Khoo, S. Y., Sciascia, J. M., Pettorelli, A., Maddux, J.-M. N., & Chaudhri, N. (2019). The medial prefrontal cortex is required for responding to alcohol-predictive cues but only in the absence of alcohol delivery. Journal of Psychopharmacology. https://doi.org/10.1177/0269881119844180

The medial prefrontal cortex is required for responding to alcohol-predictive cues but only in the absence of alcohol delivery

Short Title: Pavlovian conditioned approach and the mPFC

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Abstract

Background: The prelimbic medial prefrontal cortex is implicated in promoting drug-seeking in relapse tests. However, drug-seeking behaviour is typically extinguished before a test and tests normally occur without drug delivery.

Aims: We investigated the involvement of the prelimbic and the infralimbic cortex in responding elicited by a non-extinguished cue for alcohol that was presented without alcohol in an alcohol-associated context or a neutral context, and in responding to the same cue when it was paired with alcohol.

Methods: Male, Long-Evans rats (220–240 g on arrival) were acclimated to 15% ethanol (v/v; 'alcohol') and then trained to associate a conditioned stimulus (10 s white noise; 15 trials/session) with alcohol delivery into a fluid port (0.2 mL/conditioned stimulus, 3 mL per session) for oral intake. Conditioning sessions occurred in a specific 'alcohol context' and were alternated daily with exposure to a second 'neutral' context that contained neither the conditioned stimulus nor alcohol.

Results: At test, functional prelimbic cortex inactivation using baclofen/muscimol reduced fluid port entries elicited by a non-extinguished conditioned stimulus that was presented without alcohol, but had no subsequent impact on port entries when the conditioned stimulus was paired with alcohol. Similar results were obtained following infralimbic cortex inactivation; however, infralimbic cortex inactivation also non-specifically reduced port entries in the absence of alcohol.

Conclusions: These data indicate that the prelimbic and infralimbic cortex are involved in responding to cues for alcohol

Key words

Prelimbic cortex, infralimbic cortex, ethanol, Pavlovian conditioning, context, goal-tracking, reinstatement, relapse, reward

Introduction

Instrumental and Pavlovian conditioning are fundamental learning processes that contribute to drug-seeking and drug-taking behaviours (Everitt and Robbins, 2005; Everitt et al., 2001). Animal modelsbased on these processes allow for a greater understanding of theneurobiological mechanisms that regulate drug use and relapse(Janak and Chaudhri, 2010; Perry et al., 2014; Valyear et al., 2017).

In one such model, responding on an operant device is paired with a drug reinforcer, and then the reinforcer is withheld to induce extinction. Instrumental responding can then be reinstated in the absence of reinforcement by exposure to stress, a prime or a response-contingent, discrete stimulus that was previously paired with the reinforcer. Using these relapse models, converging evidence across multiple drug classes indicates that the prelimbic (PL) medial prefrontal cortex is required for reinstatement (Ball and Slane, 2012; Brown et al., 2016; Capriles et al., 2003; Fuchs et al., 2005; McFarland and Kalivas, 2001; McLaughlin and See, 2003; Rocha and Kalivas, 2010; Stefanik et al., 2013).

Studies using a version of the reinstatement procedure that was adapted to investigate the impact of context in relapse provide a more nuanced view of PL function. In one such model, instrumental conditioning and test are conducted in the same context, whereas extinction is conducted in a second, distinct context (ABA renewal). Studies with non-drug or drug reinforcers indicate that functional inactivation of the PL cortex attenuated ABA renewal of instrumental behaviour (Eddy et al., 2016; Fuchs et al., 2005; Palombo et al., 2017; Trask et al., 2017a; Willcocks and McNally, 2013), suggesting that this region is required for controlling instrumental behaviour that is guided by contextual stimuli. Interestingly, PL inactivation had no effect on renewal triggered by exposure to a novel context, following conditioning and extinction in separate, distinct contexts (ABC renewal), suggesting more specifically that the PL cortex may control instrumental behaviour that is guided by the conditioning context (Trask et al., 2017a).

In Pavlovian conditioning tasks that involve learning an association between a discrete conditioned stimulus (CS) and an unconditioned stimulus (US), PL neurons showed sustained activation during a shock-predictive CS (Burgos-Robles et al., 2009), as well as c-Fos expression in tests where a shock-predictive CS was experienced in a context that was distinct from the extinction context (Knapska and Maren, 2009). Rats with PL lesions or rats that received pharmacological inactivation of the PL cortex also failed to show ABA renewal of responding to a shock-predictive CS (Sharpe and Killcross, 2015a). These findings are mirrored in the appetitive domain, where PL neurons showed increased c-Fos expression in response to a food-predictive CS that was experienced in the conditioning context, relative to a different, extinction context (Anderson and Petrovich, 2018; Keefer and Petrovich, 2017). Thus, the PL cortex also appears to be involved in the context-dependent expression of conditioned responding to discrete Pavlovian cues.

In the studies reviewed above, responding was extinguished before the test by withholding the reinforcer or US, and responding at test was assessed under extinction conditions (i.e. without the reinforcer or US present). While these experimental conditions are germane to the relapse model, they leave open the questions of whether or not the PL cortex is needed for responding that has not previously been systematically extinguished, and if it is involved in responding when the reinforcer or US is present.

A handful of studies have indirectly addressed these questions. For example, functional inactivation of the PL cortex had no impact on operant responding on a cocaine-associated lever in the absence of prior extinction (Fuchs et al., 2006; Koya et al., 2009). Similarly, functional inactivation of the PL cortex had no impact on responding in the first session of extinction when sucrose (solution) was withheld in either Pavlovian or instrumental conditioning tasks (Mendoza et al., 2015). These data suggest that the PL cortex is not engaged unless systematic extinction has previously occurred. However, another study using instrumental conditioning procedures found that pressing a lever associated with sucrose (pellet) delivery was reduced following PL inactivation in the first

session of extinction (Trask et al., 2017a). Interestingly, this effect only occurred in a context that was associated with prior sucrose self-administration, and did not occur in a context in which sucrose self-administration had not previously been performed (Trask et al., 2017a). The latter findings further support a role for the PL cortex in guiding context-specific instrumental behaviour.

In operant studies in which the reinforcer was present at test, PL neurons showed electrophysiological responses that correlated with responding on a sucrose-paired lever (Burgos-Robles et al., 2013; Moorman and Aston-Jones, 2015). However, PL inactivation had no effect on the initial acquisition of operant alcohol selfadministration (Willcocks and McNally, 2013) or on stable sucrose self-administration (Burgos-Robles et al., 2013). These data suggest that while PL neurons are responsive during reinforced operant behaviour, functional activity in this structure is not critical for performing a reinforced operant response. This hypothesis is supported by the findings that following outcome devaluation, rats with PL lesions showed selectively reduced instrumental responding in the presence of the reinforcer, but failed to show this effect when instrumental responding occurred in the absence of the reinforcer (Corbit and Balleine, 2003). Thus, the PL cortex may be particularly necessary under conditions in which behaviour is guided by the memory of a learned response-outcome association.

