Behavioral/Cognitive

Anterior Insular Cortex is Critical for the Propensity to Relapse Following Punishment-Imposed Abstinence of Alcohol Seeking

Erin J. Campbell,[1,2] Jeremy P.M. Flanagan,[1,2] Leigh C. Walker,[1,2] Mitchell K.R.I. Hill,[1,2] Nathan J. Marchant,[1,2,3] and Andrew J. Lawrence[1,2]

[1]The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria 3052, Australia, [2]Florey Department of Neuroscience and Mental Health, The University of Melbourne, Victoria 3010, Australia, and [3]Department of Anatomy and Neurosciences, VU University Medical Center, Amsterdam, 1081 HZ, The Netherlands

Humans with alcohol use disorder typically abstain because of the negative consequences associated with excessive drinking, and exposure to contexts previously associated with alcohol use can trigger relapse. We used a rat model that captures a characteristic of this human condition: namely voluntary abstinence from alcohol use because of contingent punishment. There is substantial variability in the propensity to relapse following extended periods of abstinence, and this is a critical feature preventing the successful treatment of alcohol use disorder. Here we examined relapse following acute or prolonged abstinence. In male alcohol preferring P rats, we found an increased propensity to relapse in Context B, the punishment context after prolonged abstinence. Next, we found that neither alcohol intake history nor the motivational strength of alcohol predicted the propensity to relapse. We next examined the putative circuitry of context-induced relapse to alcohol seeking following prolonged abstinence using Fos as a marker of neuronal activation. The anterior insular cortex (AI) was the only brain region examined where Fos expression correlated with alcohol seeking behavior in Context B after prolonged abstinence. Finally, we used local infusion of $GABA_A$ and $GABA_B$ receptor agonists (muscimol + baclofen) to show a causal role of the AI in context-induced relapse in Context B, the punishment context after prolonged abstinence. Our results show that there is substantial individual variability in the propensity to relapse in the punishment-associated context after prolonged abstinence, and this is mediated by activity in the AI.

Key words: **alcohol use disorder; anterior insular cortex; context; iP rat; punishment; relapse**

Significance Statement

A key feature of alcohol use disorder is that sufferers show an enduring propensity to relapse throughout their lifetime. Relapse typically occurs despite the knowledge of adverse consequences including health complications or relationship breakdowns. Here we use a recently developed rodent model that recapitulates this behavior. After an extended period of abstinence, relapse propensity is markedly increased in the "adverse consequence" environment, akin to humans with alcohol use disorder relapsing in the face of adversity. From a circuitry perspective, we demonstrate a causal role of the anterior insular cortex in relapse to alcohol seeking after extended abstinence following punishment imposed voluntary cessation of alcohol use.

Introduction

Individual variation in the expression of particular traits contributes to the onset of neuropsychiatric disease states, including drug addiction (Piazza et al., 1989). Variation in the propensity to relapse following extended abstinence is a critical feature preventing successful treatment of addiction (Gossop et al., 1989). In abstinent alcoholics, environments previously associated with alcohol use provoke relapse (Wikler, 1973; O'Brien, 1997). In rodents, this is modeled using ABA renewal (Bouton and Bolles, 1979) where self-administration of alcohol occurs in one context (Context A) followed by extinction (experimenter-imposed) of alcohol-reinforced responding in a difference context (Context B). Renewal is observed when the rodent is returned to the orig-

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Correspondence should be addressed to Andrew J. Lawrence at andrew.lawrence@florey.edu.au or Erin J. Campbell at erin.campbell@florey.edu.au.

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inal alcohol-associated context (Context A; Crombag and Shaham, 2002; Hamlin et al., 2007). However, abstinence in humans is often voluntary because of the adverse consequences of alcohol use (Klingemann, 1991; Blume et al., 2006) without any treatment intervention.

We modified a variation of the ABA renewal model where abstinence is self-imposed in Context B because of adverse consequences (punishment; Marchant et al., 2013) by examining alcohol seeking following extended abstinence (30 d). First, we determined the propensity to relapse to context-induced alcohol seeking following prolonged abstinence after punishment-imposed suppression of alcohol use. We found an increased propensity to relapse in Context B, the punishment context after prolonged abstinence. Second, we determined the predictability of this propensity to relapse using alcohol intake history and the motivational strength of alcohol. Neither factor predicted relapse. Third, we examined the putative circuitry of context-induced relapse to alcohol seeking following prolonged abstinence using Fos expression (Dragunow et al., 1987). The anterior insular cortex (AI) was the only brain region examined where Fos expression correlated with alcohol seeking in the punishment context after prolonged abstinence. Finally, we demonstrated a causal role of the AI in context-induced relapse using local infusion of muscimol and baclofen to reversibly inactivate the AI which essentially prevented relapse in Context B, the punishment context after prolonged abstinence, but not acute abstinence.

Materials and Methods

Ethics statement

All procedures performed were in accordance with the Prevention of Cruelty to Animals Act (2004), under the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Experimental Purposes (2013) and approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

Animals

Inbred male iP rats (~8 weeks old, total $n = 129$) were obtained from the breeding colony at the Florey. Parental stock was previously obtained from T. K. Li (while at Indiana University). All rats were pair-housed except during Experiments 4 and 5 where they were single-housed. Food (Barastoc rat and mouse, Ridley) and water were available *ad libitum* and all rats were maintained on a normal 12 h light/dark cycle (07:00 lights on).

Apparatus

Standard operant chambers (Med Associates) enclosed in a ventilated sound-attenuating cubicle were used for self-administration. Each chamber was equipped with two retractable levers and grid floors were connected to shockers. An active lever press resulted in the delivery of 20% ethanol (0.1 ml/delivery) into the receptacle. An inactive lever press had no consequence. Contexts A and B were manipulated as in our previous study (Campbell et al., 2018): illumination level (white/no house light), background (stripes/none), bedding (saw dust/recycled paper), background noise (fan off/on).

Experiment 1: effect of context-induced relapse to alcohol seeking following acute or prolonged abstinence

The behavioral procedure (Fig. 1*A*) was the same as previously published (Campbell et al., 2018).

Behavioral procedure (4 phases)

Phase 1: home-cage alcohol intake. An intermittent access (3–4 times/week) alcohol procedure (Wise, 1973; Simms et al., 2008) was used where rats received 8 × 24 h sessions of access to 20% v/v alcohol. In Experiments 4 and 5, rats received 12 × 24 h home-cage sessions. Alcohol solutions were prepared in tap water from 100% (v/v) ethanol. Daily sessions began at 09:00. After 24 h, the alcohol was replaced with a second water bottle for the subsequent 24–48 h alcohol-free period. The following day, the second water bottle was replaced with 20% alcohol, and the location of the alcohol was alternated from the previous session. Total alcohol consumption was calculated for each session, using the weight difference between the beginning and end of the session, minus 1 g for spillage, multiplied by 0.97 (density of 20% ethanol), and divided by 2 (number of rats per cage).

Phase 2: operant self-administration: Context A. All rats were given one 16 h overnight training session where only the active lever was presented. An active lever press resulted in the delivery of 0.1 ml of 20% alcohol into a receptacle followed by a 2 s light cue above the active lever. Food and water was provided *ad libitum*. Rats were then trained for seven 20 min self-administration sessions under a fixed-ratio 1 (FR-1) schedule. Responding on the active lever resulted in the delivery of 0.1 ml of 20% alcohol and the 2 s light cue followed by a 20 s timeout period where lever presses were recorded but not reinforced. Inactive lever presses were recorded but had no consequence. Following FR-1 training, rats progressed to a variable-interval 30 s (VI-30) schedule for six 20 min sessions where alcohol delivery was available after an active lever press at pseudo-random intervals (1–59 s) after the preceding alcohol delivery.

