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ARTICLE

Blocking μ-opioid receptors attenuates reinstatement of responding to an alcohol-predictive conditioned stimulus through actions in the ventral hippocampus

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The μ-opioid system is involved in the reinstatement of responding that is immediately evoked by alcohol-predictive cues. The extent of its involvement in reinstatement observed in a new model that evaluates the delayed effects of re-exposure to alcohol, however, is unclear. The current study investigated the role of μ-opioid receptors (MORs) in the delayed reinstatement of an extinguished, Pavlovian conditioned response that was evoked 24 h after alcohol re-exposure. Female and male Long-Evans rats received Pavlovian conditioning in which a conditioned stimulus (CS) was paired with the delivery of an appetitive unconditioned stimulus (US; Experiments 1, 2, 4: 15% *v/v* alcohol; Experiment 3: 10% *w/v* sucrose) that was delivered into a fluid port for oral intake. During subsequent extinction sessions, the CS was presented as before but without the US. Next, the US was delivered 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 systemic naltrexone (0.3 or 1.0 mg/kg) attenuated reinstatement of port entries elicited by an alcohol-CS, but not those elicited by a sucrose-CS. Finally, blocking 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 alcohol-CS port entries. 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 in the ventral hippocampus are necessary for responding to an alcohol-predictive cue.

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INTRODUCTION

An established theory in the research on alcohol use disorders is that environmental stimuli which accompany alcohol intake can become cues that predict alcohol availability. Consequently, exposure to alcohol-predictive cues can influence human behaviour, such as precipitating craving and relapse [1–3]. Similarly in animal studies, exposure to alcohol-predictive contexts [4, 5], discrete cues [6, 7], discriminative stimuli [8], and alcohol-primes [9, 10] prompts the reinstatement of extinguished, conditioned responding for alcohol. These reinstatement models are valuable tools that provide insight into how maladaptive behaviours in response to cues contribute to relapse [1]. As such, 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 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 alcohol intake and probability of relapse [11, 12]. It is posited that naltrexone's efficacy is in part due to a reduction in cue-evoked craving for alcohol [13, 14]. Similarly, in animal models, systemic MOR antagonist treatment attenuates reinstatement of operant alcohol-seeking evoked by an alcohol-predictive context [15–17], discrete cue [18], discriminative stimulus [19–21], and alcohol-prime [22–24]. Blocking MORs does not affect other motivated behaviour such as alcohol-seeking reinstated by stressful stimuli [18, 22] or reinstatement of sucrose-seeking [25], thereby demonstrating that the involvement of MORs in reinstatement is alcohol-specific.

While the systemic effects of MOR antagonists on cue-evoked alcohol-seeking are well documented, the neural loci of this effect are less understood. The strongest evidence implicates substrates of the reward neurocircuitry, like the nucleus accumbens [26, 27], basolateral amygdala [16, 28], and ventral pallidum [29, 30]. The extent to which MORs in other brain regions contribute to reinstatement of responding, however, remains largely unknown despite there being promising options. The ventral subregion of the hippocampus is a likely target as it is an integral part of the reward neurocircuitry [30], has rich expression of MORs [31, 32], and inactivation of this region attenuates reinstatement of drug-seeking evoked by drug-predictive cues and contexts [33–37]. Despite this evidence, the role of ventral hippocampal MORs in responding to alcohol-predictive cues has not yet been investigated.

The involvement of MORs in responding to alcohol-predictive cues has, overwhelmingly, been studied using traditional reinstatement models. While these models provide insight into how reinstating stimuli immediately precipitate relapse-like behaviour, they do not address the delayed impact that these stimuli have on

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behaviour. A novel reinstatement model addresses this delayed effect. 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 is significantly reinstated [38, 39]. This novel model has great translational value, as it illustrates how a lapse in alcohol use can influence relapse at a future point in time. Indeed, momentary lapses in drug use are powerful predictors of relapse months in the future [40]. The delayed reinstatement model captures this distinct aspect of human addiction that traditional models do not. Interestingly, this model is also driven by a psychological process, specifically a context-alcohol association [39], which is distinct from the processes proposed to drive traditional reinstatement [41]. These methodological and mechanistic differences highlight the novelty of the delayed reinstatement model, which also brings into question if different neural processes may govern this distinct behaviour. Investigating these potential differences would help develop a greater understanding of the complex phenomenon that is relapse.