Based on this literature, we evaluated the role of the PL cortex in responding to a non-extinguished cue for alcohol and in responding elicited by the cue when it was paired with alcohol delivery. For this, we used an animal model of Pavlovian conditioning that engages brain regions that are interconnected with the PL cortex (Khoo et al., 2019; Millan et al., 2015; Sciascia et al., 2015; Valyear et al., 2017). Briefly, rats were trained to associate a discrete auditory conditioned stimulus (CS) with alcohol that was delivered into a fluid port for oral consumption. Training sessions occurred in a specific context (the ‘alcohol context’) and were alternated daily with sessions of exposure to a different context (called the ‘neutral context’) where neither the CS nor alcohol was presented. After an equal number of

sessions in each context, CS port entries elicited by the non-extinguished CS presented without alcohol were tested in the alcohol context and/or the neutral context. In this task, CS port entries at test are higher in the alcohol context relative to the neutral context (Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2015; Valyear et al., 2018). We predicted that if the PL cortex was necessary for utilising contextual information to guide conditioned responding, then we might observe a reduction in CS port entries at test in the alcohol context, but not in the neutral context following functional inactivation of the PL cortex. Alternately, a reduction in both contexts following PL inactivation would suggest that the PL cortex was necessary for responding elicited by a discrete cue, regardless of the context in which the cue is experienced. In the same subjects, we then examined the role of the PL cortex in responding to the CS when it was paired with alcohol during a Pavlovian conditioning session in the alcohol context.

An additional objective of the present research was to examine the involvement of the adjacent infralimbic (IL) prefrontal cortex in CS port entries in response to an alcohol cue. While the PL cortex has been implicated in promoting conditioned responding, the IL cortex has been implicated in the extinction of appetitive conditioned behaviour in both instrumental (Eddy et al., 2016; Peters et al., 2008) and Pavlovian (Lay et al., 2018; Mendoza et al., 2015; Villaruel et al., 2018) tasks. That said, functional inactivation of the IL cortex has also been shown to reduce (Bossert et al., 2011; Bossert et al., 2012; Rocha and Kalivas, 2010) or have no impact (Capriles et al., 2003; Rocha and Kalivas, 2010; Willcocks and McNally, 2013) on responding in relapse models based on instrumental learning procedures. The IL cortex is involved in the extinction of alcohol-seeking in an instrumental learning task (Pfarr et al., 2015), but no study to our knowledge has examined the role of the IL cortex in alcohol-seeking elicited by a discrete CS. We addressed this gap by examining the role of the IL cortex in responding to an alcohol-predictive CS presented without alcohol in a neutral context, or with alcohol during a Pavlovian conditioning session.

Methods

Subjects

Male Long-Evans rats (n=60, 220–240 g on arrival, Harlan Laboratories/Envigo, Indianapolis, USA) were maintained in a temperature (21°C) and humidity (44%) controlled vivarium. Rats were individually housed in polycarbonate cages (44.5 cm×25.8 cm×21.7 cm) containing sani-chip bedding and a nylabone (Cat#: K3580, Bio-Serv, Flemington, New Jersey, USA) with unrestricted access to food and water throughout the experiments. Before the start of experiments, rats had a week to acclimate to the vivarium, during which time they were weighed and handled daily. All procedures were approved by the Concordia University Animal Research Ethics Committee and performed in accordance with the guidelines of the Canadian Council on Animal Care.

Home-cage alcohol exposure

In order to acclimate them to the taste and pharmacological effects of 15% ethanol (v/v, prepared by mixing 95% ethanol and tap water; henceforth called ‘alcohol’), rats received 12–15 sessions of intermittent exposure to alcohol in their home-cages (Simms et al., 2008; Sparks et al., 2014; Wise, 1973). Rats had free access to regular tap water at all times. However, three times a week they also received 24 h of access to a 100 mL cylinder containing 15% alcohol. To measure water and alcohol consumption, water bottles and alcohol cylinders were weighed before and after exposure sessions and rats were weighed before each exposure session. To control for spillage, water bottles and alcohol cylinders were also placed on two empty home-cages and the mean difference in the weight of the controls was subtracted from each rat’s consumption. To mitigate the impact of side preferences, the alcohol cylinders and water bottles were placed on alternating sides of the cage in each session. After session 6, rats (n=4, 2 and 1 for experiments 1, 2 and 3, respectively) with mean consumption of less than 1 g/kg alcohol for the preceding three

sessions were given a solution of 15% ethanol/2% sucrose for a maximum of two sessions before being returned to 15% ethanol.

Surgery

Rats were then deeply anaesthetised using isoflurane (5% for induction and 2% for maintenance, 0.8 L/min oxygen) and surgically implanted with bilateral guide cannula (26 ga, Plastics One, Roanoke, Virginia, USA) targeting the PL or IL cortex at coordinates (in mm from bregma): +2.7 mm AP, ±0.6 mm ML, and −1.7 or −3.4 mm DV for the PL and IL cortex, respectively. Injectors extended beyond the cannula by 2 mm so that final target coordinates were −3.7 (PL cortex) and −5.4 (IL cortex) mm DV. Cannulae were secured to the skull with screws and dental acrylic. Dummies were inserted to ensure cannula patency, but these did not project beyond the cannula. Rats were given saline (0.9%, 10 mL/kg, subcutaneous (s.c.)) for rehydration, buprenorphine (0.1 mg/kg, s.c.) for post-operative analgesia, and one week for recovery.

Apparatus

Behavioural training was performed in 12 identical conditioning chambers (ENV-009A, Med Associates Inc., St Albans, Vermont, USA) housed in individual sound-attenuating boxes with a house fan to provide ventilation and mask external noise. Each chamber was connected to a personal computer (PC) running Med-PC IV and was composed of a clear polycarbonate ceiling and front and back walls with stainless-steel sides. On the left side was a houselight (ENV-215M) and white noise generator (ENV-225SM, calibrated to a volume 8 dB higher than background), while the centre of the right side had a fluid port (ENV-200R3AM) with infrared sensor (ENV-254CB) connected by polyethylene tubing to a syringe pump (PHM-100) placed outside the sound-attenuating box.

Contexts were differentiated across visual, tactile and olfactory sensory modalities. Context 1 had a black cardboard cover over the clear polycarbonate front and back walls and ceiling, a solid plexiglass floor, and a 10% suspension of lemon oil (CAS#: 8008-56-8,

Cat#: W262528, Sigma-Aldrich, Ontario, Canada) applied to brown paper in the waste pan (ENV-007A3) beneath the chamber floor (ENV-009A-GF). Context 2 had no cover over the polycarbonate walls or ceiling, a grid metal floor, and a 10% suspension of bitter almond (benzaldehyde, CAS#: 100-52-7, Cat#: B6259, Sigma-Aldrich) applied to white paper in the waste pan.

Behavioural training

After recovery from surgery, rats were habituated to the apparatus over three days. Rats were first habituated to transport and the behaviour room. They were placed on a trolley and taken to the behaviour room where they were left, with the house fans on, for 20 min. For the next two days, they were placed in the chambers, set up as context 1 on day 1 and context 2 on day 2, for a 20-minute habituation session. Rats were handled and weighed prior to placement in the chamber. Habituation consisted of a two-minute delay to allow experimenters to leave the behavioural room before a 20-minute session in which the houselight was on and port entries were counted.

Rats were then given 22 training sessions that alternated daily between context 1 and context 2 (11 sessions in each context). Pavlovian conditioning in which a discrete auditory CS was paired with alcohol was conducted in one context, whereas in the second context neither the CS nor alcohol was presented. Contexts were counterbalanced such that Pavlovian conditioning occurred in context 1 for half the rats and context 2 for the remainder. The context in which Pavlovian conditioning was conducted was designated as the ‘alcohol context’.