Phase 3: punishment: Context B. During 20 min sessions, rats self-administered alcohol in an alternate context (Context B) under the same VI-30 schedule. Active lever presses resulted in the delivery of 0.1 ml of alcohol paired with the 2 s light cue. 50% of the reinforced active lever presses randomly resulted in a 0.5 s footshock (0.2–0.7 mA). Punished active lever presses resulted in footshock, 2 s light cue, and alcohol delivery. Inactive lever presses had no consequence. All rats were punished in Context B for up to 6 d, and footshock intensity was increased by 0.2 mA per session up to 0.6 mA, or to 0.7 mA if rats made >25 active lever presses after three punishment sessions.

Phase 4: context-induced relapse test following either acute or prolonged abstinence. Rats in the acute abstinence protocol were tested for alcohol seeking the day after punishment-imposed abstinence (i.e., Day 1 Context A alcohol group, $n = 12$; Day 1 Context B punishment group, $n = 12$). Rats in the prolonged abstinence protocol were moved to a separate holding room for 29 d and tested for alcohol seeking on Day 30 (i.e., Day 30 Context A alcohol group, $n = 23$; Day 30 Context B punishment group, $n = 21$). All rats were tested under extinction conditions during 20 min sessions in either Context A or B in a counterbalanced order. During test, an active lever press under a VI-30 schedule resulted in delivery of the 2 s light cue but no alcohol or footshock.

Experiment 2: predicting the propensity to relapse in the punishment context following prolonged abstinence

On the third last day of Context A training, 14 rats were tested on a progressive ratio (PR)3–4 schedule for a single 2 h session. For the PR3–4 schedule, 32 active lever presses were required for the 10th infusion of alcohol (Farid et al., 2012), breakpoint was defined as the final ratio completed within the 2 h session. Subsequently, all rats received two further Context A sessions. Alcohol-reinforced responding was then punished in Context B. All rats were placed in a separate room for the prolonged abstinence phase. On abstinence Day 30, all rats were tested for alcohol seeking behavior in Context B.

Experiment 3: effect of context-induced alcohol seeking following prolonged abstinence on Fos-protein expression

Ninety minutes following the initiation of the relapse test, a subset of rats from Experiment 1 (i.e., Day 30 Context A alcohol group, $n = 6$; Day 30 Context B punishment low relapsing group, $n = 7$; Day 30 Context B punishment high relapsing group, $n = 8$) were anesthetized (sodium pentobarbitone100 mg/kg, i.p.; Virbac). Rats were transcardially perfused with ~100 ml of 0.1 M PBS followed by ~400 ml of 4% paraformaldehyde (PFA). Brains were removed and postfixed in PFA (2 h), then transferred to 30% sucrose in PBS (48 h at 4°C). Brains were frozen over dry ice and stored at −80°C. Serial (40 μm) coronal sections were cut using a Leica Microsystems cryostat and stored in 0.1 M PBS containing 0.1% sodium azide at 4°C.

A 1-in-4 series of the whole brain was processed for Fos-immunoreactivity, as published (Campbell et al., 2015, 2017a). Free-

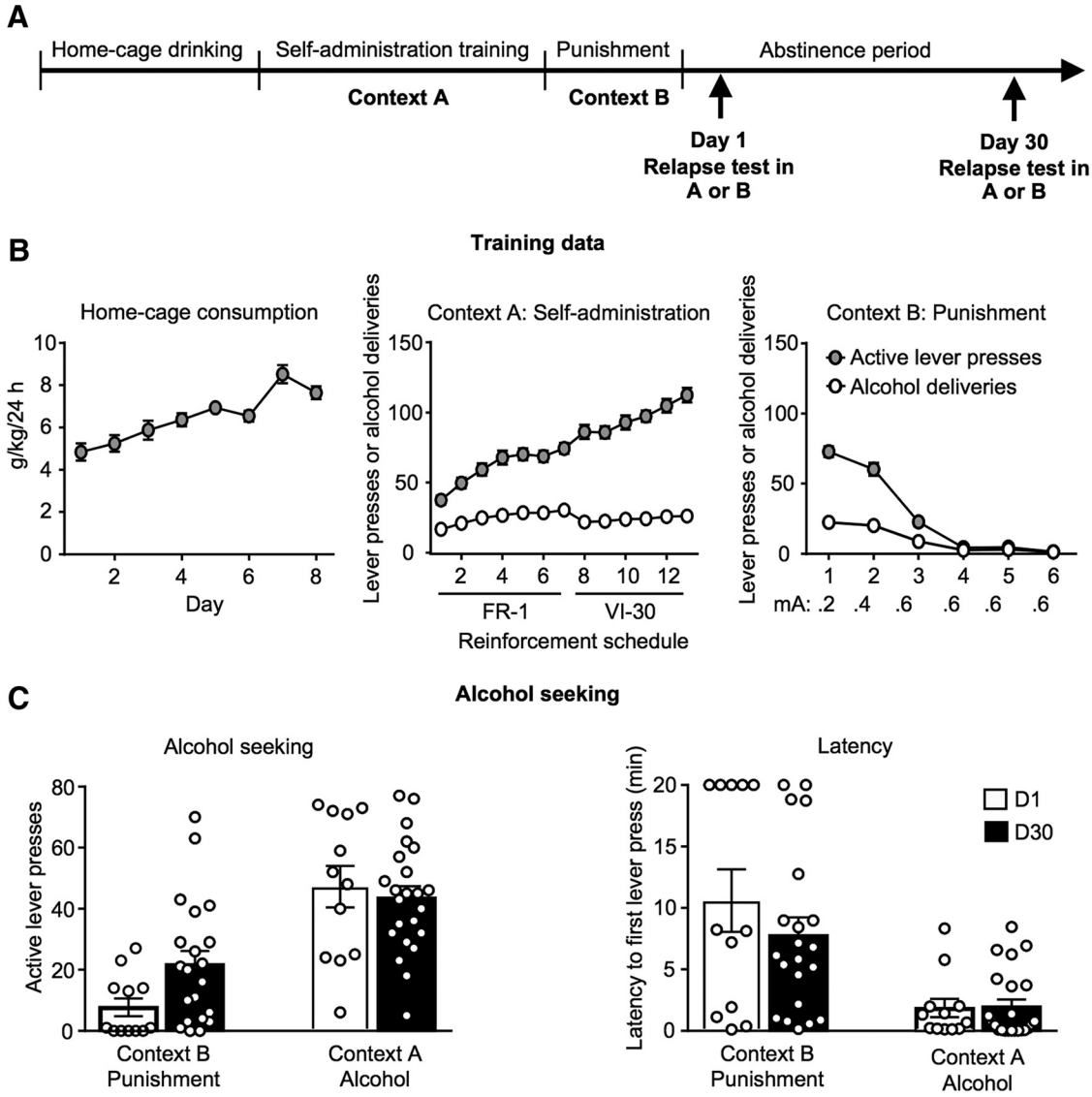

Figure 1. Home-cage intake, alcohol self-administration in Context A, and punishment of alcohol self-administration in Context B and alcohol seeking behavior after acute and prolonged abstinence. Outline of the behavioral procedure (***A***). Alcohol intake (grams per kilogram) during the home-cage access phase. Context A active lever presses and alcohol deliveries under the FR-1 and VI-30 schedules of reinforcement. Context B active lever presses and alcohol deliveries with the foot shock punishment range from 0.2 to 0.6 mA (***B***). Number of active lever presses (left) and latency to first lever press (right) during the relapse test at either Day 1 abstinence or Day 30 abstinence (***C***). Data are presented as mean ± SEM. Total $n = 68$, $n = 12$/group Day 1 abstinence, $n = 21$ Context B, punishment Day 30 abstinence group, $n = 23$ Context A, alcohol Day 30 abstinence group. D1, Day 1 abstinence; D30, Day 30 abstinence.

