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RESEARCH ARTICLE

Compulsive alcohol seeking and relapse: Central role of conditioning factors associated with alleviation of withdrawal states by alcohol

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Background and Purpose: Learned associations between environmental stimuli and drugs of abuse represent a major factor in the chronically relapsing nature of drug addiction. In drug dependent subjects these associations must be presumed to include associations linked to reversal of adverse withdrawal states by drug use—"withdrawal-associated learning" (WDL). However, their significance in drug seeking has received little experimental scrutiny.

Experimental Approach: Using alcohol as a drug of abuse, the behavioural consequences of WDL were investigated in animal models of relapse and compulsive drug seeking by comparing the effects of WD L-associated stimuli versus stimuli associated with alcohol without WDL experience in nondependent and post-dependent rats. Brain sites activated by exposure to the respective stimuli were identified by *c-fos* immunohistochemistry.

Key Results: (1) WDL-associated stimuli elicited significant alcohol seeking. In rats with WDL experience, stimuli associated with alcohol in the nondependent state no longer elicited robust alcohol seeking. (2) Responding elicited by WDL-associated stimuli, but not stimuli conditioned to alcohol in the nondependent state, was resistant to footshock punishment and increased response effort requirements for presentation of WDL-related stimuli. (3) Stimuli conditioned to alcohol in rats with a dependence but not WDL history did not sustain punished responding or tolerance of increased effort. (4) The central nucleus of the amygdala was identified as a site selectively responsive to WDL stimulus exposure.

Conclusion and Implications: Environmental stimuli associated with reversal of adverse withdrawal states by alcohol elicit compulsive-like alcohol seeking and establish WDL as a major, not well-recognized factor, in relapse vulnerability.

Abbreviations: CS, conditioned stimulus; EXT, extinction; FR, fixed ratio schedule (FR1, means that reinforcement is given after each response; FR 5 means that reinforcement is given after every 5 responses and so on); N-WDL, absence of withdrawal-related learning; SC-"W", stimulus context associated with alcohol availability without prior dependence history, serving as a control for the SC-W condition (N-WDL); SC-POST-D, stimulus context associated with alcohol availability in the post-dependent state (i.e. following a history of alcohol dependence that did not include WDL); SC-POST-ND, stimulus context associated with alcohol availability without prior dependence history, serving as a nondependent control for the SC-POST-D condition; SC-PRE, stimulus context associated with alcohol availability in the nondependent state before subsequent dependence induction; SC-W, stimulus context associated with alcohol availability during withdrawal (WDL); WDL, withdrawal-related learning.

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KEYWORDS
alcohol, negative reinforcement, rats, withdrawal-related learning

1 | INTRODUCTION

Alcoholism is a chronically relapsing disorder characterized by compulsive alcohol seeking and use. A critical factor in this behavioural profile are learned responses evoked by environmental stimuli that have become associated with the subjective actions of alcohol. Both retrospective and controlled laboratory studies show that such stimuli evoke alcohol craving that can lead to the resumption of drinking in abstinent individuals suffering from alcohol use disorder (Witteman et al., 2015; for review, Valyear et al., 2017).

The "relapse-inducing" effects of stimuli associated with alcohol have been extensively corroborated by animal studies using the reinstatement model (Burattini et al., 2006; Ciccocioppo et al., 2003; Janak & Chaudhri, 2010; Katner et al., 1999; Le & Shaham, 2002; Radwanska et al., 2008). However, evidence on the role of conditioning factors in alcohol seeking is limited to that from reinstatement studies in nondependent subjects or, in animals with dependence histories, the effects of stimuli conditioned to the reinforcing effects of alcohol before dependence induction rather than conditioning effects related directly to alcohol consumption during withdrawal (e.g. Ciccocioppo et al., 2003; Hansson et al., 2018; Liu & Weiss, 2002). The findings that alcohol-associated stimuli elicit alcohol seeking in nondependent animals are consistent with evidence that even light drinkers show conditioned cue reactivity and mild craving in response to alcohol cue exposure (Greeley et al., 1993; McCusker & Brown, 1990; Streeter et al., 2002). However, in subjects experiencing alcohol use disorder, craving states increase with the duration and severity of alcohol use as reflected by significant positive correlations between history (or severity) of dependence and the intensity of craving induced by alcohol-related stimuli (Greeley et al., 1993; Laberg, 1986; Myrick et al., 2004; Sjoerds et al., 2014; Streeter et al., 2002). One explanation for this observation is that consumption of alcohol during withdrawal states—experiences inextricably linked to alcohol addiction—results in learning of the negative contingency between alcohol consumption and withdrawal symptoms. This modifies an individual's reinforcement history to include learning about amelioration of adverse withdrawal states as an essential aspect of alcohol's reinforcing actions. These learning experiences presumably increase the incentive salience of alcohol over that produced by its positive reinforcing effects, rendering the drug a qualitatively different and more potent reinforcer (Roberts et al., 2000; Vendruscolo & Roberts, 2014).