In all 22 sessions, following initiation of the computer program there was a two-minute delay, after which the houselight was illuminated and remained lit for the duration of the session (71 min and 30 s). Pavlovian conditioning occurred across 15 trials per session, and a trial consisted of a 10 s pre-CS interval, a 10 s white noise CS that co-terminated with six seconds of pump operation to deliver 0.2 mL of 15% ethanol (‘alcohol’) and a 10 s post-

CS interval. Alcohol was delivered into a fluid port for oral consumption (3 mL per session). Ports were checked at the end of each session to ensure that all the alcohol was consumed. The mean ingested alcohol dose during the final session of training ranged from 0.8–1.2 g/kg, which we showed previously resulted in blood alcohol concentrations of 40–60 mg/dL (Cofresí et al., 2018; LeCocq et al., 2018). Inter-trial intervals (which excluded the pre-CS, CS and post-CS intervals) were randomly selected from a list of possible durations with a mean of 240 s (120, 240 or 360 s). In the second context type that was designated as the 'neutral context', no CS and no alcohol were delivered. The purpose of these sessions was to familiarise rats to a second environment where they would never receive alcohol.

Rats were habituated to microinjection procedures prior to sessionsin each context with sham microinjections. Dummies were removed and injectors were inserted into the cannulae. Polyethylene tubing connected injectors to a 10 µL Hamilton syringe located on a syringe pump (PHD 2000, Harvard Apparatus, Holliston, Massachusetts, USA). On the first two habituation days (sessions 13–14), a simulated microinjection was performed using injectors that were cut to the same length as the cannulae. Prior to sessions 17 and 18 saline microinjections were performed using injectors that protruded 2 mm beyond the cannulae. Microinjection volumes were 0.3 µL delivered over one minute, with injectors left in place for two minutes to allow for diffusion before injectors were removed and dummies were replaced. Additionally, on the day before the first test, full length injectors (projecting 2 mm beyond the cannula) were briefly inserted and removed. This procedure was conducted in the vivarium several hours after the behavioural training session for that day had ended, and was done to pierce any scar tissue that may have formed at the base of the cannulae.

Testing

Before test sessions, rats received either 0.9% saline vehicle or a mixture of 1 mM baclofen/0.1 mM muscimol (Baclofen, CAS#: 1134-47-0, Cat#: B5399, Muscimol, CAS#: 2763-96-4, Cat#: M1523, Sigma-Aldrich). Approximately 5–20 min after microinjections, rats were tested for responding to the CS. The order of microinjections was counterbalanced

across treatment and test. For tests that occurred in the absence of alcohol, no syringes were placed in the pumps and although CS trials occurred in a manner that was identical to Pavlovian conditioning sessions, no alcohol was delivered. For tests in which the CS was paired with alcohol, the test session was identical to a Pavlovian conditioning session. Rats had 1–5 days of retraining between tests. Specific tests that were conducted in each experiment are described in detail below.

Histology

After the conclusion of behavioural testing, rats were deeply anaesthetised with an overdose of sodium pentobarbital (>100 mg/kg, intraperitoneal). To aid visualisation, rats were given a microinjection of 0.3 µL 4% fast green before decapitation. Brains were sectioned at 60 µm, stained with cresyl violet, and a person who was blind to the behavioural data examined the tissue to verify injection sites.

Experiment 1: effect of PL inactivation on CS port entries in the absence of alcohol

Following training (see Figure 1(a) for protocol summary), we tested the role of the PL cortex in CS port entries in the absence of alcohol in both the alcohol context and the neutral context (within-subjects, counterbalanced). Before tests, rats received either vehicle (n=9) or baclofen/muscimol (n=11) microinfusions into the PL cortex (two tests per rat, treatment was between-subjects). At test, the CS was presented as during Pavlovian conditioning but without alcohol.

Experiment 2: effect of PL inactivation on CS port entries in the absence or presence of alcohol

This study sought to first replicate experiment 1, and then to examine the impact of PL inactivation on responding to the CS in the presence of alcohol. As in experiment 1, following training, rats were tested in both the alcohol context and the neutral context (within-subjects, counterbalanced) for responding to the CS without alcohol after allocation to vehicle (n=7) or baclofen/muscimol (n=8) treatment conditions (between-subjects).

Rats then received a Pavlovian conditioning session in the alcohol context, after which they were re-allocated to receive either vehicle (n=7) or baclofen/muscimol (n=8) before a Pavlovian conditioning session in which the CS was paired with alcohol.

Experiment 3: effect of IL inactivation onCS port entries in the absence or presence of alcohol

This experiment examined the role of the IL cortex in responding to an alcohol-predictive CS. Rats were trained in a manner that was identical to experiments 1 and 2. Next, they received tests in which the CS was presented without alcohol in the neutral context, following a microinfusion into the IL cortex. These tests were followed by a Pavlovian conditioning session in the alcohol context, and then tests in the alcohol context that examined the role of the IL cortex in responding to the CS when it was paired with alcohol.

Unlike in experiments 1 and 2, treatment was a within-subjects factor in this experiment. Thus, rats (n=7) received counterbalanced microinfusions of either vehicle or baclofen/muscimol before each of the tests described above (four tests per rat).

Data, statistical analysis and material availability

To estimate CS port entries independent of baseline exploratory activity, normalised-CS port entries were calculated by subtracting the Pre-CS port entries from the CS port entries (LeCocq et al., 2018; Panayi and Killcross, 2018; Villaruel et al., 2018). Pre-CS port entries were low throughout all phases of each experiment, with means±standard error of the mean (SEM) ranging between 0.67±0.2 and 4.58±1.5 during training and 0±0 and 2.5±0.8 at test. During training in the neutral context no CS and no alcohol were delivered. The measurement of port entries during trials in the neutral context occurred during intervals that were yoked to corresponding intervals (e.g., pre-CS, CS, post-CS) in boxes that were running Pavlovian conditioning sessions. We also report port entries during the inter-trial interval (ITI), which is the random interval between CS trials that excludes the pre-CS, CS

and post-CS. Finally, we examined the number of port entries made during each CS presentation at test.

Data were analysed using the free open source statistics package JASP (JASP Team, 2018: https://jasp-stats.org/) and SPSS 23 (IBM, Armonk, New York, USA). Statistical analyses included mixed-design and repeated measures analysis of variance (ANOVA), independent t-tests and paired t-tests. Sphericity violations were corrected for using the Greenhouse-Geisser correction when $\varepsilon<0.75$. Data are presented as means±SEM. Raw data and underlying Med-PC code are available on Figshare (Khoo et al., 2019: doi: 10.6084/m9.figshare.6274112).

Results

Histology

Experiment 1 started with 27 rats and 20 were included in the final analysis (Figure 1(b)). Rats were excluded due to sickness(n=1), low (<1 g/kg) home cage alcohol consumption (n=2), failure to acquire Pavlovian conditioning (n=1) or misplaced or indeterminate cannula placements (n=3).

Experiment 2 started with 19 rats and 15 were included in the final analysis (Figure 1(c)). Rats with low home-cage alcohol consumption (n=2) or that died in surgery (n=2) were excluded.

Experiment 3 started with 14 rats and seven were included in the final analysis (Figure 1(d)). Rats with low home-cage alcohol consumption (n=2) or misplaced cannulae (n=5) were excluded.

