ingested dose of alcohol increased across sessions of intermittent-access to alcohol and water in the home cage. Data represent mean (± SEM) ingested alcohol dose (g/kg) for rats whose data were included and analysed. Experiment 4 is not depicted here or in S. Fig. 3. because these rats were used for analysis of blood ethanol content followed by a pilot behavioural study that was not included in this article. ANOVA indicated a significant main effect of Session in each experiment [Exp. 1, [F (5.775, 132.815) = 8.91, p < .001: Exp. 2, F (2.740, 63.025) = 7.59, p < 0.001: Exp. 3, F (6.230, 218.050) = 6.47, p < .001].

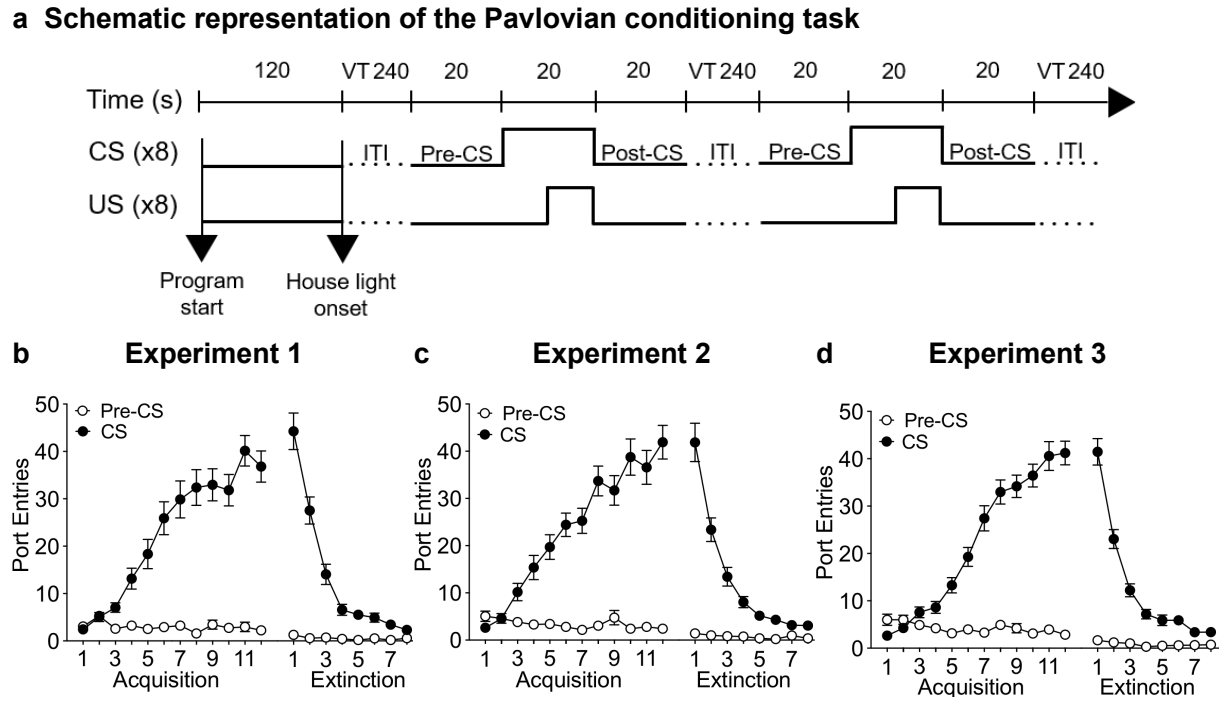

Figure A.3. Acquisition and extinction of Pavlovian conditioning. **A** Schematic depicting the experimental parameters used for Pavlovian conditioning. Conditioned stimulus (CS) presentations were paired with the alcohol unconditioned stimulus (US). US delivery lasted for 10 sec. The inter-trial interval (ITI) occurred on a variable-time 240 s schedule, which did not include the 20 s pre-CS and post-CS intervals. Parameters were identical during extinction and test, except that no alcohol was delivered. **B-D** Mean (± SEM) number of port entries (collapsed across group) made during CS presentations increased across acquisition sessions and decreased across extinction sessions, whereas port entries made during the pre-CS interval remained stable across training.