The present experiments were designed to test the hypothesis that, due to *withdrawal-related learning* (WDL), environmental stimuli conditioned to amelioration of withdrawal (i.e. negative reinforcement by alcohol) acquire more powerful control over alcohol-seeking behaviour than stimuli conditioned to the drug's positive reinforcing effects alone. To accomplish this, the effects of WDL-associated stimulus contexts on alcohol seeking were established in animal models of relapse and compulsive drug seeking, including comparison to the effects of stimulus contexts associated with alcohol in nondependent and in alcohol post-dependent rats without WDL experience (N-WDL). A parallel objective was to identify brain sites showing neuronal activation by exposure to WDL-associated stimulus contexts. This was carried out to test the hypothesis that WDL-motivated behaviour is regulated by brain regions different from those mediating behaviour induced by stimuli conditioned to the positive reinforcing effects of alcohol in the nondependent state. This was focused on the central and basolateral amygdala, given the established roles of these nuclei in the development of alcohol dependence and alcohol seeking induced by alcohol related environmental stimuli (de Guglielmo et al., 2016; Merlo Pich et al., 1995; Radwanska et al., 2008). The focus on the central amygdala has been guided in particular by recent findings that **corticotropin-releasing factor (CRF)** neurons in this nucleus which become hyperactive during alcohol withdrawal and, via projections to the bed nucleus of the stria terminalis, mediate both the somatic signs of withdrawal and excessive self-administration in dependent rats (de Guglielmo et al., 2019).

What is already known

- Alcoholism is a chronically relapsing disorder.
- Environmental stimuli conditioned to the subjective effects of alcohol elicit craving and relapse.

What does this study add

- Conditioning of environmental stimuli to amelioration of withdrawal is a dominant factor in compulsive alcohol-seeking/relapse.
- In subjects with this conditioning experience, stimuli associated with alcohol in the nondependent state diminish in efficacy to induce alcohol seeking.

What is the clinical significance

- Conditioning to alcohol's negative reinforcing effects during withdrawal is a major, previously not well recognized factor in relapse.
- Such conditioning has implications for alcoholism treatment and medication development.

2 | METHODS

2.1 | Animals

Male Wistar rats (Charles River, Wilmington, MA.; average weight 150 g at time of arrival, 480 g at time of testing), pair-housed (two per cage) on a 12-h/12-h reverse light–dark cycle (lights off, 8:00 am) in a temperature (22°C) and humidity-controlled vivarium with *ad libitum* food and water. All training and experimental procedures were conducted during the dark phase (10:00 AM to 4:00 PM) and in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

2.2 | Apparatus

Behavioural procedures were conducted in standard sound-attenuated operant conditioning chambers (Med Associates, St. Albans, VT) equipped, as described previously (Kufahl et al., 2011), with two retractable levers, house and cue lights, and a speaker for presentation of auditory stimuli. A syringe pump, activated by responses at a designated lever, delivered 0.1 ml of liquid reinforcers into a 0.15-ml drinking reservoir.

2.3 | Withdrawal ratings

The presence of alcohol withdrawal signs was verified using a subjective rating scale as previously described (Liu & Weiss, 2002). Rated withdrawal signs including ventromedial limb retraction, vocalization (irritably to touch), tail rigidity, abnormal gait and body tremors were recorded using a subjective rating. Withdrawal ratings were scored by a researcher blind to the experimental conditions during the final week of dependence induction, 7–12 h following removal from alcohol when rats exhibit somatic and motivational signs of withdrawal (Vendruscolo & Roberts, 2014).