Experiment 1. PL inactivation reduced CS port entries in the absence of alcohol

Over the course of Pavlovian training, norm-CS port entries (calculated as CS port entries minus pre-CS port entries) increased across sessions only in the alcohol context (Figure 2(a)). Due to a significant Mauchly's test of sphericity for the main effect of session

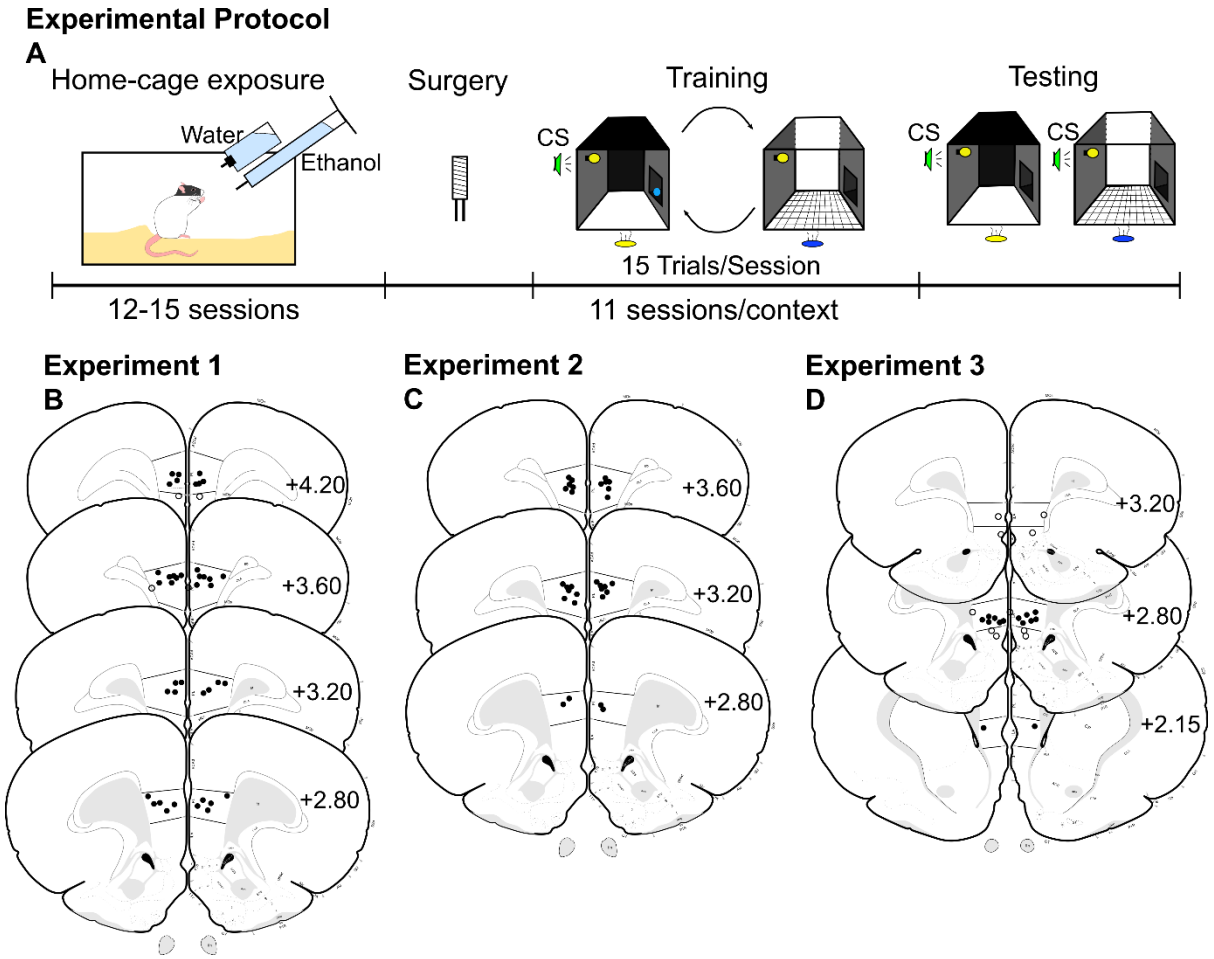


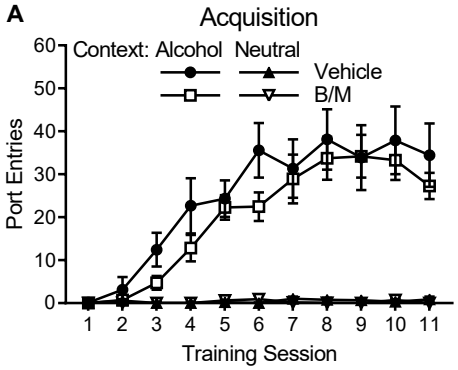
Figure 1. Overview of behavioural procedures and histological verification of injection sites. (A) Rats received home-cage exposure to 15% ethanol followed by surgery to implant guide cannulae into the PL or IL. After recovery, rats were given Pavlovian conditioning sessions in which a white noise conditioned stimulus (CS) was paired with 0.2 mL deliveries of 15% ethanol in a specific context (alcohol context). These sessions were alternated with sessions of exposure to a different (neutral) context in which neither the CS nor alcohol was presented. Next, port entries triggered by the CS alone were tested in the alcohol context and the neutral context. (B) Histological verification of PL injection sites in Experiment 1, with 20 accurate placements, two inaccurate placements, and one rat having an indeterminate cannula placement. (C) Histological verification of PL injection sites for Experiment 2, with 15 accurate cannula placements. (D) Histological verification of IL injection sites for Experiment 3, with seven accurate and five excluded placements. Solid circles (●) indicate accurate and open circles (○) indicate inaccurate cannula placements. AP coordinates are given in mm from bregma. Atlas figures are adapted from Swanson (2018) under a CC-BY-NC 4.0 license.

(W=9.69×10^{-5}, p<0.001) and context×session interaction (W=5.58×10^{-5}, p<0.001), Greenhouse-Geisser corrections were applied to session (ε=0.32) and context×session interactions (ε=0.34). Mixed-design ANOVA with context and session as within-subjects factors and treatment as a between-subjects factor showed significant main effects of context ($F_{(1,18)}$=134.2, p<0.001), session ($F_{(3.19,57.4)}$=22.8, p<0.001), and a significant context×session interaction ($F_{(3.35,60.3)}$=20.97, p<0.001). There was no main effect of treatment ($F_{(1,18)}$=1.68, p=0.21), context×treatment interaction ($F_{(1,18)}$=1.39, p=0.25), session×treatment interaction ($F_{(3.19,57.4)}$=0.47, p=0.72) or context×session×treatment interaction ($F_{(3.35,60.3)}$=0.58, p=0.65), indicating that groups were well-matched prior to test.

At test, rats received CS presentations without alcohol in the alcohol context or the neutral context. While there was no impact of context on norm-CS port entries in vehicle treated rats, baclofen/muscimol inactivation of the PL reduced norm-CS port entries in both contexts (Figure 2(b)). Mixed-design ANOVA with context as a within-subjects factor and treatment as a between-subjects factor showed a significant main effect of treatment ($F_{(1,18)}$=7.13, p=0.016), but no main effect of context ($F_{(1,18)}$=1.85, p=0.19), or context×treatment interaction ($F_{(1,18)}$=0.41, p=0.53). This reduction was specific for CS port entries, because there was no effect of PL inactivation on port entries made during the ITI (Figure 2(c)). A mixed-design ANOVA on ITI port entries showed no main effect of treatment($F_{(1,18)}$=2.92, p=0.104), context ($F_{(1,18)}$=3.14, p=0.093) or context×treatment interaction ($F_{(1,18)}$=0.22, p=0.65).