Table A.2. ANOVA table for CS-elicited port entries across Pavlovian conditioning and extinction training shown in Supplementary Figure 3.

Experiment 1	**ANOVA**	***df***	***F***	***p***
Pavlovian Conditioning	Session	11, 480	18.255	< 0.001
	Interval	1, 480	530.255	< 0.001
	Session x Interval	11, 480	20.524	< 0.001
Extinction	Session	7,320	56.399	< 0.001
	Interval	1, 320	331.136	< 0.001
	Session x Interval	7,320	52.164	< 0.001
Experiment 2				
Pavlovian Conditioning	Session	11, 528	22.256	< 0.001
	Interval	1, 528	690.591	< 0.001
	Session x Interval	11, 528	26.965	< 0.001
Extinction	Session	7, 352	55.590	< 0.001
	Interval	1, 352	336.723	< 0.001
	Session x Interval	7, 352	52.006	< 0.001
Experiment 3				
Pavlovian Conditioning	Session	11, 768	41.461	< 0.001
	Interval	1,768	831.270	< 0.001
	Session x Interval	11, 768	49.936	< 0.001
Extinction	Session	7, 512	82.922	< 0.001
	Interval	1, 512	508.767	< 0.001
	Session x Interval	7, 512	73.463	< 0.001

Note: ANOVA conducted on Session (number of sessions in a given phase) and Interval (Pre-CS, CS) for each experiment shown in Supplementary Figure 3. During Pavlovian conditioning, port entries increased across sessions, specifically during the CS interval. During extinction, port entries decreased across sessions, specifically during the CS interval.

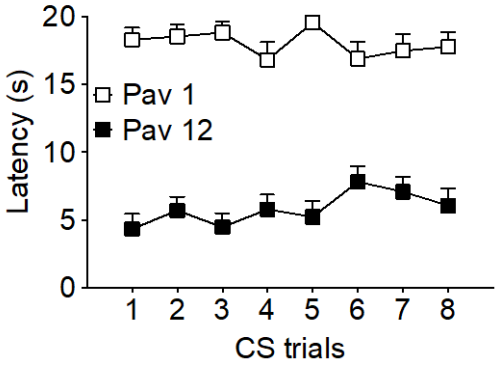

Figure A.4. Rats learned to make port entries after CS onset faster during Pavlovian conditioning session 12 compared to session 1. This supports the inference that alcohol became associated with CS presentations despite having unrestricted access to the fluid port throughout the session. Data represent mean (± SEM) total latency to initiate CS-elicited port entries across trials during Pavlovian conditioning session 1 (white squares) and session 12 (black squares).

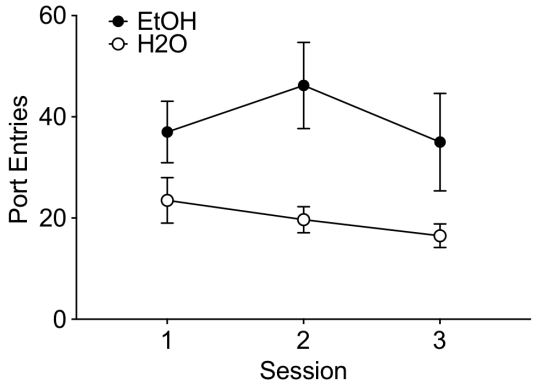

Figure A.5. Alcohol is a more effective reinforcer in a Pavlovian procedure than water. At the end of the study, a subset of rats from Experiment 1 received three Pavlovian conditioning sessions in which CS presentations were paired with alcohol (EtOH, n = 5) or water (H2O, n = 6) in separate groups. Data represent mean (± SEM) normalized CS-elicited port entries across these sessions. ANOVA indicated a significant main effect of Group [F (1, 9) = 7.69, p = 0.022], confirming that water was less effective as a reinforcer than alcohol in this task. There was no main effect of Session [F (2, 18) = 1.89, p = 0.179] or Session x Group interaction [F (2, 18) = 1.55, p = 0.202].