floating sections were washed in PBS followed by blocking in 5% normal horse serum (NHS). Sections were incubated (48 h at 4°C) in PBS containing 0.5% Triton-X with 2% NHS and Fos primary antibody (1:3000; rabbit polyclonal, sc-52, Santa Cruz Biotechnology). Following primary incubation, sections were incubated (2 h) in biotinylated horse anti-rabbit secondary (1:300; BA-1100, Vector Laboratories). Subsequently, sections were incubated (1 h) in ABC reagent (Vector Laboratories) followed by incubation (10 min) in 0.1 M sodium acetate with 0.025% diaminobenzidine in 2% nickel sulfate containing 2 mg/ml D-glucose, and 0.4 mg/ml ammonium chloride. Glucose oxidase (0.2 μl/ml) was added to visualize Fos. Sections were mounted onto gelatin-coated slides, air dried, and coverslipped.

Bright-field images of Fos-immunoreactive cells were captured using a MBF Biosciences Color 12-Bit (QImaging) camera attached to an upright Leica DMLB-2 microscope using a 10× objective. Images were quantified using iVision (BioVision) by an observer blinded to experimental conditions. Bilateral cell counts were quantified for each rat across 17 brain regions. These brain regions included the AI, posterior insular cortex (AIp), prelimbic cortex (PrL), infralimbic cortex (IL), lateral orbitofrontal cortex (LOFC), entorhinal cortex (EC), ventral subiculum (vSub), paraventricular thalamus (PVT), lateral hypothalamus (LH), lateral habenula (LHb), basolateral amygdala (BLA), central amygdala (CeA), medial amygdala (MeA) dorsal dentate gyrus (DDG), paraventricular nucleus of the hypothalamus, medial parvicellular part (PVN), nucleus accumbens core (NAcC), and nucleus accumbens shell (NAcSh). Please refer to Table 1 for more detail on bregma levels quantified. All brain coordinates were from Paxinos and Watson (2007).

Experiment 4: effect of AI inactivation on context-induced relapse to alcohol seeking in Context B, the punishment context, following prolonged abstinence

Surgery. Eighteen rats underwent behavioral training as per Experiment 1. During the abstinence period, rats were anesthetized with isoflurane (5% induction, 2% maintenance) before being placed in a stereotaxic frame (Stoelting Instruments). Bilateral guide cannula (26-gauge; Plastics One) were positioned 1.5 mm above the AI (anteroposterior: +2.8 mm, mediolateral: ±4.4 mm, and dorsoventral: −4.7 mm from bregma;

Table 1. Bregma levels and references for Fos quantification across several brain regions

Brain region	Bregma level, mm	References
AI	+3.08 to +2.76	Arguello et al., 2017; Venniro et al., 2017; Nasser et al., 2018
AIp	−1.68 to −2.00	Contreras et al., 2012
PrL	+3.72 to +2.76	Perry and McNally, 2013; Brown et al., 2016; Burgos- Robles et al., 2017
IL	+3.72 to +2.76	Perry and McNally, 2013
LOFC	+3.72 to +2.76	Perry and McNally, 2013
EC	−5.72 to −6.24	Ge et al., 2017
vSub	−5.72 to −6.24	Marchant et al., 2016
PVT	−1.68 to −3.12	Marchant et al., 2014; Campbell et al., 2017b
LH	−1.68 to −3.12	Marchant et al., 2014; Campbell et al., 2017b
LHb	−2.96 to −3.44	Marchant et al., 2014; Zuo et al., 2017
BLA	−2.48 to −2.80	Campbell et al., 2017a
CeA	−2.48 to −2.80	Campbell et al., 2017a
MeA	−2.48 to −2.80	Campbell et al., 2017a
DDG	−3.24 to −3.62	Ge et al., 2017
PVN	−1.52 to −1.84	James et al., 2014
NAcC/NAcSh	+2.04 to +1.72	Marchant et al., 2014; Campbell et al., 2017a

Bregma levels and references for Experiment 3, which examined the effect of context-induced alcohol seeking following prolonged abstinence on Fos-protein expression.

Venniro et al., 2017; Nasser et al., 2018). Cannulae were anchored to the skull with screws and dental cement. After surgery, rats were administered meloxicam (3 mg/kg, i.p.; Troy Laboratories) and Baytril (3 mg/kg, i.p.; Bayer Health Care) for 3 d. All rats were given 7 d recovery from surgery before experimentation.

Intracranial infusions. Three days preceding the test, rats underwent three daily habituation injections. These occurred in the operant training room and involved the connection of 40 cm polyethylene connectors (Plastics One) to both cannulae and activation of an automated syringe pump (Harvard Apparatus) for 2 min. Following this, connectors were left in place for an additional 2 min. Connectors were then removed and rats were left in their home cages for 10 min before being moved back to their holding room. After 30 d abstinence, rats were tested for alcohol seeking (under extinction conditions) in Context B. Muscimol + baclofen [M+B; Tocris Bioscience; (50 + 50)ng/0.5 μl/hemisphere] was dissolved in sterile saline. The injectors extended 1.5 mm below the guide cannula tips. 0.5 μl/hemisphere of M+B ($n = 8$) or vehicle ($n = 10$, 0.9% saline) was infused bilaterally into the AI over 2 min (0.25 μl/min) using a Harvard Apparatus syringe pump connected to two 1 μl microsyringes (SGE Analytical Science) via polyethylene tubing. Injectors were left in place for a further 2 min. Rats were tested in Context B 5–10 min after infusions. Following this, rats were anesthetized (pentobarbitone 100 mg/kg, i.p.; Virbac) and methylene blue (0.5 μl/hemisphere) was infused into the AI. Brains were removed and frozen over super-cooled isopentane, then cut into 40 μm coronal sections using a Leica Microsystems cryostat and counterstained with neutral red to verify cannula placements (see Fig. 4*C*). One rat was excluded due to misplaced cannula.

Experiment 5: effect of AI inactivation on context-induced relapse to alcohol seeking following acute abstinence

Surgery. Twenty-nine rats underwent behavior training as per Experiment 1. After 11 Context A self-administration sessions, rats received AI intracranial guide cannula surgery as described in Experiment 4. All rats were given at least 7 d to recover from surgery before experimentation. Following recovery, all rats were exposed to six additional Context A sessions followed by punishment in Context B as described in Experiment 1.

Intracranial infusions. All rats received two habituation injections in Context A and two habituation injections in Context B before the relapse test. This involved the connection of 40 cm polyethylene connectors (Plastics One) to both cannulae and activation of an automated syringe pump (Harvard Apparatus) for 2 min. Following this, connectors were left in place for an additional 2 min. After 1 d abstinence, rats were tested for alcohol seeking (under extinction conditions) in either Context A or B. M+B [Tocris Bioscience (50 + 50)ng/0.5 μl/hemispher] was dissolved in sterile saline. The injectors extended 1.5 mm below the guide cannula tips. 0.5 μl/hemisphere of M+B (Context A: $n = 6$, Context B: $n = 5$) or vehicle (Context A: $n = 6$, Context B: $n = 5$; 0.9% saline) was infused bilaterally into the AI over 2 min (0.25 μl/min) using a Harvard Apparatus syringe pump connected to two 1 μl microsyringes (SGE Analytical Science) via polyethylene tubing. Injectors were left in place for a further 2 min. Three rats were excluded due to misplaced cannula, three rats were excluded due to ill health following surgery and 1 rat was excluded due to a program operational error.