PL inactivation did not significantly alter the within-session pattern of responding to consecutive CS trials at test in either the alcohol context or the neutral context (Figure 2(d)). A mixed-design ANOVA confirmed that PL inactivation reduced CS port entries overall (treatment; $F_{(1,18)}$=6.651, p=0.019) and that there was a reduction in the number of CS port entries across the session (trial: $F_{(3.351,60.316)}$=9.364, p<0.001, ε=0.239). However, there was no trial×treatment interaction ($F_{(3.351,60.316)}$=2.244, p=0.086, ε=0.239) or context×trial×treatment interaction $F_{(5.259,94.659)}$=0.894, p=0.492, ε=0.376). Similarly, there

Prelimbic Cortex



Test: CS, no alcohol

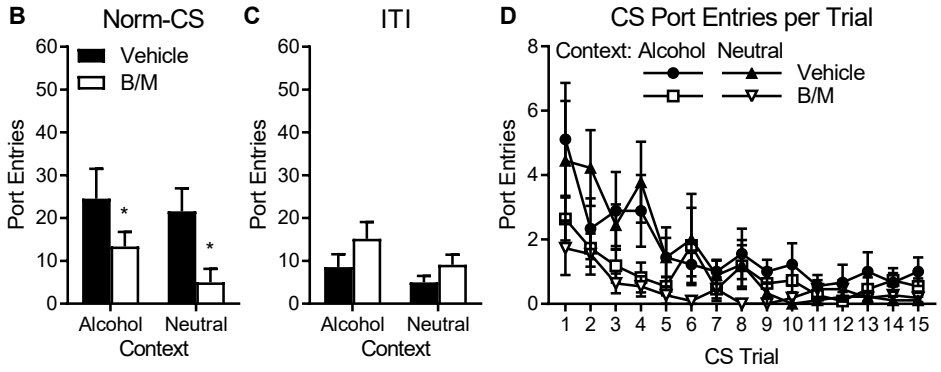


Figure 2. Prelimbic medial prefrontal cortex inactivation with baclofen/muscimol (B/M) reduced conditioned stimulus (CS) port entries at test when the CS was presented without alcohol. (A) Rats acquired conditioned responding to a 10 s white noise, paired with 0.2 mL of 15% ethanol in the alcohol context with no pre-existing differences between treatment groups. (B) At test, rats received 0.3 µL/side microinjections of 1 mM baclofen/ 0.1 mM muscimol (n =11) or saline vehicle (n = 9) and were presented with the CS in the absence of alcohol. Rats treated with B/M showed significantly reduced CS port entries in both contexts (norm-CS port entries = CS port entries minus pre-CS port entries). (C) The effect of B/M treatment was specific to the CS because there was no effect on port entries made during the ITI. (D) Non-normalised CS port entries on a per-trial basis showed an overall reduction due to PL inactivation, but no change in the pattern of responding. Data are means ± SEM, * $P < 0.05$ for main effect of treatment.

was no main effect of context ($F_{(1,18)}$=1.369, p=0.257), context×treatment interaction ($F_{(1,18)}$=0.207, p=0.655) or context×trial interaction ($F_{(5.259,94.659)}$=0.787, p=0.567, ε=0.376).

Experiment 2. PL inactivation reduced CSport entries in the absence of alcohol but not when the CS was paired with alcohol

As shown in Figure 3(a), norm-CS port entries increased only in the alcohol context, and there were no pre-existing differences between treatment groups. Due to a significant Mauchly's test of sphericity for the main effect of session (W=6.62 x 10^{-6}, p<0.001) and context×session interaction (W=4.996 x 10^{-6}, p<0.001), Greenhouse-Geisser corrections were applied to session (ε=0.33) and context×session interactions (ε=0.35). Mixed-design ANOVA with context and session as within-subjects factors and treatment as a between-subjects factor showed significant main effects of context ($F_{(1,13)}$=74.9, p<0.001), session ($F_{(3.32,43.1)}$=19.3, p<0.001) and a significant context×session interaction ($F_{(3.47,45.1)}$=19.3, p<0.001). There was no main effect of treatment ($F_{(1,13)}$=0.23, p=0.64), context×treatment interaction ($F_{(1,13)}$=0.2, p=0.66), session×treatment interaction ($F_{(3.32,43.1)}$=1.49, p=0.23) or context×session×treatment interaction ($F_{(3.47,45.1)}$=1.95, p=0.13).

Experiment 2 replicated the results of experiment 1. Context had no overall impact on norm-CS port entries. However, PL inactivation reduced norm-CS port entries at test in both the alcohol context and the neutral context when the CS was presented without alcohol (Figure 3(b)). Mixed-design ANOVA with context as a within-subjects factor and treatment as a between-subjects factor showed a significant main effect of treatment ($F_{(1,13)}$=89.5, p<0.001), but no main effect of context ($F_{(1,13)}$=0.62, p=0.45) or context×treatment interaction ($F_{(1,13)}$=0.03, p=0.86). Furthermore, there was no impact of PL inactivation on port entries during the ITI (Figure 3(c)). A mixed-design ANOVA on ITI port entries showed no main effect of treatment ($F_{(1,13)}$=0.83, p<0.38), context ($F_{(1,13)}$=1.58, p=0.23) or context×treatment interaction ($F_{(1,13)}$=0.81, p=0.38).