To better understand the neural mechanisms underlying the delayed reinstatement model, we assessed the role of MORs in this behaviour. The effects of systemic naltrexone administration on reinstatement of responding to an alcohol-CS were tested in male (Experiment 1) and female rats (Experiment 2). A separate experiment tested the effects of naltrexone on reinstatement of responding to a sucrose-predictive CS (Experiment 3). Then, to determine the role of ventral hippocampal MORs in delayed reinstatement of responding for alcohol, the effects of administering the MOR antagonist, CTAP, into the vHipp on the reinstatement were tested (Experiment 4).

METHODOLOGY

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. Cages containing unrestricted access to chow (Purina Agribrands, Charles River), water, and environmental enrichment (see Supplementary Materials), were held in a colony room following a 12 h light/dark cycle (0700 h lights on; experiments conducted during the light phase). All procedures followed the Canadian Council on Animal Care guidelines and were approved by the Concordia University Animal Research Ethics Committee.

Apparatus

Behavioural procedures were conducted in 12 conditioning chambers (ENV-009A; Med Associates Inc., St-Albans, VT) that are described in the Supplementary Materials.

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 on the day of use by dissolving naltrexone hydrochloride (Sigma Aldrich) in sterile saline (0.9%) to obtain a 0.3 or 1.0 mg/ml concentration that was administered at a volume of 1 ml/kg. D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP; Tocris) was dissolved in sterile saline (0.9%) to obtain a 2.5 or 5.0 µg/0.3 µl concentration which was administered at a volume of 0.3 µl/hemisphere. Aliquots were stored at −20 °C until use.

Surgery

After 12 intermittent alcohol access sessions, rats underwent stereotaxic surgery using standard procedures [42] to bilaterally implant stainless steel cannulae (26 gauge; Plastics One C235G-1.2-SPC) into the ventral hippocampus (vHipp). Coordinates used were −5.5 mm anterior-posterior, ±5.4 mm medial-lateral, and −3.0 ventral from the skull surface [43, 44]. During intracranial drug microinfusions, 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. Guide cannulae were occluded with 7.5 mm dummy canulae. Postsurgical pain was managed with buprenorphine (Buprenex; 0.1 mg/kg, subcutaneous). Three additional intermittent alcohol access sessions were conducted 1 week after surgery.

Intracranial microinfusions

Bilateral microinfusions were conducted using standard procedures [42]. Microinfusions were administered with a 33 gauge injector attached to polyethylene tubing (PE20, VWR, CA-63 018-645) connected to a 10 µL Hamilton syringe (Hamilton, 1701N). Microinfusions were delivered by syringe pump (Pump 11 Elite, Harvard Apparatus, 704 501) at a rate of 0.3 µl/min; injectors remained in place for 2 min to ensure proper drug diffusion.

Behavioural procedures

Intermittent alcohol access and sucrose habituation. Fifteen intermittent alcohol access sessions (see Supplementary Materials) were conducted. In Experiment 3, rats received 48 h access to 10% (*w/v*) sucrose in the home-cage to familiarise them with sucrose.

Pavlovian conditioning. Sessions began with the house lights illuminating, followed by eight trials of a 20 s continuous white-noise conditioned stimulus (CS) paired with 10 s activation of the fluid pump which delivered 0.3 ml of the US into the fluid port which co-terminated with the CS (Experiments 1, 2, 4: alcohol; Experiment 3: sucrose). Trials were presented on a variable time 240 s schedule. Fluid ports were checked at the end of each session to verify that the US was ingested. The number of Pavlovian conditioning sessions conducted for each experiment are detailed in the Supplementary Materials.

Extinction. Sessions were identical to Pavlovian conditioning parameters except that CS presentations were paired with the activation of empty syringe pumps (i.e. US was not delivered). The number of extinction sessions conducted for each experiment are detailed in the Supplementary Materials.

US Re-exposure. During this session, 2.4 ml of the US (Experiments 1, 2, 4: alcohol; Experiment 3: sucrose) was delivered into the fluid port according to the same delivery schedule as Pavlovian conditioning; however, the CS was not presented.

Reinstatement test. Reinstatement tests were conducted 24 h after the US re-exposure session, during which the CS was presented under extinction conditions.

Experiment 1: Effects of systemic naltrexone on reinstatement of responding to an alcohol-CS

The effects of systemic naltrexone administration on the reinstatement of responding to an alcohol-CS were tested. After intermittent alcohol access, naïve male rats ($n = 15$) 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 test [15, 18, 20]. Rats were habituated with saline injections before the second-last extinction session and the alcohol-US re-exposure sessions. The experimental procedure is illustrated in Fig. 1A.