2.4 | Experimental design

2.4.1 | Experiment 1: Motivating effects of stimuli associated with alcohol availability in the nondependent state versus alcohol availability during withdrawal

This experiment was designed to establish (1), whether stimulus contexts selectively associated with alleviation of alcohol withdrawal (withdrawal-related learning [WDL]) elicit stronger alcohol seeking than stimulus contexts selectively associated with the positive reinforcing actions of alcohol in the nondependent state and (2), whether WDL-related conditioning modifies the motivating effects of stimuli that have become associated with the positive reinforcing actions of alcohol earlier in the subjects' alcohol history. A second, parallel set of experiments controlled for a history of alcohol dependence per se as an explanation for any presumptive effects of WDL (for experimental design and sequence, see Figure 1).

Alcohol self-administration training

Rats were trained to self-administer oral **ethyl alcohol** on a continuous reinforcement schedule [fixed ratio (FR) 1] in daily 30-min sessions using a sweet solution (3% glucose/0.125% saccharin) fading procedure (Ji et al., 2008; Walker & Koob, 2008). During the first five training days, lever responses resulted in delivery of the sweet solution only. Starting on day 6, training continued with 10% (w/v) alcohol added to the sweet solution for 4 days. During the following four training days, glucose was gradually eliminated from the solution, followed by removal of saccharin after completion of 14 training days.

Context conditioning in the nondependent state (SC-PRE)

Following completion of self-administration training, alcohol availability was conditioned to a compound contextual stimulus as previously described (Gonzalez-Cuevas et al., 2018). Briefly, rats continued to self-administer 10% (w/v) alcohol in daily 30-min continuous reinforcement schedule sessions in the presence of compound olfactory and auditory alcohol-predictive stimulus context (SC-PRE) consisting of either anise or orange extract (McCormick, Hunt Valley, MD) paired with either a continuous 7-kHz tone or illumination of a white cue light. Olfactory and auditory stimulus combinations were counterbalanced with one half of rats receiving anise-tone and the other half orange-cue light stimulus combinations.

Following completion of SC-PRE conditioning sessions, rats were divided into four groups. Two of these four groups, SC-W (W: withdrawal) and SC-POST-D (D: dependent), were subjected to alcohol dependence induction for 6 weeks via an intermittent (14 h on; 10 h off) alcohol vapour inhalation procedure (Vendruscolo & Roberts, 2014). Remaining two groups (SC-"W" and SC-POST-ND) remained on room air and served as nondependent controls (See Figure 1).

Three weeks into the dependence induction period, rats in the SC-W group were removed from the vapour chambers and, following 6 h of withdrawal, allowed to self-administer 10% (w/v) alcohol for 30 min in the presence of compound contextual stimuli (SC-W) distinct from those conditioned to alcohol availability in the nondependent state (i.e. the stimulus components described previously were conditioned in a counterbalanced manner to alcohol availability in the nondependent vs. withdrawal state [see Figure 1a, SC-PRE vs. SC-W]). Nondependent controls (SC-"W") received access to alcohol under the same contingencies and stimulus conditions as the SC-W group. Dependent rats undergoing SC-W conditioning were returned to alcohol vapour and nondependent rats undergoing SC-"W" were returned to room air at the end of conditioning sessions. Training under these conditions was conducted over 10 sessions