Locomotor testing. Twenty-four hours after the relapse test, a subset of rats from Experiment 5 received either M+B ($n = 8$) or vehicle ($n = 8$) infusions into the AI and locomotor activity was recorded for 1 h (Med Associates; 43.2 × 43.2 × 30.5 cm). Distance traveled (m) was recorded using photobeam detectors. Following this, rats were anesthetized (pentobarbitone 100 mg/kg, i.p.; Virbac) and methylene blue (0.5 μl/hemisphere) was infused into the AI. Brains were removed and frozen over super-cooled isopentane, then cut into 40 μm coronal sections using a Leica Microsystems cryostat and counterstained with neutral red to verify cannula placements (see Fig. 5*C*).

Statistical analysis

Data were analyzed separately for the four behavioral phases: home-cage alcohol intake, Context A training, Context B punishment, and context-induced relapse tests. For Experiment 1, training and punishment data were analyzed using repeated-measures ANOVA examining a main effect of session. For the relapse test, between-subjects factors were Context (Context A or Context B) and Abstinence Day (Days 1 or 30). The dependent variables were total number of active lever presses (inactive lever presses as covariate) or number of minutes to first active lever press (latency). The relapse test data were also analyzed using a χ^2 test to examine the relationship between Abstinence Day and the relapse status of rats (low vs high relapsing).

Pearson's correlations examined the relationship between alcohol seeking in Context B after 30 d abstinence and average home-cage consumption (g/kg/24 h), average self-administration alcohol deliveries, active lever presses and timeout lever presses for an early time point (first 5 min), late time point (first 10 min), or the total for the 20 min operant session. These data were divided into the first seven sessions when the reinforcement schedule was FR-1 and the last six sessions when the reinforcement schedule was VI-30. Alcohol seeking was also correlated with average punishment alcohol deliveries, active lever presses and timeout lever presses for an early time point (first 5 min), late time point (first 10 min) or the total for the 20 min operant session. For Experiment 2, Pearson's correlations examined the relationship between alcohol seeking and breakpoint as well as alcohol seeking and the number of alcohol deliveries, active lever presses and timeout lever presses for an early time point (first 5 min), late time point (first 10 min), or the total for the 2 h PR session.

In Experiment 3, immunohistochemical data were analyzed as cell counts/mm^2 of a given brain region as the dependent variable. To compare Fos expression between rats with a heightened propensity to relapse versus rats with a low propensity to relapse in Context B, a median split was performed on the Day 30 Abstinence Context B relapse data (James et al., 2011). This resulted in a relapse score of 20; rats that had relapse scores <20 were allocated to the "Low relapsing" group and rats that had relapse scores 20 were allocated to the "High relapsing" group. One-way ANOVAs then assessed the effect of abstinence Day 30 treatment condition (Alcohol, Low relapsing, High relapsing) on the number of Fos cells/mm^2. *Post hoc* comparisons were assessed using least significant differences tests. Pearson's correlations examined the relationship between alcohol seeking in Context B and Fos expression.

In Experiment 4, one-way ANOVAs assessed the effect of Treatment (vehicle, M+B) on alcohol seeking (inactive lever presses as covariate) and latency in Context B after 30 d abstinence. In Experiment 5, a two-way between-subjects ANOVA assessed the effect of Drug Treatment (vehicle, M+B) or Context (Contexts A or B) on alcohol seeking (inac-

tive lever presses as covariate) and latency after 1 d abstinence. A one-way ANOVA assessed the effect of M+B or vehicle on distance traveled (m) in the locomotor arena. All analyses were performed using SPSS v25 (α 0.05). Data are presented as mean ± SEM.

Results

Experiment 1: context-induced relapse to alcohol seeking following acute or prolonged abstinence

Rats consumed high quantities of alcohol during the home-cage period and reliably self-administered alcohol in Context A (Fig. 1*B*). In Context B, rats reduced alcohol self-administration with increasing shock intensity (Fig. 1*B*).

Alcohol seeking behavior following acute or prolonged abstinence

There was a significant interaction between Test Context × Abstinence Day ($F_{(1,63)} = 4.578$, $p = 0.036$; Fig. 1*C*). There was also a significant main effect of Context ($F_{(1,63)} = 40.081$, $p < 0.0001$) with rats tested in Context A having a greater number of active lever presses compared with rats tested in Context B. There was no significant main effect of Abstinence Day ($F_{(1,63)} = 0.021$, $p = 0.884$). In Context B there was variability in relapse data on Day 30, therefore rats were divided using a median split (James et al., 2011), which resulted in a criterion Relapse Score of 20 active lever presses. Rats with relapse scores <20 formed the "Punishment low relapsing" group and rats with relapse scores ≥20 formed the "Punishment high relapsing" group. Using this criterion, there was a ~threefold increase in the number of rats relapsing in Context B after 30 d abstinence, from 16% at Day 1 compared with 52% at Day 30 (Fig. 1*C*). Chi-square analyses revealed a significant relationship between Abstinence Day and the relapse status of rats in Context B ($\chi^2 = 4.080$, $p = 0.043$). There was no significant relationship between Abstinence Day and relapse status in Context A ($\chi^2 = 0.001$, $p = 0.971$). Analysis of latency to first lever press revealed no interaction between Test Context × Abstinence Day ($F_{(1,64)} = 1.082$, $p = 0.302$). There was a significant main effect of Context on latency to first lever press ($F_{(1,64)} = 26.715$, $p < 0.0001$) with rats tested in Context B having a greater latency to first lever press compared with Context A. There was no significant main effect of Abstinence Day on latency ($F_{(1,64)} = 0.911$, $p = 0.343$). These data indicate that 30 d abstinence following punishment-imposed abstinence increases relapse propensity in the punishment context (Fig. 1*C*).

Experiment 2: predicting the propensity to relapse in Context B, the punishment context following prolonged abstinence

Retrospective Pearson's correlations were performed between home-cage drinking, self-administration, punishment and propensity to relapse after 30 d abstinence. There were no significant correlations between alcohol drinking history and relapse propensity in Context B (p values >0.05; Table 2). Evidence suggests that a history of high alcohol intake does not necessarily predict future alcohol seeking behavior, rather there is a relationship between motivation for alcohol and compulsive alcohol seeking (Giuliano et al., 2015, 2018). Therefore, 14 rats were tested using a PR task (Richardson and Roberts, 1996) during self-administration to assess motivation for alcohol (Fig. 2*A*). There was no significant correlation between Context B active lever presses after prolonged abstinence and PR breakpoint, $r = 0.153$, $p = 0.602$ (Fig. 2*B*). Additionally, there were no significant correlations between Context B active lever presses after prolonged abstinence and the number of alcohol deliveries ($r = -0.034$, $p = 0.909$) or the number of active lever presses $r = 0.063$, $p = 0.830$) in the first 20 min of the PR task. Thus motivation for alcohol does not predict propensity for relapse in Context B in this paradigm.