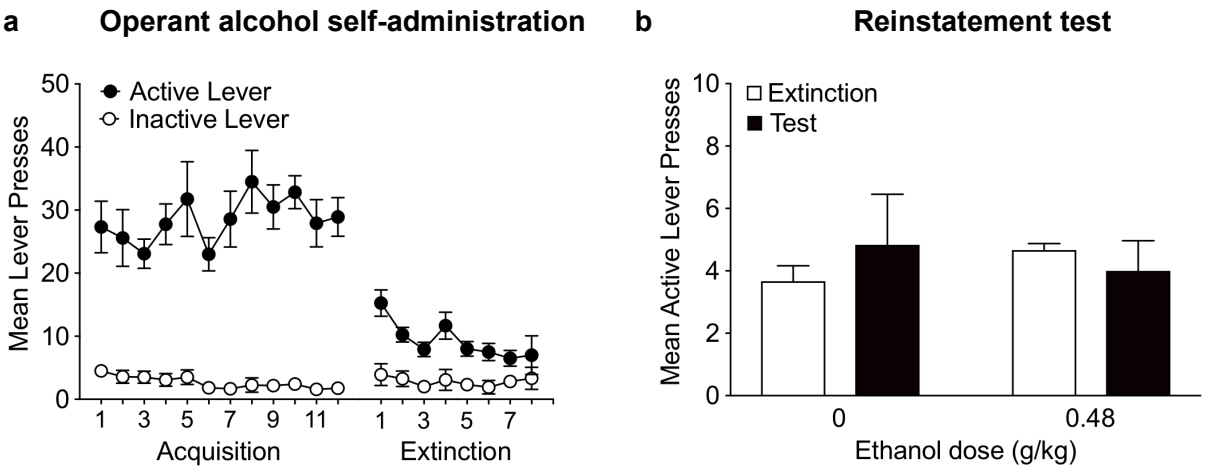

Figure A.6. Operant alcohol-seeking behaviour was not reinstated by an intraperitoneal injection of alcohol. In this pilot study, we attempted to replicate the findings of Le et al., (1998, Psychopharmacology) in which intraperitoneal injection of alcohol administered immediately before an extinction session reinstated extinguished operant alcohol-seeking behaviour. Using a subset of rats from Experiment 3, we conducted a lever-shaping session in which pressing an active lever was reinforced by alcohol (0.1 mL) on a continuous reinforcement schedule until 200 reinforcers had been earned. Subsequently, we conducted 12 operant conditioning sessions followed by eight extinction sessions. During operant conditioning, responding on an active lever produced alcohol (0.1 mL) on a continuous reinforcement schedule, whereas responding on a second, inactive lever was recorded but not reinforced. Alcohol delivery was paired with the 5 s onset of a white cue light located above the active and the inactive levers. During extinction, active lever pressing produced the cue lights but no alcohol. Operant conditioning and extinction sessions lasted 40 min or until 200 reinforcers had been earned. **A** Mean (± SEM) active and inactive lever presses across operant conditioning and extinction sessions for all 12 rats. **B** Following extinction, rats were tested 10-15 min after receiving an intraperitoneal injection of saline (n = 6) or alcohol (0.48 g/kg; n = 6). Data represent mean (± SEM) active lever presses during the last extinction session and the test session for the for both groups. There was no significant reinstatement effect [Phase, F (1, 10) = 0.06, p = 0.812; Phase x Group, F (1, 10) = 0.78, p = 0.392; Group, F (1, 10) = 0.01, p = 0.931].

Appendix B: Chapter 3 Supplementary Material

Table B.1. Number of rats receiving different alcohol concentrations during Pavlovian conditioning and alcohol re-exposure sessions.