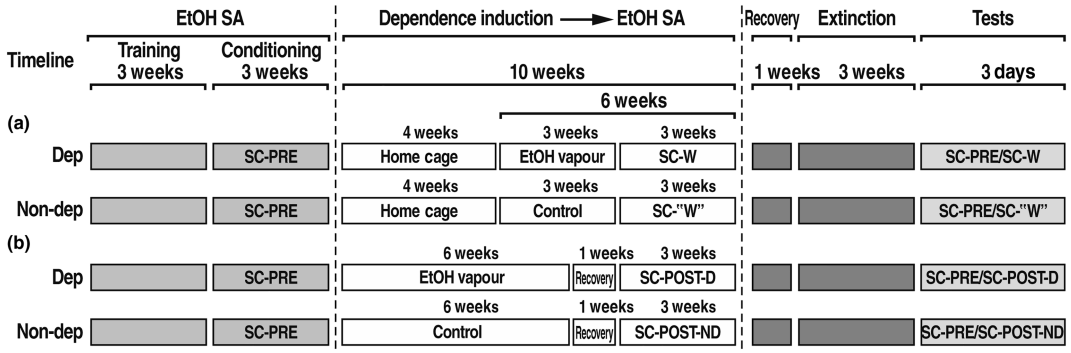

FIGURE 1 Experimental design. (a) Withdrawal-related learning (WDL). The purpose of this condition was to establish the effects of contextual stimuli (SC) conditioned to alcohol reinforcement during withdrawal (SC-W) on reinstatement, as well as the effects of this WDL experience on alcohol seeking induced by stimuli conditioned to alcohol earlier in the nondependent state (SC-PRE). Following alcohol self-administration training, alcohol was available in the presence of distinct contextual stimuli (SC-PRE). Rats then were subjected to alcohol vapour inhalation (Dep) or remained nondependent (Non-dep). After 3 weeks, rats were transiently removed from the vapour (or control) chambers and, after 6 h of withdrawal, given the opportunity to operantly self-administer alcohol in the presence of distinctly different SC (SC-W) stimuli in 30-min sessions. Training in this phase continued for 10 sessions, separated by 1–2 days with rats remaining undisturbed in the vapour chambers (or room air). Note that comparison of SC-"W" versus SC-PRE effects in nondependent rats (Non-dep) provided a control for recency effects on reinstatement. (b) No withdrawal-related learning (N-WDL). The purpose of this condition was to provide a control/comparison for the effects of a dependence history alone without alcohol reinforcement during withdrawal (and to provide a further control for recency effects via comparison of SC-PRE versus SC-POST effects on reinstatement in the nondependent group). Conditioning in the nondependent state (SC-PRE) was identical to that in the WDL condition (i.e. 10 sessions, separated by 1–2 days during which rats remained undisturbed in the home cages). Following dependence induction, rats were withdrawn from alcohol for 1 week and then given the opportunity to operantly self-administer alcohol in the presence of distinctly different contextual stimuli (SC-POST-D). After completion of procedures in the "dependence induction" phase, rats remained in their home cages for 1 week, followed by daily re-exposure to the self-administration chamber under extinction conditions. Subsequent reinstatement tests were conducted by exposure to the SC-PRE/SC-W (or SC-PRE/SC-POST) in counterbalanced order, separated by one home-cage day. Note that the duration of alcohol vapour exposure was held constant to 6 weeks in the WDL and N-WDL conditions, with the only difference that the final 3 weeks of alcohol vapour exposure included daily self-administration/conditioning sessions in the WDL condition

separated by 1–2 days during which rats remained undisturbed on their regular intoxication cycle in the vapour chambers (or room air). Rats designated for testing the effects of dependence history alone without WDL experience on subsequent alcohol seeking (SC-POST-D) and their respective nondependent controls (SC-POST-ND) were given access to alcohol 1 week after completion of alcohol withdrawal. This was done under the same contingencies and stimulus conditions as rats in the withdrawal learning (SC-W) and corresponding control (SC-"W") groups (see Figure 1b, SC-POST-D vs. SC-POST-ND). After completion of the respective conditioning phases, all rats were subjected to daily 30-min extinction (EXT) sessions for a total of 16 days. Levers were presented without the contextual stimuli and responses had no scheduled consequences.

Reinstatement testing

Following completion of extinction training, rats were tested in 30 min sessions for reinstatement induced by the contextual stimuli conditioned to alcohol availability in the nondependent (SC-PRE) versus withdrawal learning (SC-W or SC-"W″) state (Figure 1a, SC-PRE/SC-W and SC-PRE/SC-"W") or nondependent (SC-PRE) versus post-withdrawal (SC-POST-D or SC-POST-ND) state (Figure 1b, SC-PRE/SC-POST-D and SC-PRE/SC-POST-ND). The respective context effects were tested in two separate sessions, conducted in counterbalanced order separated by 1 day.

2.4.2 | Experiment 2: Effects of a withdrawal-related learning (WDL) on compulsive-like alcohol seeking

Experiment 2A: Withdrawal-related learning (WDL) and alcohol seeking under conditions of motivational and environmental challenge

Alcohol self-administration training

Rats were initially acclimated to 20% (w/v) alcohol for 3 weeks under an intermittent 2-bottle alcohol/water free-choice contingency (Simms et al., 2008) and then trained to orally self-administer 10% (w/v) alcohol as in Experiment 1, with modifications. To expedite the transition from 2-bottle free-choice drinking to operant self-administration, the first two sessions were conducted on a FR1 schedule and lasted 12 h (4:00 PM/6:00 AM) with only the active lever present. The next three sessions continued on an FR1 schedule but lasted 1 h with both active and inactive levers present. Subsequently, all sessions lasted 30 min and the response requirement was gradually raised to and maintained at FR3.