Experiment 2: Effects of systemic naltrexone on reinstatement of responding to an alcohol-CS in female and male rats

The effects of systemic naltrexone on reinstatement of responding to an alcohol-CS was replicated in female and male rats. A group

of naïve rats ($n = 18$ females, $n = 18$ males) underwent the identical reinstatement and pharmacological manipulations described in Experiment 1.

Experiment 3: Effects of systemic naltrexone on reinstatement of responding to a sucrose-CS

The capacity for naltrexone to attenuate reinstatement in an alcohol-specific manner was examined by testing the effects of systemic naltrexone administration on reinstatement of responding to a sucrose-CS. After sucrose habituation, naïve 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 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. The experimental procedure is illustrated in Fig. 3A.

Experiment 4: Effects of intra-ventral hippocampal CTAP on reinstatement of responding to an alcohol-CS

The role of ventral hippocampal MORs in the reinstatement of responding to an alcohol-CS was tested via intra-ventral hippocampal administration of the MOR antagonist CTAP. CTAP was used to specifically target MORs and not delta or kappa opioid receptors [45]. After stereotaxic surgery and intermittent alcohol access, naïve rats ($n = 13$ females, $n = 13$ males) received Pavlovian conditioning with an alcohol-US, extinction, alcohol-US re-exposure, followed 24 h later with a test for reinstatement. CTAP (0, 2.5, 5.0 µg/hemisphere; counterbalanced across tests) was bilaterally microinfused into the ventral hippocampus 5 min before the reinstatement test [26, 29, 46, 47]. Rats were habituated to intracranial microinfusions of saline (0.3 µl/hemisphere) before the second-last extinction session and the US re-exposure session. The experimental procedure is illustrated in Fig. 4A.

Histology

Coronal sections (40 µm) were collected from paraformaldehyde-fixed brains using a cryostat (−20 °C) for Nissl staining using a standard protocol [42]. Ventral placements of injector tips were identified using light microscopy and the Paxinos and Watson rat brain atlas [48].

Data management

Exclusion criteria. Rats were excluded if they did not learn the Pavlovian task, did not have extinguished conditioned responding, had a difference score of ≤0 ΔCS port entries (reinstatement test minus last extinction session) under saline treatment as these rats were deemed to not reinstate under control conditions, detached headcaps, obstructed cannulae, or injury (see Supplementary Table 1 for sample sizes).

Variables. ΔCS port entries (CS port entries minus pre-CS port entries) and intertrial interval port entries (port entries outside of the CS interval) were analysed. Responding at reinstatement test was compared to a baseline, which was average responding during the last two extinction sessions.

Statistical analyses

All experiments used within-subjects designs and were analysed using analysis of variance (ANOVA). Analyses included the Phase and Drug within-subjects' factors and the Sex between-subjects factor. Experiment 4 data were not analysed with a Sex factor because of the limited number of females, and the lack of sex differences observed in Experiment 2.

Huynh-Feldt corrections were applied when Mauchly's test of sphericity was violated. 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 $p < 0.05$. Non-significant statistics are provided in the Supplementary Materials. Graphs were created with Graphpad Prism (Version 8; La Jolla, CA).

RESULTS

Acquisition and extinction of conditioned responding

Alcohol intake increased, or remained elevated, across intermittent alcohol access sessions (Supplementary Fig. 1). Rats learned to associate the CS with the US as ΔCS port entries increased across Pavlovian conditioning sessions, whereas ΔCS port entries decreased across extinction sessions (Supplementary Fig. 2).

Experiment 1. Naltrexone reduced reinstatement of responding to an alcohol-CS

Relative to extinction, ΔCS port entries (Fig. 1B) significantly increased at test [Phase: $F_{(1,10)} = 49.249$, $p < 0.001$]; however, this increase differed by naltrexone dose [Phase × Dose: $F_{(2,20)} = 10.959$, $p < 0.001$; Dose: $F_{(2,20)} = 3.673$, $p = 0.044$]. Post-hoc analyses revealed that reinstatement of ΔCS port entries occurred following saline ($p < 0.001$) and 0.3 mg/kg of naltrexone ($p = 0.002$), whereas reinstatement was prevented by 1.0 mg/kg of naltrexone ($p = 0.166$). Moreover, relative to saline, ΔCS port entries at test were reduced by 0.3 mg/kg ($p = 0.005$) and 1.0 mg/kg ($p = 0.002$) of naltrexone.