Experiment	Number of rats on 5% EtOH	Number of rats on 15% EtOH
1A	*n* = 0	*n* = 36
1B	*n* = 0	*n* = 36
2A	*n* = 1	*n* = 27
2B	*n* = 1	*n* = 27

Intermittent alcohol access

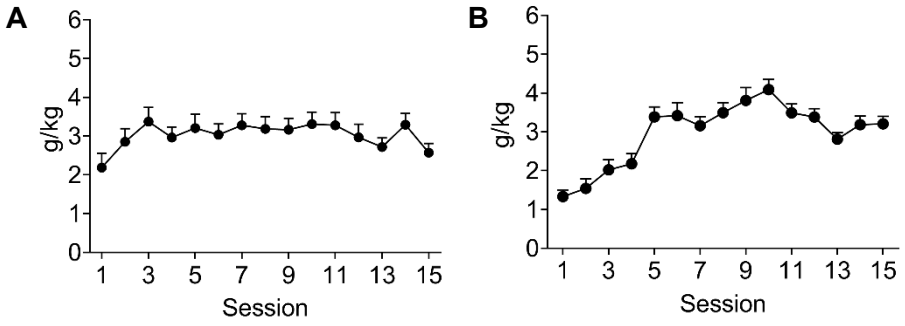

Figure B.1. A repeated measures ANOVA revealed that alcohol intake (g/kg) increased across intermittent alcohol access sessions **(A)** Experiment 1 [$F_{(6.725,\ 235.358)} = 16.343$, $p < 0.001$], and (**B)** Experiment 2 [$F_{(5.291,\ 142.856)} = 2.276$, $p = 0.047$]. Data represent mean (± SEM) g/kg obtained across sessions.