Table 2. Correlations between alcohol drinking history and the propensity to relapse after 30 d abstinence in the punishment context

Alcohol drinking history measure	Pearson correlation with propensity to relapse	FR-1 schedule Pearson correlation with propensity to relapse	VI-30 schedule Pearson correlation with propensity to relapse	Punishment Pearson correlation with propensity to relapse
Home-cage consumption	0.000			
Early alcohol deliveries		0.236	0.389	0.361
Early active lever presses		0.201	0.397	0.309
Early timeout lever presses		0.182	0.275	0.282
Late alcohol deliveries		0.286	0.223	0.396
Late active lever presses		0.283	0.329	0.337
Late timeout lever presses		0.394	0.341	0.305
Total alcohol deliveries		0.268	0.294	0.404
Total active lever presses		0.242	0.374	0.309
Total timeout lever presses		0.226	0.381	0.303

Retrospective Pearson's correlations were performed between alcohol seeking behavior in Context B after 30 d abstinence and average home-cage consumption (g/kg/24 h), average self-administration alcohol deliveries, active lever presses and timeout lever presses for an early time point (first 10mins), late time point (last 10 min) or the total for the 20 min operant session. These data were divided into the first seven sessions when the reinforcement schedule was FR-1 and the last six sessions when the reinforcement schedule was VI-30. Alcohol seeking was also correlated with average punishment alcohol deliveries, active lever presses and timeout lever presses for an early time point (first 10 min), late time point (last 10 min) or the total for the 20 min operant session. Data presented as the Pearson correlation coefficient (r). $n = 21$.

Experiment 3: Fos expression associated with context-induced relapse to alcohol seeking following prolonged abstinence

There was a significant effect of Abstinence Group (Day 30 Alcohol $n = 6$, Day 30 Punishment low relapsing $n = 7$, Day 30 Punishment high relapsing $n = 8$) on the number of Fos-positive cells in the LH ($F_{(2,18)} = 4.112$, $p = 0.034$), PVT ($F_{(2,18)} = 4.494$, $p = 0.026$), LHb ($F_{(2,17)} = 4.943$, $p = 0.020$), and AI ($F_{(2,17)} = 5.682$, $p = 0.016$). *Post hoc* analyses showed significant differences between groups Day 30 Alcohol and Day 30 Punishment high relapsing in the LH, PVT, LHb, and AI (p values <0.05; Table 3). In the AI and LHb, there was also a significant difference between groups Day 30 Punishment low relapsing and Day 30 Punishment high relapsing (p values <0.05; Fig. 3; Table 3). Only the AI had a significant correlation between alcohol seeking in the punishment context and Fos counts, $r = 0.609$, $p = 0.047$ (Fig. 3*B*; Table 4).

Experiment 4: effect of AI inactivation on context-induced relapse to alcohol seeking in the punishment context following prolonged abstinence

Experiment 4 tested a causal role of AI in context-induced relapse to alcohol seeking in Context B, the punishment context after prolonged abstinence (Fig. 4*A*), using reversible inactivation with intra-AI injections of muscimol + baclofen. AI inactivation reduced context-induced relapse to alcohol seeking in the punishment context following prolonged abstinence [1/8 rats relapsed (12.5%), compared with 5/9 for vehicle (56%); Fig. 4B]. There was a significant main effect of Drug ($F_{(1,14)} = 5.700$, $p = 0.032$). Analysis of latency to first lever press revealed no significant effect of Drug ($F_{(1,15)} = 2.542$, $p = 0.132$). These results suggest the AI plays a critical role in context-induced relapse to alcohol seeking in Context B, the punishment context after prolonged abstinence.

Experiment 5: effect of AI inactivation on context-induced relapse to alcohol seeking following 1 d abstinence

Experiment 5 tested a causal role of AI in context-induced relapse to alcohol seeking after 1 d abstinence (Fig. 5), using reversible

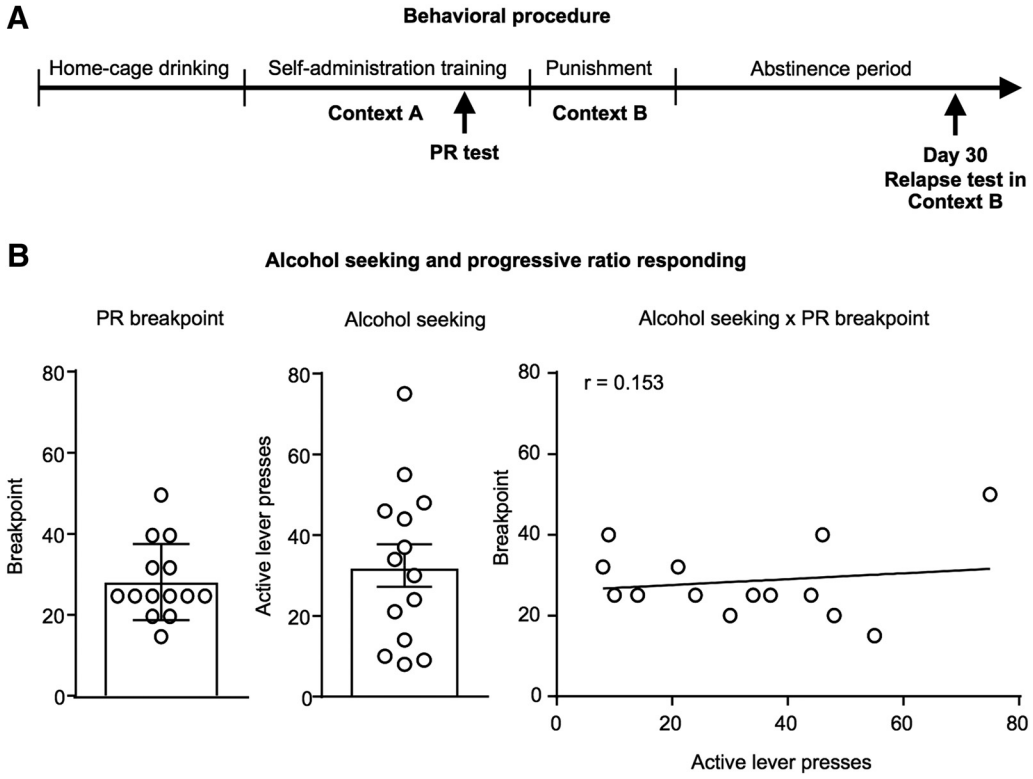

Figure 2. Predicting the propensity to relapse in Context B, the punishment context following prolonged abstinence. Outline of the behavioral procedure (***A***). PR breakpoint and number of active lever presses during the relapse test in the punishment context (***B***, left). Correlation between PR breakpoint and alcohol seeking active lever presses in the punishment context (***B***, right). Data are presented as mean ± SEM. $n = 14$.