CS port entry as a function of trial was analysed to examine the effects of naltrexone on the pattern of responding at test. During the test session, ΔCS port entries (Fig. 1C) significantly decreased across CS trials due to within-session extinction [Trial: $F_{(7,70)} = 16.757$, $p < 0.001$]; however, this responding differed by naltrexone dose [Trial × Dose: $F_{(14,140)} = 4.887$, $p < 0.001$; Dose: $F_{(2,20)} = 7.546$, $p = 0.004$]. Post-hoc analyses revealed that, relative to saline, 0.3 mg/kg ($p < 0.001$) and 1.0 mg/kg ($p < 0.001$) of naltrexone reduced ΔCS port entries during the first CS trial. ITI port entries (Fig. 1D) were unaffected by naltrexone [Phase, Phase x Dose, Dose: $p > 0.05$].

Experiment 2. Naltrexone reduced reinstatement of responding to an alcohol-CS in female and male rats

Collapsed across Sex and relative to extinction, ΔCS port entries (Fig. 2A) significantly increased at test [Phase: $F_{(1,22)} = 65.994$, $p < 0.001$]; however, this differed by naltrexone dose [Phase × Dose: $F_{(2,44)} = 7.552$, $p = 0.002$; Dose: $F_{(2,44)} = 12.887$, $p < 0.001$]. ΔCS port entries reinstated following administration of saline ($p < 0.001$), 0.3 mg/kg ($p < 0.001$) and 1.0 mg/kg ($p = 0.001$) of naltrexone. However, relative to saline, reinstatement of ΔCS port entries was reduced by 0.3 mg/kg ($p = 0.016$) and 1.0 mg/kg ($p < 0.001$) of naltrexone. This effect did not differ between sex [Sex, Sex × Phase, Sex × Dose, Sex × Dose × Phase: $p > 0.05$].

ΔCS port entries (Fig. 2B) significantly decreased across CS trials [Trial: $F_{(5.717,125.770)} = 12.141$, $p < 0.001$]; however, this again differed by naltrexone dose [Trial × Dose: $F_{(8.991,197.809)} = 3.543$, $p < 0.001$; Dose: $F_{(2,20)} = 12.838$, $p < 0.001$]. Relative to saline, 0.3 mg/kg ($p < 0.001$) and 1.0 mg/kg ($p < 0.001$) of naltrexone reduced ΔCS port entries during the first CS trial. This effect did not differ by sex [Sex, Sex × Trial, Sex × Dose, Sex × Dose × Trial: $p > 0.05$]. ITI port entries (Fig. 2C) were unaffected by naltrexone in female and male rats [Phase, Phase x Dose, Dose, Sex, Sex x Dose, Sex x Phase, Sex x Dose x Phase: $p > 0.05$].

Experiment 3. Naltrexone did not affect reinstatement of responding to a sucrose-CS

Relative to extinction, ΔCS port entries (Fig. 3B) significantly increased at test [Phase: $F_{(1,17)} = 143.912$, $p < 0.001$] similarly across naltrexone doses [Dose, Phase × Dose: $p > 0.05$]. Reinstatement did, however, significantly differ between sex groups

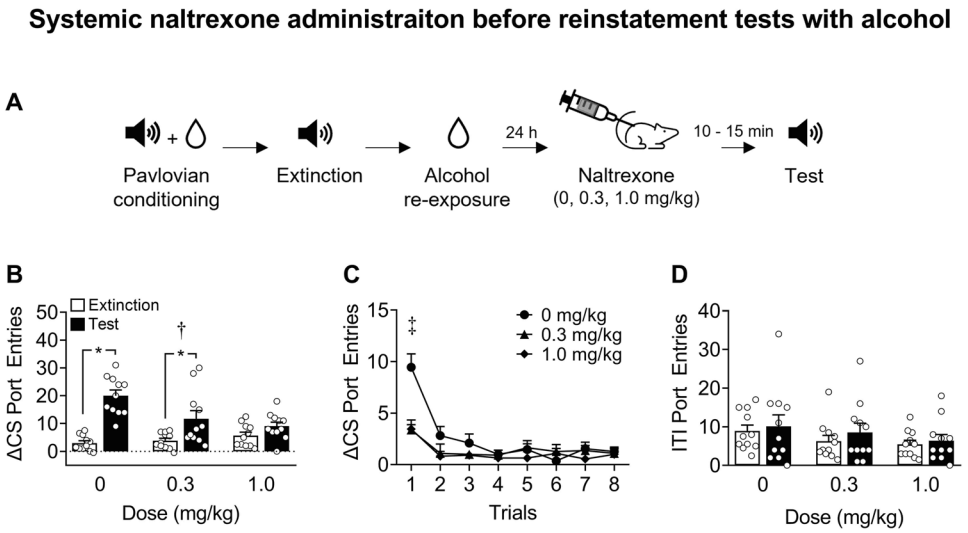

Fig. 1 Systemic naltrexone attenuated reinstatement of responding to an alcohol-CS. A Schematic representation of the behavioural design. Data are from rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone. **B** Mean (±SEM) ΔCS port entries made during extinction and test. **C** Mean (±SEM) 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. *$p < 0.05$, Phase post-hoc (Extinction < Test) †$p < 0.05$, Phase × Dose post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg at Test) ‡$p < 0.05$, Trial × Dose post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg on CS trial 1).