Prelimbic Cortex

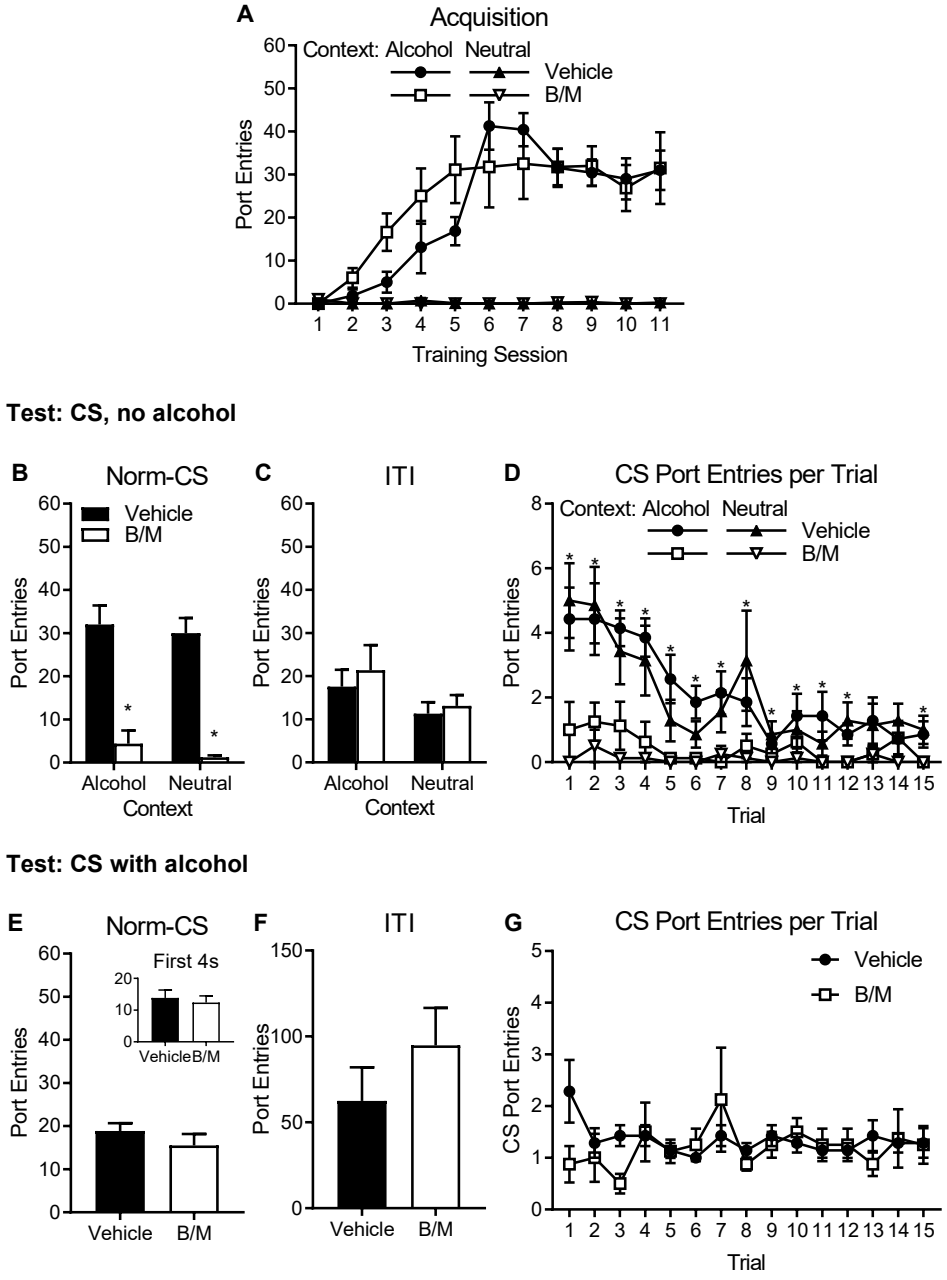


Figure 3. Prelimbic (PL) medial prefrontal cortex inactivation with baclofen/muscimol (B/M) had no effect on conditioned stimulus (CS) port entries when the CS was paired with alcohol. (A) Rats acquired conditioned responding to a 10 s white noise, paired with 0.2 mL of 15% ethanol in the alcohol context with no pre-existing differences between treatment groups. (B) Rats treated with 0.3 µL/side 1 mM baclofen/0.1 mM muscimol (n=8) or saline vehicle (n=7) showed significantly reduced CS port entries in both contexts in tests in which the CS was presented without alcohol (norm-CS port entries=CS port entries minus pre-CS port entries), with no effect of PL inactivation on (C) ITI responding. (D) Non-normalised CS port entries were significantly reduced in trials 1–12 and 15 by PL inactivation, regardless of context. (E) Rats then received a retraining session and were allocated to receive treatment with B/M (n=8) or vehicle (n=7) before a test session in the alcohol context in which the CS was paired with alcohol. PL inactivation had no effect on CS port entries during the whole CS presentation or during the first four seconds before alcohol delivery (see inset). (F) PL inactivation had no effect on ITI port entries. (G) The within-session pattern of CS port entry responding was not affected by PL inactivation in the presence of alcohol. Data are means ± standard error of the mean (SEM), *p<0.05 for main effect of treatment or Bonferroni-adjusted post-hoc comparisons.

PL inactivation reduced CS port entries from the first trial in both contexts (Figure 3(d)). Mixed-design ANOVA showed a main effect of treatment ($F_{(1,13)}$=84.935, p<0.001), trial ($F_{(14,182)}$=10.896, p<0.001) and a trial×treatment interaction ($F_{(14,182)}$=6.049, p<0.001). Bonferroni-adjusted post-hoc comparisons showed that there were significant differences following vehicle and baclofen/muscimol in trials 1–12 and 15 (p's from <0.001 to 0.019). However, there was no main effect of context ($F_{(1,13)}$=1.279, p=0.279), context×treatment interaction ($F_{(1,13)}$=0.246, p=0.628), context×trial interaction ($F_{(14,182)}$=0.402, p=0.973) or context×trial×treatment interaction ($F_{(14,182)}$=0.648, p=0.822).

Rats then received retraining and were tested during a Pavlovian conditioning session in the alcohol context in which the CS was paired with alcohol. Under these conditions, there was no effect of PL inactivation on either norm-CS port entries ($t_{(13)}$=1.002, p=0.335; Figure 3(e)) or ITI port entries ($t_{(13)}$=−1.087, p=0.297; Figure 3(f)). Because the last six seconds of the CS were paired with alcohol delivery in these tests, port entries during the first four seconds of the CS are shown as an inset in Figure 3(e). There was no significant difference in norm-CS port entries in the first four seconds ($t_{(13)}$=0.461, p=0.653).

There was no impact of PL inactivation on the within-session pattern of responding to the CS when it was paired with alcohol (Figure 3(g)). Mixed-design ANOVA showed no main effect of treatment ($F_{(1,13)}$=0.573, p=0.463) or trial×treatment interaction ($F_{(14,182)}$=1.079, p=0.379). The number of port entries made in each CS trial remained at a constant level throughout the session, as there was no significant effect of trial ($F_{(14,182)}$=0.773, p=0.698).

Experiment 3. IL inactivation non-specifically reduced port entries in the absence of alcohol delivery at test

Rats acquired norm-CS responding over the course of Pavlovian training in the alcohol context but not the neutral context (Figure 4(a)). Repeated measures ANOVA revealed significant main effects of context ($F_{(1,6)}$=110.8, p<0.001), session ($F_{(10,60)}$=9.37, p<0.001) and a context×session interaction ($F_{(10,60)}$=8.73, p<0.001). CS port entries in the absence of

alcohol delivery were then tested in the neutral context following intra-IL vehicle or baclofen/muscimol using a within-subjects design. Intra-IL baclofen/muscimol appeared to produce a non-specific decrease in responding, because paired *t*-tests showed a significant reduction in both norm-CS port entries ($t_{(6)}$=3.52, p=0.013; Figure 4(b)) and ITI port entries ($t_{(6)}$=2.53, p=0.045; Figure 4(c)) following IL inactivation.

IL inactivation reduced CS port entries primarily at the start of the test (Figure 4(d)). Repeated-measures ANOVA revealed a main effect of treatment ($F_{(1,6)}$=12.839, p=0.012), trial ($F_{(14,84)}$=4.173, p<0.001) and treatment×trial interaction ($F_{(14,84)}$=2.137, p=0.017). Bonferroni-adjusted post-hoc comparisons showed significant differences following IL inactivation during trials 2-4 (p's=0.01, 0.023 and 0.007 respectively).

Rats were then tested during a Pavlovian conditioning session in the alcohol context in which the CS was paired with alcohol. There was no effect of IL inactivation on CS port entries in this test. Due to a significant Shapiro-Wilk's test of normality (W=0.613, p<0.001), a Wilcoxon signed-rank test was performed for norm-CS port entries, which showed no difference between vehicle and muscimol/baclofen treated rats (V=13, p=0.67, Figure 4(e)). Similarly, a paired *t*-test showed no effect of IL inactivation on ITI responding ($t_{(6)}$=−0.162, p=0.877, Figure 4(f)). Because the last six seconds of the CS also included alcohol delivery, port entries during the first four seconds of the CS are shown as an inset in Figure 4(e). Due to a significant Shapiro-Wilk test (W=0.655, p<0.001), a Wilcoxon signed-rank test was performed. There was no significant difference in norm-CS port entries in the first four seconds (V=13.5, p=0.136).

IL inactivation had no impact on the pattern of responding elicited by consecutive CS trials at test in the presence of alcohol (Figure 4(g)). Mixed-design ANOVA showed no main effect of treatment ($F_{(1,12)}$=0.388, p=0.545) or trial×treatment interaction ($F_{(14,168)}$=1.07, p=0.388). CS port entries remained at a constant level throughout the session, as there was no significant effect of trial ($F_{(14,168)}$=0.77, p=0.7).