Table 3. Fos-protein expression across several brain regions following 30 d abstinence

Brain region	Context A, alcohol	Context B, punishment low relapsing	Context B, punishment high relapsing
AI	32.102 ± 10.460	48.679 ± 13.150#	108.517 ± 23.707**
AIp	16.064 ± 5.467	24.793 ± 5.639	34.041 ± 6.944
PrL	32.645 ± 7.045	30.354 ± 15.082	73.565 ± 26.275
IL	36.773 ± 9.896	24.377 ± 12.281	61.105 ± 17.219
LOFC	120.322 ± 32.167	110.660 ± 34.262	148.329 ± 33.813
EC	44.752 ± 10.964	38.191 ± 9.895	73.703 ± 18.003
vSub	67.540 ± 17.905	56.231 ± 8.594	71.599 ± 5.483
PVT	60.854 ± 7.797	101.111 ± 21.192	131.584 ± 15.626**
LH	19.615 ± 2.517	27.611 ± 4.990	37.364 ± 4.561*
LHb	12.300 ± 3.617	16.474 ± 3.929	39.292 ± 9.577*
BLA	12.862 ± 4.768	9.913 ± 2.630	20.926 ± 4.162
CeA	26.909 ± 9.721	54.359 ± 19.369	38.956 ± 9.851
MeA	40.849 ± 11.937	37.668 ± 9.557	62.679 ± 14.002
DDG	12.218 ± 2.844	16.454 ± 4.637	23.966 ± 3.917
PVN	43.589 ± 12.772	71.525 ± 40.149	67.019 ± 19.133
NAcC	22.970 ± 7.921	19.236 ± 8.475	29.519 ± 11.549
NAcSh	15.894 ± 4.935	17.451 ± 7.186	38.213 ± 11.570

Data presented as average number of Fos-positive cells per mm^2 ± SEM. $n = 6–8$/group.

*$p < 0.05$ versus Alcohol group, **$p < 0.01$ versus Alcohol group, #$p < 0.05$ versus Punishment high relapsing group.

inactivation with intra-AI injections of muscimol + baclofen. AI inactivation did not change alcohol seeking behavior in either context following acute abstinence (Fig. 5*B*). There was no significant interaction between Drug × Test Context ($F_{(1,17)} = 0.010$, $p = 0.921$). There was a significant main effect of Context ($F_{(1,17)} = 8.959$, $p = 0.008$) with rats tested in Context A having a greater number of active lever presses compared with rats tested in Context B. There was no significant main effect of Drug ($F_{(1,17)} = 0.004$, $p = 0.952$). Analysis of latency to first lever press revealed no significant interaction between Drug × Test Context ($F_{(1,17)} = 2.626$, $p = 0.124$). There was a significant main effect of Context ($F_{(1,17)} = 10.543$, $p = 0.005$) with rats tested in Context B having a greater latency to first lever press compared with rats tested in Context A. There was no significant main effect of Drug ($F_{(1,17)} = 1.589$, $p = 0.224$). There was also no significant effect of AI inactivation on spontaneous locomotor activity ($F_{(1,14)} = 2.302$, $p = 0.151$; Fig. 5*D*). These results suggest the AI does not play a critical role in context-induced relapse to alcohol seeking after acute abstinence.

Discussion

We extended a recently developed model of context-induced relapse to alcohol seeking following punishment-imposed abstinence (Marchant et al., 2013), by examining relapse after prolonged abstinence. We found an increased propensity to relapse in Context B, the punishment context after prolonged abstinence. Neither prior alcohol use history, nor the motivational strength of alcohol, predicted relapse vulnerability after prolonged abstinence. Increased propensity to relapse in the punishment context was correlated with activation of the AI, and reversible inactivation of the AI dramatically reduced the proportion of rats relapsing in the punishment context after prolonged abstinence but not following acute abstinence. Our results demonstrate a critical role of the AI in context-induced relapse to alcohol seeking in Context B, the punishment context, following prolonged abstinence.

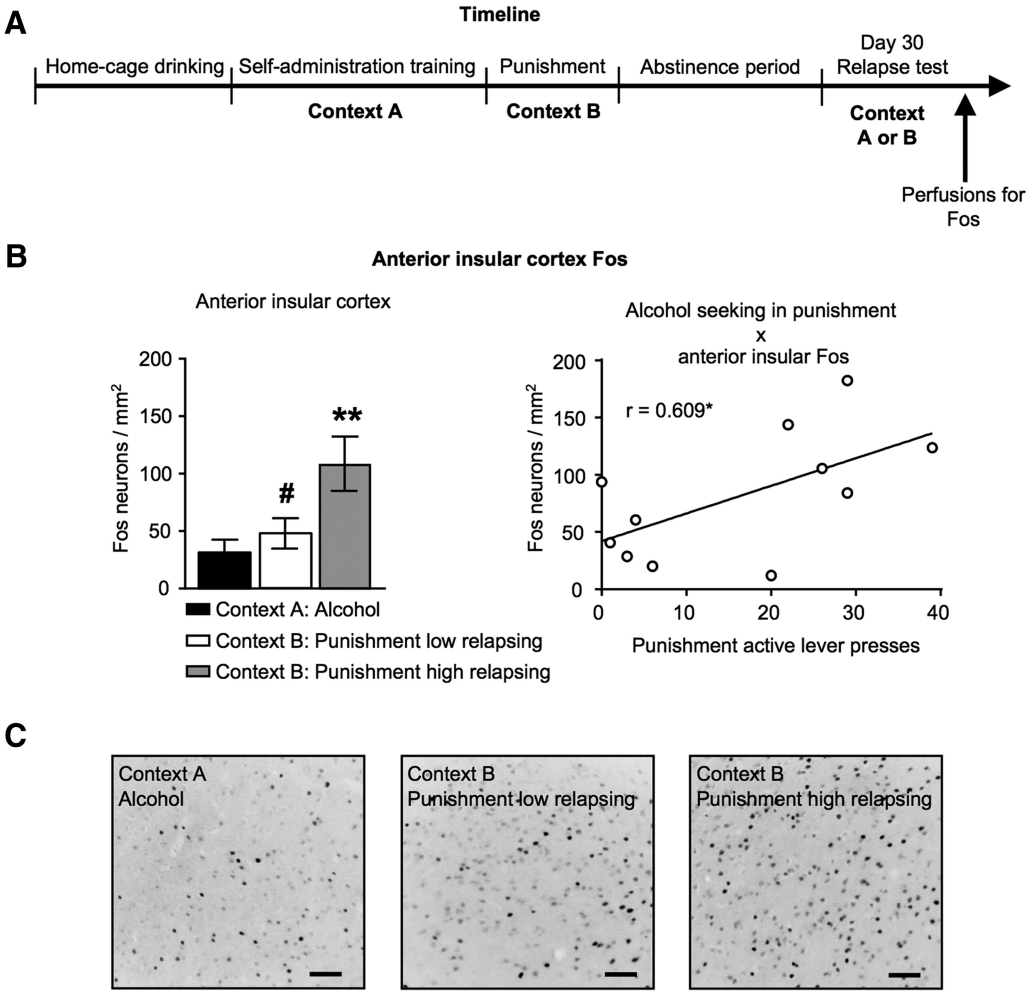

Figure 3. Context-induced relapse to alcohol seeking is associated with selective activation of the AI. Outline of the experimental procedure (***A***). Number of Fos-positive neurons per square millimeter in the AI in rats tested in Context A: alcohol context, or in Context B: punishment context who were either low relapsing or high relapsing. Correlation between alcohol seeking behavior in Context B: punishment context and active lever presses on relapse test (***B***). Photomicrographs representing Fos-protein expression across each treatment group (***C***). Data are presented as mean ± SEM. $n = 6–8$/group. Scale bar, 200 μm. $^{**}p < 0.01$ versus Alcohol group, $\#p < 0.05$ versus Punishment high relapsing group, $^{*}p < 0.05$.