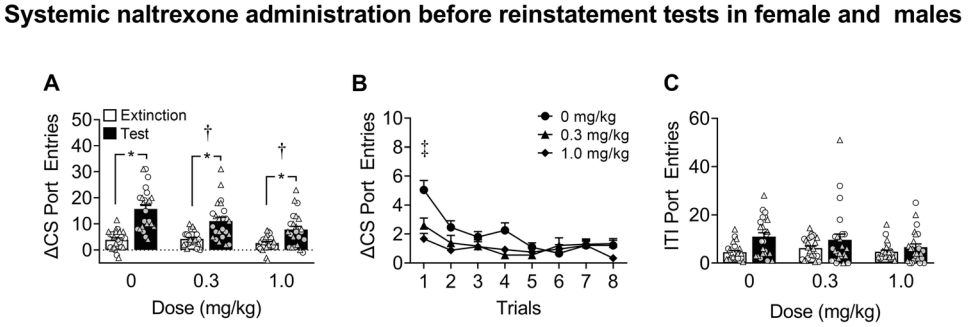

Fig. 2 Systemic naltrexone attenuated reinstatement of responding to an alcohol-CS in both female and male rats. Data are from rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone before reinstatement tests. **A** Mean (±SEM) ΔCS port entries made during extinction and test. **B** Mean (±SEM) 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. *$p < 0.05$, Phase post hoc (Extinction < Test) † $p < 0.05$, Phase × Dose post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg at Test) ‡ $p < 0.05$, Trial x Dose post-hoc (0.3 mg/kg and 1.0 mg/kg < 0 mg/kg on CS trial 1).

[Phase x Sex: $F_{(1,17)} = 6.518$, $p = 0.021$; Sex: $F_{(1,17)} = 11.771$, $p = 0.003$], regardless of dose [Sex × Dose, Sex × Dose × Phase, $p > 0.05$]. Both female ($p < 0.001$) and males ($p < 0.001$) reinstated port entries during the test; however, ΔCS port entries were higher in females compared to males ($p < 0.001$).

ΔCS port entries (Fig. 3C) decreased across CS trials [Trial: $F_{(7,119)} = 20.050$, $p < 0.001$], similarly across doses [Dose, Trial × Dose: $p > 0.05$]. Again, ΔCS port entries significantly differed between sex [Sex: $F_{(1,17)} = 9.453$, $p = 0.007$], regardless of CS trial or dose [Sex × Trial, Sex × Dose, Sex × Dose × Trial: $p > 0.05$], where females made significantly more ΔCS port compared to males ($p < 0.001$).

Experiment 4. Intra-ventral hippocampal CTAP prevented reinstatement of responding to an alcohol-CS

Relative to extinction, ΔCS port entries (Fig. 4B) significantly increased at test [Phase: $F_{(1,9)} = 5.668$, $p = 0.041$]; however, this differed by CTAP dose [Phase × Dose: $F_{(2,18)} = 4.455$, $p = 0.027$; Dose: $p > 0.05$]. Reinstatement of ΔCS port entries occurred following microinfusions of saline ($p = 0.002$), however, reinstatement was prevented by 2.5 µg ($p = 0.970$) and 5.0 µg ($p = 0.970$) of CTAP. Moreover, relative to saline, reinstatement was reduced by 2.5 µg ($p = 0.037$) and 5.0 µg ($p = 0.016$) of CTAP.

ΔCS port entries (Fig. 4C) decreased across CS trials at test [Trial: $F_{(7,63)} = 5.402$, $p < 0.001$], however, this differed across CTAP dose [Trial × Dose: $F_{(14,126)} = 2.430$, $p = 0.005$; Dose: $p > 0.05$]. Relative to saline, 2.5 µg ($p < 0.001$) and 5.0 µg ($p < 0.001$) of CTAP reduced ΔCS port entries during the first CS trial. ITI port entries (Fig. 4D) remained unaffected by intra-ventral hippocampal administration of CTAP [Phase, Phase x Dose, Dose: $p > 0.05$].