Infralimbic Cortex

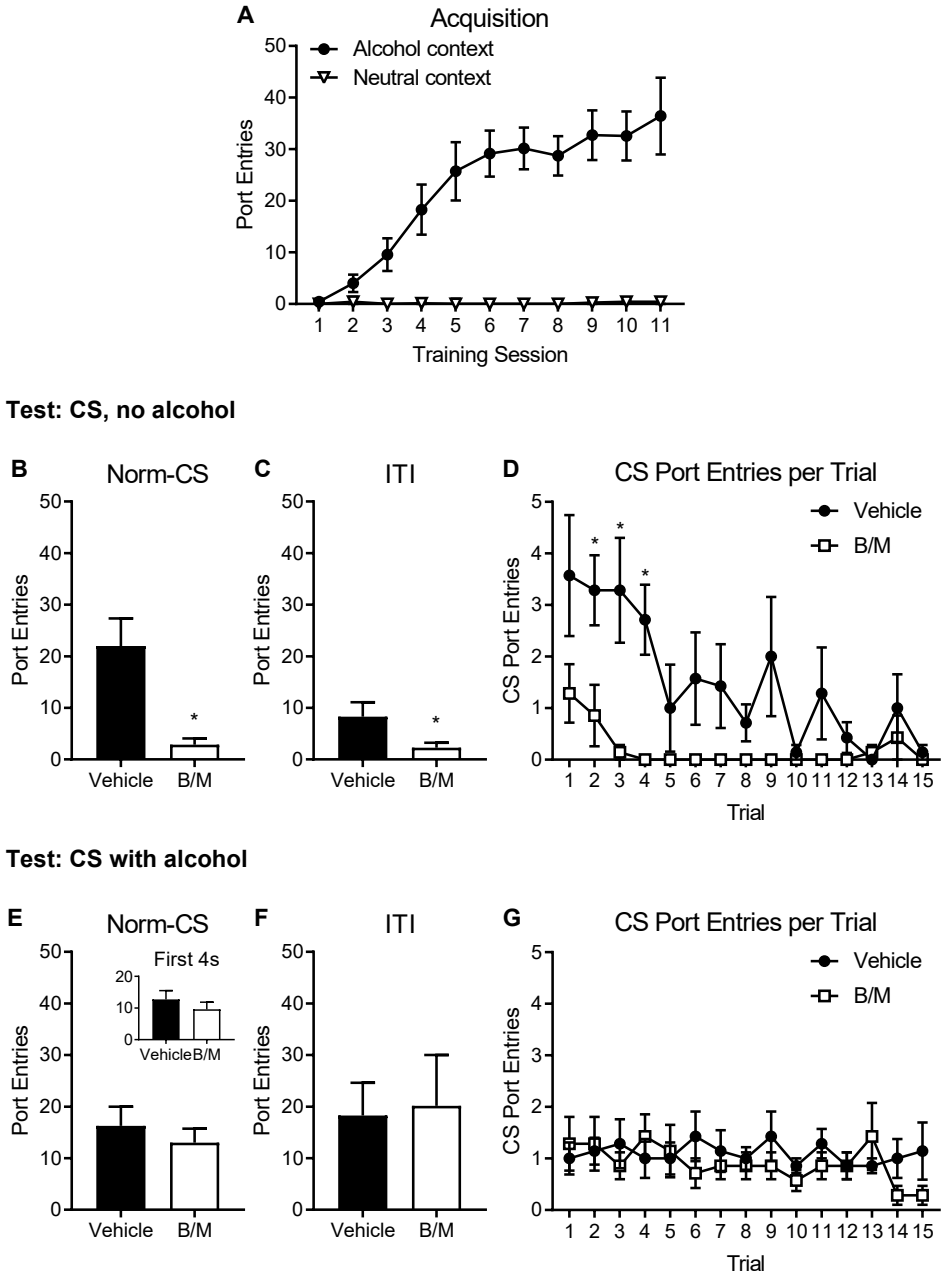


Figure 4. Infralimbic (IL) medial prefrontal cortex inactivation with baclofen/muscimol (B/M) produced a non-specific reduction in responding in the neutral context. (A) Rats (*n*=7) acquired responding to the conditioned stimulus (CS) only in the alcohol context where the CS and alcohol were paired. (B) Rats were then administered 0.3 µL/side 1 mM baclofen/0.1 mM muscimol or saline vehicle in counterbalanced order in a test session in the neutral context in which the CS was presented without alcohol. IL inactivation significantly reduced CS port entries (norm-CS port entries=CS port entries–pre-CS port entries). (C) Intra-IL B/M also produced a non-specific reduction in ITI port entries. (D) IL inactivation significantly reduced non-normalised CS port entries in trials 2–4. (E) Rats were then given a retraining session and retested in the alcohol context with CS presentations that were paired with alcohol delivery. IL inactivation had no effect on CS port entries during the whole CS presentation or during the first four seconds before alcohol delivery (see inset). (F) IL inactivation had no effect on ITI responding. (G) IL inactivation had no effect on the within-session pattern of CS port entry responding. Data are means±standard error of the mean (SEM), **p*<0.05 for paired *t*-test or Bonferroni-adjusted post-hoc comparisons.

Discussion

In the present research, functional inactivation of either the PL or IL cortex reduced port entries elicited by an alcohol-predictive CS that had not previously been systematically extinguished. This effect only occurred when the CS was presented without alcohol. In the absence of alcohol, PL inactivation selectively reduced CS port entries in both an alcohol context and a neutral context, with no effect on ITI port entries, whereas IL inactivation non-specifically reduced CS and ITI port entries at test in the neutral context. Interestingly, neither PL nor IL inactivation affected CS port entries when the CS was paired with alcohol. These results support existing data showing that the PL and IL cortex are required for responding in relapse models and extend this literature by suggesting that the contribution of these regions does not depend on prior, systematic extinction of responding. However, when the alcohol is delivered at test and conditioned responding is not reliant on memory, brain regions other than the PL and IL cortex likely mediate responding to an alcohol-predictive CS.

These experiments used a behavioural task that was designed to examine the impact of context on CS port entries. However, contrary to our expectations based on multiple studies (Khoo et al., 2019; Millan et al., 2015; Remedios et al., 2014; Sciascia et al., 2014; Sciascia et al., 2015; Valyear et al., 2018), vehicle treated rats did not make more CS port entries at test in the alcohol context relative tothe neutral context. Previous studies have shown that context does not always sum with (or modulate) the CS, especially if there is a temporal delay between the animal being placed in the context and the first CS onset (Trask et al., 2017b). Such a delay might have been a subtle, inadvertent component of the present research. Because there was no context-modulation of CS port entries in the vehicle group, we were unable to determine a role for the PL cortex in context-dependent modulation of this behavioural effect. Nonetheless, we replicated the finding that regardless of context, PL inactivation reduced CS port entries, which strengthens the conclusion that this region

mediates responses triggered by a discrete alcohol-predictive cue, particularly one that has not previously undergone systematic extinction.

Although we did not observe a modulation of CS port entries by context, the present data corroborate a vast literature based on instrumental and Pavlovian conditioning procedures that implicates the PL cortex in promoting appetitive behaviour (Anderson and Petrovich, 2018; Ball and Slane, 2012; Brown et al., 2016; Capriles et al., 2003; Eddy et al., 2016; Fuchs et al., 2005; Keefer and Petrovich, 2017; McFarland and Kalivas, 2001; McLaughlin and See, 2003; Palombo et al., 2017; Rocha and Kalivas, 2010; Sharpe and Killcross, 2015a; Stefanik et al., 2013; Trask et al., 2017a; Willcocks and McNally, 2013). A prior study in mice found that exposure to an alcohol-associated context increased cAMP response element-binding protein phosphorylation in the PL and the IL cortex (Groblewski et al., 2012). The present data support these findings and are the first to reveal a functional role for the PL cortex in responding elicited by a discrete, alcoholpredictive CS.

In the present research CS port entries were not systematically extinguished before test, and we found that PL inactivation reduced CS port entries in the absence of alcohol delivery at test in an alcohol context and in a neutral context, with no effect on ITI port entries. This selective reduction occurred early in the session, as early as the first CS trial in experiment 2, which supports a role for the PL in the expression of Pavlovian associations (Corcoran and Quirk, 2007). The observation of a reduction in both contexts suggests that the PL region is integral in brain circuits that mediate conditioned responding triggered by discrete, alcohol-predictive cues. These circuits could include medial prefrontal cortex projections to the nucleus accumbens shell, which are required for cue-induced reinstatement of alcohol-seeking in an operant task (Keistler et al., 2017). They could also include PL projections to the nucleus accumbens core, a region that is necessary for responding to an alcohol-predictive CS (Chaudhri et al., 2010), or PL projections to the basolateral amygdala (Khoo et al., 2019; Millan et al., 2015; Sciascia et al., 2015) that are

important for responding to Pavlovian cues (Reppucci and Petrovich, 2016; Song et al., 2015).