Table 4. Correlations between Fos expression and alcohol seeking behavior in either Context B: Punishment context, or Context A: Alcohol context following prolonged abstinence

	Pearson correlation	
Brain region	Context B, punishment	Context A, alcohol
AI	0.609*	−0.033
AIp	0.193	−0.122
PrL	0.277	−0.241
IL	0.393	−0.362
LOFC	0.414	−0.556
EC	0.190	−0.150
vSub	0.175	0.480
PVT	0.193	−0.088
LH	0.446	−0.291
LHb	0.324	0.310
BLA	0.277	−0.431
CeA	−0.226	−0.198
MeA	0.365	−0.780
DDG	0.315	0.159
PVN	−0.118	0.239
NAcC	−0.031	−0.707
NAcSh	0.083	0.017

Data presented as the Pearson correlation coefficient (r). $n = 6–8$/group.
$^{*}p < 0.05$.

Individual variability in the propensity to relapse and predicting relapse vulnerability following prolonged abstinence

In humans with alcohol use disorder, relapse can occur following weeks, months, or years of abstinence, and environments previously associated with alcohol availability are often potent precipitants of relapse (Wikler, 1973; Collins and Brandon, 2002; Ferri et al., 2006). In preclinical models, drug seeking progressively increases during abstinence (Shalev et al., 2001) termed incubation of craving (Grimm et al., 2001). Time-dependent increases in cue-induced reinstatement of alcohol seeking have also been demonstrated (Bienkowski et al., 2004), as well as after punishment-imposed abstinence of methamphetamine and food seeking behavior (Krasnova et al., 2014). Interestingly, in follow-up studies this group found that the incubation effect was predominantly due to enhanced cue-induced methamphetamine seeking after withdrawal (Torres et al., 2017). We did not observe increased alcohol seeking in Context A after 30 d abstinence. This lack of "incubation" has also been demonstrated for context-induced reinstatement of alcohol and methamphetamine seeking following extinction (Zironi et al., 2006; Adhikary et al., 2017). Our findings suggest that alcohol seeking in the alcohol-associated context following voluntary abstinence does not in-

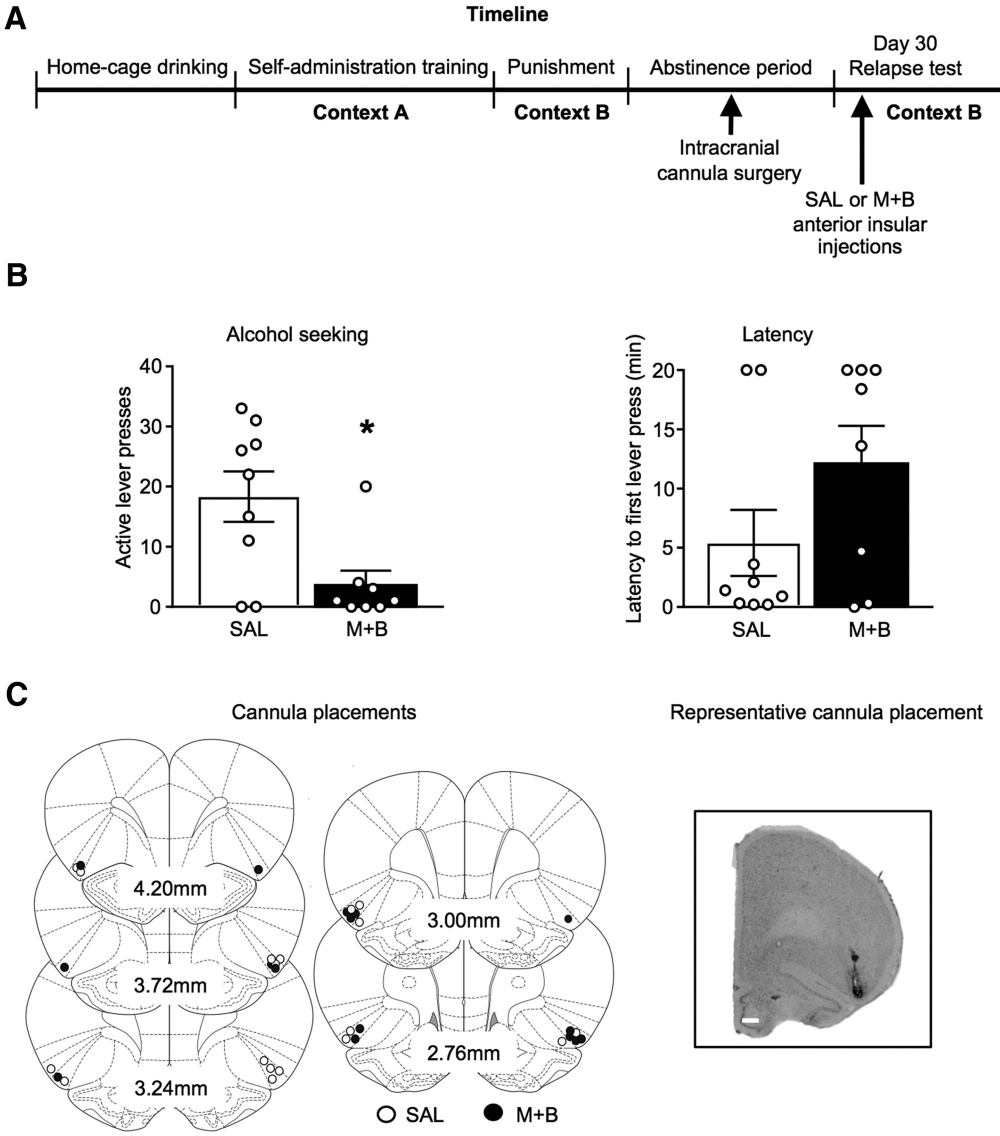

Figure 4. Anterior insular cortex inactivation prevents context-induced relapse to alcohol seeking in Context B, the punishment context following prolonged abstinence. Outline of the experimental timeline (***A***). Number of active lever presses (left) and latency to first lever press (right) in the punishment context following prolonged abstinence (***B***). Approximate cannula placements (mm from bregma) of the injector tips for the AI (***C***). SAL, saline, M+B, muscimol + baclofen. Data are presented as mean ± SEM. $n = 8–9$/group. Scale bar, 1000 μm. *$p < 0.05$.

crease over the time span of our procedure, or if it does, the peak of relapse behavior may occur either before or sometime after 30 d. Indeed, a potential criticism of the punishment model used is the relatively brief alcohol-free period before Day 1 relapse testing, because alcohol is still delivered and consumed during punished responding. As previously mentioned, our data do however show a clear effect of context on Day 1 relapse testing, suggesting that even after a short period of abstinence, rats can differentiate between "safe" and "dangerous" environments.

Our most striking finding is increased alcohol seeking, and increased individual variability, in the punishment context, Context B, after 30 d abstinence. This equates to increased alcohol seeking despite the knowledge of likely negative consequences. In contrast to the alcohol context, we repeatedly observed a clear increase in alcohol seeking in the punishment context, Context B, over time. Previous studies have shown that context-induced reinstatement of alcohol seeking following 15 d abstinence does not increase responding for alcohol in the extinction context (Zironi et al., 2006). Thus, our study identifies an important difference between extinction-based and punishment-based models of abstinence and relapse.

In our procedure, the punishment context is associated with alcohol availability, but alcohol seeking is suppressed by increasing shock intensity. Thus, the neural mechanisms responsible for behavioral control in this context likely involve an interaction between those processing reward and those processing the aversive stimulus (Barberini et al., 2012; Marchant et al., 2018b). In contrast, the extinction context involves experimenter-imposed extinction where the reward is no longer delivered. Although there are distinct neural substrates responsible for this, this operational difference results in vastly different learning and neurobiological mechanisms between extinction and punishment. In our model, foot shock presumably causes increased salience of the alcohol-associated cues in the punishment context for relapse vulnerable rats. In this scenario, our data suggest time-dependent increases in alcohol seeking in response to cues with strong valence in susceptible rats. Importantly, the rats that do relapse in the punishment context had higher latency to respond compared with those tested in the alcohol context. This suggests that the extended abstinence period does not completely diminish the contextual association of punishment.