DISCUSSION

Our findings demonstrate that blocking µ-opioid receptors (MORs) with the antagonist naltrexone attenuates reinstatement of responding to an alcohol-CS regardless of sex. This is an alcohol-specific effect as responding to a sucrose-CS is unaffected. Importantly, we show that blocking MORs in the vHipp prevents reinstatement of responding to an alcohol-CS, thus demonstrating for the first time that MORs located in the vHipp are necessary for responding to an alcohol-CS.

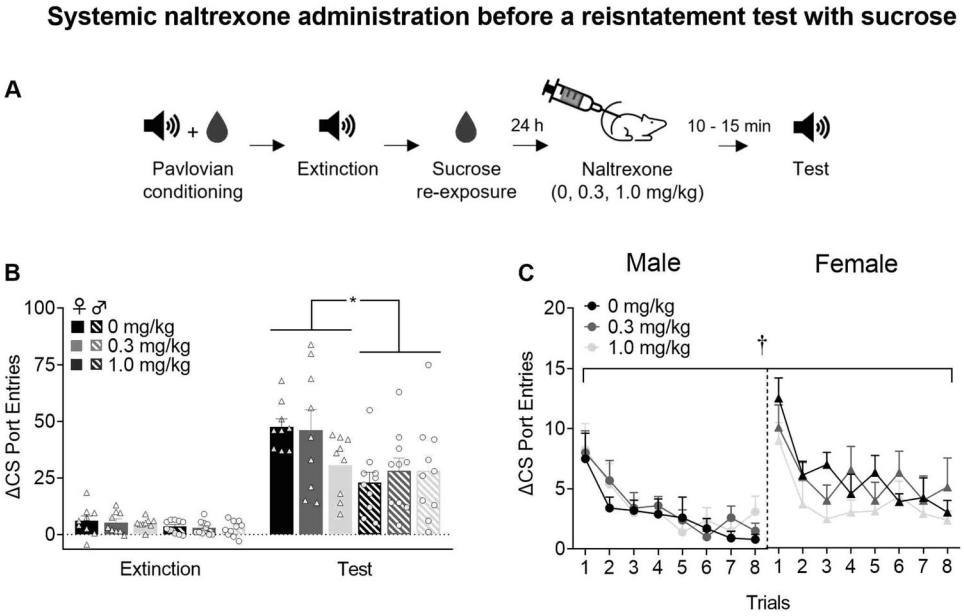

Fig. 3 Systemic naltrexone did not affect reinstatement of responding to a sucrose-CS. A Schematic representation of the behavioural design. Data are from rats that received 0 mg/kg, 0.3 mg/kg, or 1.0 mg/kg of naltrexone before reinstatement tests. **B** Mean (±SEM) ΔCS port entries made during extinction and test for female (hatched bars) and male (filled bars) rats. **C** Mean (±SEM) ΔCS port entries across CS trials at test for female (triangles) and male (circles) rats. *$p < 0.05$, Phase × Sex post-hoc (Female > Male at Test). †$p < 0.05$, Sex post-hoc (Female > Male).

Systemic naltrexone attenuated reinstatement of port entries evoked by an alcohol-CS, even at the lower 0.3 mg/kg dose, in female and male rats. These findings complement previous research showing that similar doses reduced reinstatement of operant alcohol-seeking evoked by various alcohol-predictive stimuli [16, 18–20, 22, 25]. They do, however, contrast a report that the MOR antagonist, CTOP, does not affect cue-induced reinstatement of operant alcohol-seeking [17]. This is likely due to CTOP being selective to non-opioid receptors [49]. Thus, MORs are an integral neural mechanism that mediates reinstatement of responding to an alcohol-CS in this distinct delayed reinstatement model, a finding which provides new mechanistic insight into a relapse model which has not yet been reported.

It is unlikely that the reduction in reinstatement is attributable to naltrexone producing non-specific behavioural effects that impact the ability to make a port entry, as supported by naltrexone not affecting port entries made during the intertrial interval at test, by naltrexone minimally impacting responding to a reinforced alcohol-CS (Supplementary Fig. 3), and by previous studies [16, 20, 50].

Systemic naltrexone did not impact reinstatement of port entries evoked by a sucrose-CS in either female or male rats (Supplementary Fig. 4), as previously reported [25]. Thus, naltrexone selectively attenuates reinstatement of responding to an alcohol-CS, but not a CS associated with a natural reward, which illustrates the specificity of naltrexone to reduce responding to alcohol-predictive cues. This finding also strengthens the claim that naltrexone did not reduce reinstatement of responding to an alcohol-CS through non-specific effects on behaviour.