Contrary to the present data, we previously showed that PL inactivation had no effect on CS port entries in the first session of extinction in which sucrose solution was withheld (Mendoza et al., 2015). This difference could be attributable to the US (alcohol versus sucrose), or to the behavioural procedure. In the sucrose study, Pavlovian conditioning and extinction were conducted in a single context, whereas in the current study Pavlovian conditioning was alternated with sessions of exposure to a different, neutral context in which neither the CS nor alcohol was presented.

In the present study, IL inactivation had a similar effect to PL inactivation, insofar as both reduced CS port entries only in the absence of alcohol delivery. However, IL inactivation under these conditions also reduced ITI port entries, suggesting a non-specific effect on behaviour. Interestingly, the present study replicated our prior finding that IL inactivation in the first session of extinction augmented within-session extinction of port-entries triggered by a sucrose-predictive CS (Mendoza et al., 2015). However, in that study IL inactivation had no impact on port entries that occurred between CS trials, whereas in this study IL inactivation reduced ITI port entries. This discrepancy could be related to subtle, yet important methodological factors. For example, in the present study the test occurred in a familiar but motivationally neutral context, whereas in our prior study the test occurred in the same context as Pavlovian conditioning. Another difference between these two studies, which could be explained by the different US that was used, is that in Mendoza et al. (2015) IL inactivation during a Pavlovian conditioning session in which the CS was paired with sucrose produced a non-specific increase in ITI port entries (which was not observed in the present research). There may therefore be marked differences in the contribution of the IL cortex to conditioned responding elicited by cues that predict alcohol versus sucrose, although this hypothesis needs to be methodically tested.

One possible explanation for the effect of IL inactivation in the present study is that it rendered rats better able to suppress responding in the neutral context. In the IL experiment, rats were tested without alcohol in the neutral context and previous studies have found that IL inactivation reduced context-inappropriate habitual responding (Haddon and Killcross, 2011) and that the IL cortex contains neurons sensitive to drug-associated contexts, such as heroin (Bossert et al., 2011). Consistent with this interpretation, IL inactivation reduced ITI port entries in the no-alcohol test in the neutral context, which could indicate greater sensitivity to the context. However, IL inactivation may simply suppress all responding in the absence of the US. Further studies that test the effect of IL inactivation in both the conditioning context and a neutral or extinction context are necessary to evaluate this hypothesis.

Interestingly, functional activity in the PL or the IL cortex was only required when the CS was tested without alcohol, suggesting a role for the medial prefrontal cortex in conditioned responding guided by the memory of a CS-US association. These findings are congruent with studies from an instrumental conditioning study in which rats with PL lesions showed normal devaluation of instrumental responding in the presence of a reinforcer, but not in its absence (Corbit and Balleine, 2003). They also partly correspond with data from an operant alcohol self-administration study in which inactivation of the PL or IL cortex had effects on non-reinforced operant behaviour, but not on the initial acquisition of operant alcohol self-administration (Willcocks and McNally, 2013). The difference in medial prefrontal cortex engagement in the presence versus absence of the reinforcer has implications for the clinical relevance of studies that use relapse models, which assess a return to drug-seeking behaviour after extinction but not a return to drug use. Indeed, in humans, relapse is defined as a return to drug use, and patients are frequently able to acquire alcohol and other drugs at will.

Another explanation for the present data is that the medial prefrontal cortex – particularly the PL region – is important for focusing attention on cues, and that in the

presence of the US the necessity to do so is diminished. Excitotoxic PL lesions attenuate overshadowing and enhance unblocking to an appetitive cue, suggesting that the PL cortex is involved in down-regulating attention to redundant or non-predictive cues (Sharpe and Killcross, 2014). In studies using an aversive Pavlovian conditioning paradigm, PL lesions produce a deficit in CS responding when there is a high degree of competition between contextual and discrete cues, but not when rats are extensively habituated to the context and when long ITIs are used in order to reduce competition between discrete and contextual cues (Sharpe and Killcross, 2015b). These studies also replicate, using muscimol-inactivation of the PL, an attenuation of the overshadowing effect (Sharpe and Killcross, 2015b). If the PL cortex is needed to direct attention towards predictive cues rather than for responding to the US itself, then this would explain why PL inactivation in the present study reduced CS port entries only in the absence of alcohol delivery.

In the present study, the effect of PL or IL inactivation on CS port entries was first tested without alcohol and then in the presence of alcohol. This design was based on our prior research (Millan et al., 2015; Sciascia et al., 2015); however, the order of testing may have influenced the data and should be considered when interpreting the results. Nonetheless, our data are consistent with studies showing no effect of PL or IL inactivation on operant responding for alcohol (Willcocks and McNally, 2013) or sucrose (Burgos-Robles et al., 2013) in the absence of prior extinction.

Although muscimol and baclofen inactivation is widely used, this manipulation may produce a strong inhibitory effect that overwhelms the function of discrete subsets of neurons that could regulate distinct behavioural effects. Indeed, studies have shown that behaviour can be regulated by small ensembles of neurons in a brain area such as the medial prefrontal cortex (Whitaker et al., 2017), and that eliminating different ensembles within the same region can produce dissociable effects on behaviour (Pfarr et al., 2015). A more selective approach may therefore be necessary to parse with greater specificity the

contribution of PL and IL subregions to motivated behaviour produced by appetitive Pavlovian conditioning.

In conclusion, we found that functional activity in the PL and IL cortex was required for responding triggered by a discrete, alcohol-predictive CS, but only when alcohol was not presented at test. Moreover, under these conditions PL inactivation produced a selective reduction in CS port entries in both an alcohol context and a neutral context, suggesting that this region is an important component of brain circuits that mediate this behaviour. IL inactivation in the absence of alcohol delivery produced a non-selective reduction in port entries, suggesting a more important role in general motivation or memory processes. The present conclusions are unable to address a role for the PL cortex in CS port entries regulated by context, because of a lack of contextual modulation of CS port entries in vehicle treated rats. Nonetheless, the current research extends prior evidence by establishing a role for the PL cortex in responding elicited by a non-extinguished discrete, alcohol-predictive CS. These data also suggest that the involvement of the PL and IL cortex in appetitive Pavlovian conditioning is circumscribed to tests that occur in the absence of alcohol delivery, a finding that has implications for relapse in humans, which is defined by not just a return to alcohol-seeking but a return to alcohol use and intoxication.

Acknowledgements

The authors thank Stephen Cabilio for assistance with Med-PC programming and Larry Swanson for making the Brain Maps atlas freely available for scholarly purposes.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This research was supported by the Canadian Institutes of Health Research (MOP-137030; NC), the Natural Sciences and Engineering Research Council (RGPIN-2017-04802; NC), and Fonds de la recherche du Québec-Santé (Chercheur-Boursier; NC). NC is a member of the Center for Studies in Behavioral Neurobiology/FRQS Groupe de recherche en neurobiologie comportementale (CSBN/GRNC). SYK was supported by a Concordia University Horizon Postdoctoral Fellowship. JMS was supported by graduate fellowships from the Canadian Institutes of Health Research and from Fonds de la recherche du Québec-Santé. J-MM received funding from CSBN/GRNC and Concordia University.

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