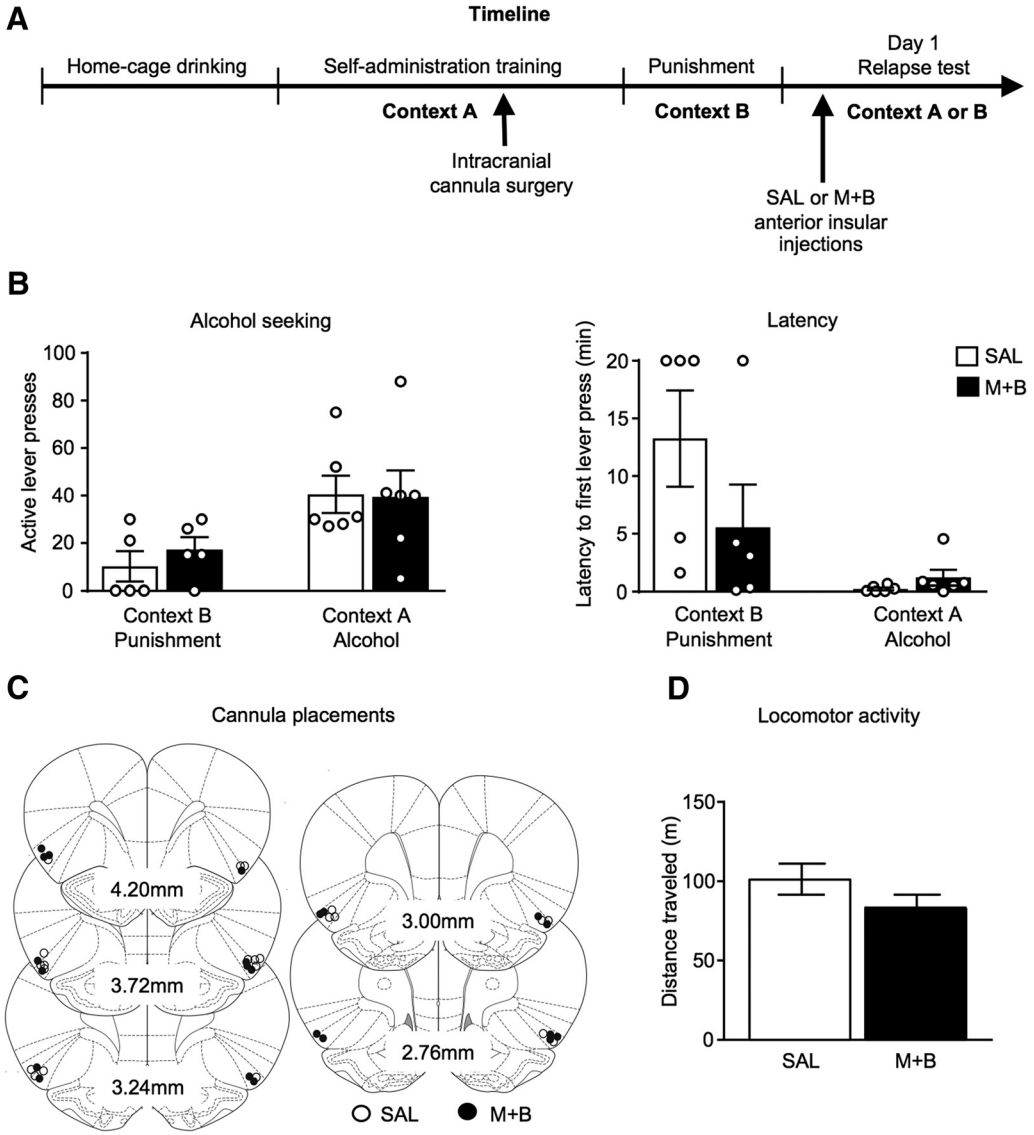

Figure 5. Anterior insular cortex inactivation has no effect on context-induced relapse to alcohol seeking in following acute abstinence. Outline of the experimental timeline (***A***). Number of active lever presses (left) and latency to first lever press (right) following acute abstinence (***B***). Approximate cannula placements (mm from bregma) of the injector tips for the AI (***C***). SAL, saline, M+B, muscimol + baclofen. There was no effect of AI inactivation on locomotor activity (***D***). Data are presented as mean ± SEM $n = 5–6$/group.

We observed significant variability in the propensity to relapse in Context B, the punishment context, after prolonged abstinence. Our attempt to predict relapse following prolonged abstinence was unsuccessful. Interestingly, these data are in line with human studies which also suggest that history of alcohol intake is not enough to predict relapse (Maisto et al., 2016; DiClemente and Crisafulli, 2017). Additionally, individual interoceptive responses to punishment might impact on alcohol relapse propensity. However, there was no correlation between relapse propensity and punishment self-administration responding in the current dataset, and we have previously shown that variability in the response to shock is not predicted by measures of alcohol self-administration (Marchant et al., 2018a).

Role of the AI in the propensity to relapse following prolonged abstinence

We found a critical role for the AI in context-induced relapse to alcohol seeking in the punishment context following prolonged abstinence, but not acute abstinence. The AI drives motivational behavior through interoception or by modulating approach versus avoidance behavior (Paulus and Stewart, 2014). Reversible inactivation of the AI reduces cue-induced reinstatement of cocaine and nicotine seeking and context-induced reinstatement of cocaine seeking (Cosme et al., 2015; Pushparaj et al., 2015; Arguello et al., 2017). Additionally, chemogenetic inhibition of AI to nucleus accumbens core projections reduces alcohol self-administration (Jaramillo et al., 2018a,b). Recently, Pelloux et al. (2018) showed increased Fos expression in the AI in Context A following context-induced relapse to cocaine seeking after punishment-imposed abstinence. Additionally, Venniro et al. (2017) showed that AI inactivation, and chemogenetic inhibition of AI to central amygdala projections, reduced relapse to methamphetamine seeking in Context A after voluntary abstinence achieved via a model of contingency management in Context B. To our knowledge, the current data are the first to link the AI with the propensity to relapse in the punishment context (B) after prolonged abstinence.

In humans, altered insular cortex volume is associated with alcohol relapse and increased activation of the anterior insular has been associated with compulsive alcohol seeking despite aversive consequences in heavy drinkers (Cardenas et al., 2011; Grodin et al., 2018). Additionally, blood oxygen level-dependent fMRI signals are increased in the insular of cocaine and methamphetamine addicts when exposed to drug-associated cues (Garavan et al., 2000; Yin et al., 2012). Finally, damage to the insular cortex reduces relapse rates in cocaine addicts (Naqvi et al., 2007; Gaznick et al., 2014). These data, combined with our preclinical studies, highlight a pivotal role for the AI in relapse.

Conclusions

In conclusion, we show an increased propensity to relapse in Context B, the punishment context, following prolonged abstinence. Neither alcohol intake history nor the motivational strength of the alcohol predicted relapse. We found increased Fos expression in the AI in rats relapsing in the punishment context, which positively correlated with alcohol seeking. Reversible inactivation confirmed a critical role for the AI in the propensity to relapse in the punishment context after prolonged abstinence. Our findings provide further evidence that alcohol use history cannot be used to accurately predict relapse. Identification of reliable biomarkers that enable precision medicine may ultimately improve relapse prevention.

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