Acquisition and extinction of conditioned responding for alcohol

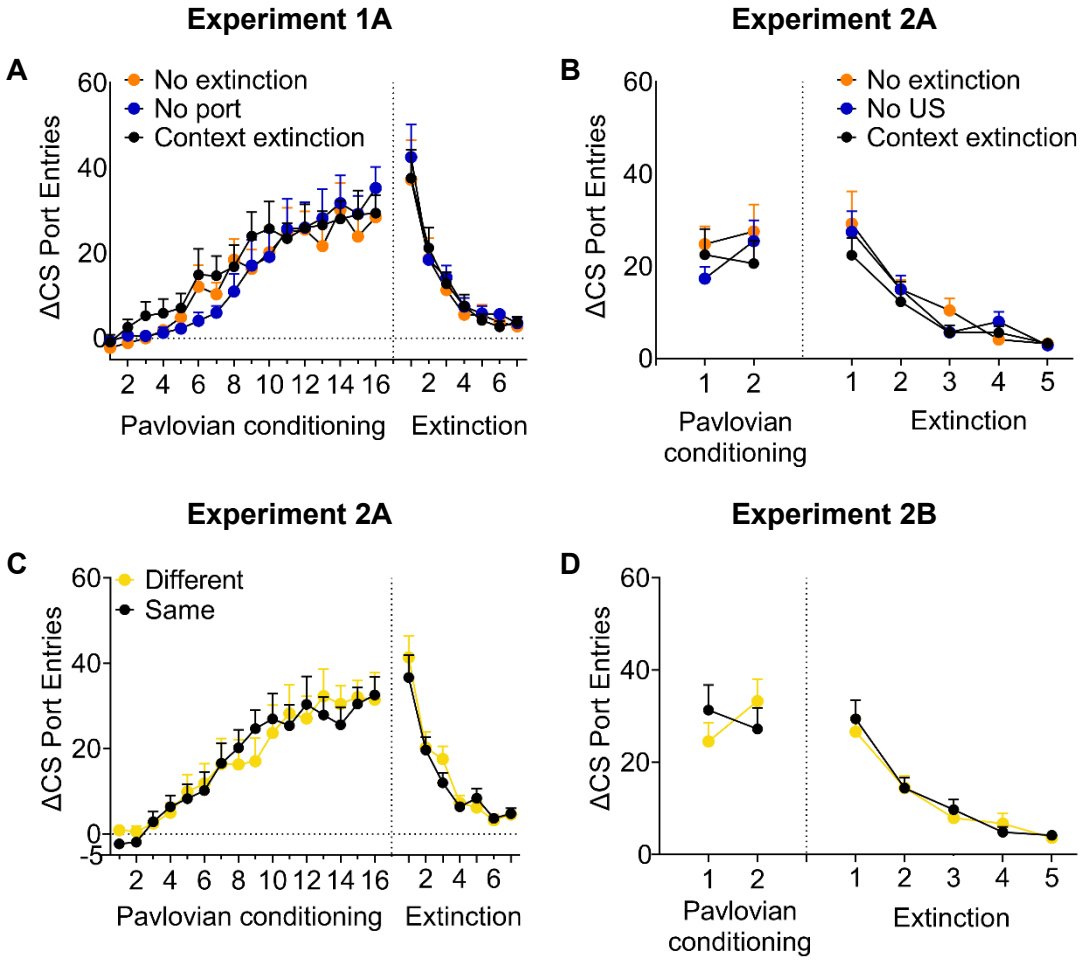

Figure B.2. Rats learned to associate the CS with alcohol in both experiments. Separate Session x Group mixed ANOVA revealed that ΔCS port entries significantly increased across Pavlovian conditioning sessions similarly in all groups in **(A)** Experiment 1A [Session: $F_{(4.380, 118.266)} = 38.760$, $p < .001$; Session x Group: $F_{(8.760, 118.266)} = 0.847$, $p = 0.572$; Group: $F_{(2, 27)} = 0.206$, $p = 0.815$], **(B)** Experiment 1B [Session: $F_{(1, 25)} = 2.076$, $p = 0.162$; Session x Group: $F_{(2, 25)} = 1.966$, $p = 0.161$; Group $F_{(2, 25)} = 0.374$, $p = 0.692$], **(C)** Experiment 2A [Session: $F_{(4.472, 89.449)} = 25.057$, $p <0.001$; Session x Group: $F_{(4.472, 89.449)} = 0.523$, $p = 0.739$; Group: $F_{(1, 20)} = 0.000$, $p = 0.984$], and **(D)** Experiment 2B [Session: $F_{(1, 21)} = 0.431$, $p = 0.518$; Session x Group: $F_{(1, 21)} = 3.283$, $p = 0.084$; Group: $F_{(1, 21)} = 0.004$, p = 0.947].

The conditioned response extinguished, as a separate Session x Group mixed ANOVA revealed that ΔCS port entries significantly decreased across extinction sessions similarly in all groups in **(A)** Experiment 1A [Session: $F_{(2.143, 57.869)} = 48.234$, $p < .001$; Session x Group: $F_{(4.287, 57.869)} = 0.195$, $p = .948$; Group: $F_{(2, 27)} = 0.166$, $p = 0.848$], **(B)** Experiment 1B [Session: $F_{(2.447, 61.165)} = 40.046$, $p < 0.001$; Session x Group: $F_{(4.893, 61.165)} = 0.676$, $p = 0.640$; Group: $F_{(2, 25)} =$

0.490, $p = 0.618$], **(C)** Experiment 2A [Session: $F_{(2.855, 57.108)} = 57.713$, $p < 0.001$; Session x Group: $F_{(2.855, 57.108)} = 0.724$, p = 0.535; Group: $F_{(1, 20)} = 0.259$, $p = 0.616$], and **(D)** Experiment 2B [Session: $F_{(2.864, 60.151)} = 41.413$, $p < 0.001$; Session x Group: $F_{(2.864, 60.151)} = 0.355$, $p = 0.777$; Group: $F_{(1, 21)} = 0.121$, $p = 0.732$]. Data represent mean (± SEM) ΔCS port entries across sessions.