An important methodological consideration is that naltrexone is a preferential MOR antagonist. As such, it also binds to δ-opioid receptors (DORs) and κ-opioid receptors (KORs), and so the reduction in reinstatement in this study could be due to actions on DORs and KORs. We reason that the observed behavioural effects are likely driven by blocking MORs, as naltrexone has substantially higher binding affinity and potency to this receptor over DORs and KORs [45, 51, 52]. Further support for this reasoning stems from evidence that selective blockade of MORs with the antagonist naloxonazine attenuates reinstatement of alcohol-seeking evoked by discriminative stimuli [19].

The current study also demonstrates that the vHipp is neural locus in which MORS mediate responding to alcohol cues, as administration of the MOR antagonist CTAP into the vHipp prevented reinstatement of port entries evoked by an alcohol-CS. Given that the vHipp is involved in cue processing [53], it is possible that MORs in the vHipp are also involved in responding to natural rewards. Future research should assess the generalisability of this neural mechanisms in responding to natural rewards and other drugs of abuse. Still, this set of data reveals, for the first time, that MORs in the vHipp are required for the reinstatement of responding to an alcohol-CS – or responding to any appetitive-cue. Ventral hippocampal MORs have been greatly implicated in epileptic- and anxiety-related behaviours [54, 55]; our findings suggest further that these receptors are involved in a diverse range behaviours.

Blocking MORs in the vHipp may reduce delayed reinstatement of responding by mediating GABAergic neuronal activity. Hippocampal MORs are predominantly localised on inhibitory GABergic interneurons [56, 57], and activating these inhibitory MORs reduces inhibitory GABAergic neurotransmission [58, 59]. Intra-ventral hippocampal administration of the antagonist CTAP may block MORs on GABAergic neurons, thus removing the inhibitory influence on GABA transmission. Such facilitation of inhibitory activity could lead to a reduction in hippocampal activity and consequently the attenuated reinstatement. This hypothesis is consistent with pharmacological inactivation of ventral hippocampal structures reducing reinstatement of drug-seeking evoked by discrete and contextual cues [34–37].

Our findings are in stark contrast to prior work in which localised administration of a MOR antagonist in the dorsal hippocampus did not affect reinstatement of operant alcohol-seeking evoked by an alcohol-context [28]. This difference is not entirely surprising given the growing evidence that the ventral and dorsal subregions of the hippocampus are functionally separate structures [53, 60]. We posit that MORs in the dorsal versus ventral hippocampus may have separable roles in the reinstatement of responding to alcohol-predictive cues; however, future studies must conduct a systematic comparison to confirm these roles.

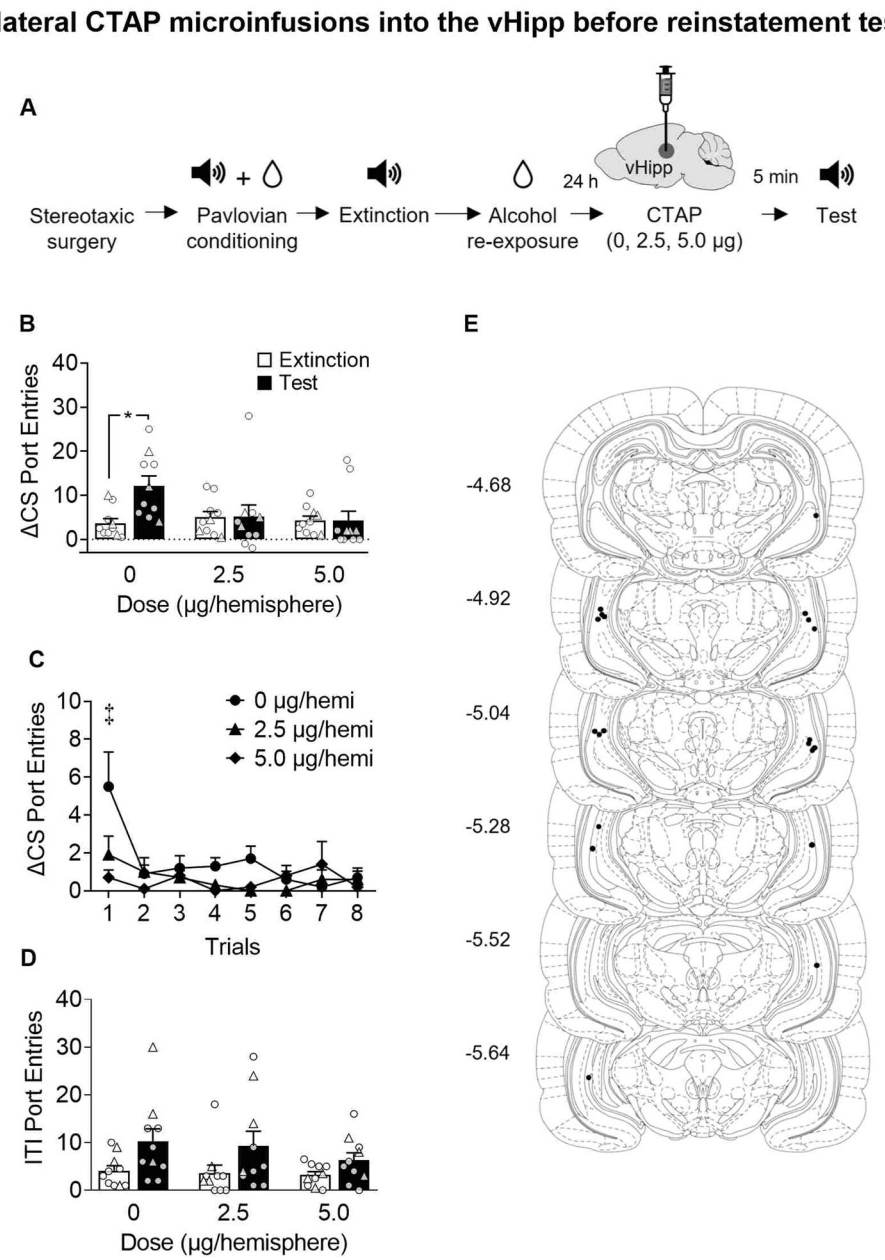

Fig. 4 Bilateral microinfusions of CTAP into the vHipp prevented reinstatement of responding to an alcohol-CS. A Schematic representation of the behavioural design. Data are from rats that received 0 µg, 2.5 µg, or 5.0 µg of CTAP before reinstatement tests. **B** Mean (±SEM) ΔCS port entries made during 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. *$p < 0.05$, Phase post-hoc (Extinction < Test) ‡$p < 0.05$, Trial × Dose post-hoc (2.5 µg and 5.0 µg < 0 µg on CS trial 1).

A unique aspect of the present study is the inclusion of female rats. Several differences have been reported between women and men living with alcohol use disorder. Relative to men, women are more sensitive to the pharmacological effects of alcohol and progress from recreational use to dependence quicker [61]. Female subjects must be included in preclinical research to capture these differences observed in clinical populations. Under saline conditions, reinstatement of port entries made during an alcohol-CS occurred similarly in female and male rats thus demonstrating a lack of sex differences in this model. Further, comparing the effects of naltrexone on reinstatement in female and male rats revealed attenuated reinstatement independent of sex. Interestingly, 1.0 mg/kg of naltrexone prevented reinstatement in the sample of males (Experiment 1), whereas the same dose only reduced reinstatement in a sample of females and males (Experiment 2). Although not statistically significant, this persistent reinstatement may be driven by greater responding at test relative to extinction in females (M Extinction = 2.29, Test = 9.08) compared to males (M Extinction = 3.00, Test = 6.58). This hypothesis is consistent with the pattern of responding observed in Experiment 3, where females showed greater reinstatement of responding to the sucrose-CS. Due to the limited number of female rats in Experiment 4, the presence or absence of sex differences in how MORs in the vHipp impact reinstatement could not be determined. Future research should pursue this question to determine if reinstatement is maintained by different structural mechanisms in females and males. Together, these findings add to the burgeoning body of literature reporting sex differences—or

lack thereof—in responding to appetitive cues, which remains relatively variable [62–67], and highlights the need to continue examining responding to appetitive cues with female samples.

In conclusion, silencing MORs attenuates reinstatement of Pavlovian conditioned responding to a CS in an alcohol-specific manner, and independent of sex. For the first time, we provide evidence that MORs in the vHipp are necessary for the delayed reinstatement of responding to an alcohol-CS. These findings build upon existing research that has predominantly studied on the role of MORs in traditional reinstatement models. Ultimately, these findings provide the basis for future studies to further investigate the role of ventral hippocampal MORs and their projections in the reinstatement of responding to alcohol-predictive cues.

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AUTHOR CONTRIBUTIONS

MRL Contributed to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work; drafting and revising the work; giving final approval of the version to be published; and agreeing to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. PC: contributed to the acquisition, analysis, and interpretation of data. NC: contributed to the conception or design of the work; and the acquisition, analysis, and interpretation of data for the work.

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COMPETING INTERESTS

The authors declare no competing interests.

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