Alcohol dependence induction and conditioning during acute withdrawal

After completion of self-administration training, animals were divided into two groups (Figure 2a). One group was subjected to alcohol

dependence induction using the same 6-week intermitted alcohol vapour inhalation procedure as in Experiment 1. The other group remained on room air to serve as a nondependent control. During the final 3 weeks of dependence induction nine WDL conditioning sessions were conducted. For this purpose, rats were removed from the vapour chambers and, following 8 h of withdrawal, allowed to self-administer alcohol on a FR3 schedule for 30 min. Sessions were separated by 1–2 days during which rats remained undisturbed on their scheduled intoxication cycle in the vapour chambers (or room air). During each session, alcohol was available in the presence of olfactory (orange or anise extract) and auditory (continuous 70 dB white noise or a 7 kHz tone) contextual stimuli as in Experiment 1, counterbalanced across the dependent (SC-W) and nondependent (SC-"W") groups (see Figure 2a). Reinforced responses were paired with 2 sec presentation of a compound conditioned stimulus (CS) consisting of a white cue light above the active lever and a 7-kHz beeping tone. Introduction of the CS in addition to the contextual stimuli was necessary to permit examination of willingness to tolerate increased effort (i.e. responding for a conditioned reinforcer). To maintain consistency, this modification was employed also for all remaining experiments. Following completion of the conditioning phase and 1 week of alcohol withdrawal in the home cage, alcohol reinforced responding was extinguished in daily 30 min sessions in which all stimuli were omitted and responses remained without scheduled consequences until rats reached a criterion of ≤10 responses over 3 days. Alcohol withdrawal ratings were conducted as described in Experiment 1.

Reinstatement—Tolerance of increased effort requirements

Once the extinction criterion was reached, rats with and without WDL history (see Figure 2a: SC-W and SC-"W") were tested for willingness to tolerate increased work requirements for presentation of the alcohol-associated CS in 30-min sessions. Rats were exposed to the alcohol-predictive contextual stimuli (SC-W or SC-"W") and the FR requirement for presentation of the CS was increased arithmetically by one step after every second presentation from FR3 to FR6 and maintained at FR6 until session termination.

Reinstatement—Resistance to punishment

Two days following completion of the effort tolerance tests, rats were tested for resistance to punishment of reinstatement responses in the presence of the alcohol-predictive contextual stimuli (see Figure 2a: SC-W and SC-"W"). Active lever responses resulted in presentation of the alcohol CS on a FR3 schedule paired with 0.5 s of electric footshock. Reinstatement tests were conducted within-subjects every other day across ascending current intensities (0.2 mA, 0.25 mA, 0.35 mA, and 0.50 mA) to characterize the profile of resistance to punishment in rats with WDL experience versus non-dependent rats without WDL history.

Simple reinstatement

To assess the potency of withdrawal-related learning despite the recent experience with motivational and environmental challenges, once effort tolerance and resistance to punishment were established, all rats were re-tested under "simple" reinstatement conditions in the presence of the alcohol-predictive contextual stimuli and each reinforced response (FR3) resulting in presentation of the alcohol CS.

Experiment 2b: Alcohol dependence history without withdrawal-related learning and compulsive-like alcohol seeking

The purpose of this experiment was to ascertain that withdrawal-related learning rather than a history of alcohol dependence alone accounts for the development of compulsive-like alcohol seeking observed in Experiment 2a (2.4.1, 2.4.2).

Design and procedures

All procedures were identical to those in Experiment 2a above, except that alcohol context and CS conditioning procedures were conducted 1 week following withdrawal and recovery from alcohol vapour exposure or the respective procedures in nondependent controls (see Figure 2b). Thus, tests in Experiment 2b were replicated in a "dependent no withdrawal-related learning group" (SC-POST-D – Dependent) and a "nondependent no withdrawal-related learning group" (SC-POST-ND – Nondependent). See Figure 2b for the sequence of experimental procedures.

2.4.3 | Experiment 3: Neuronal activation in rats with and without withdrawal learning history

The objective of this experiment was to identify brain sites showing neuronal activation following exposure to WDL-associated stimulus contexts and to test the hypothesis that behaviour motivated by withdrawal-related learning (i.e. the negative reinforcing effects of alcohol) is regulated, at least in part, by brain regions different from those mediating behaviour motivated by alcohol learning experiences in the nondependent state (i.e. the positive reinforcing effects of alcohol). For this purpose, brains of rats tested in Experiment 2 were used and brain regions associated with the "addiction circuit" were targeted. These included the basolateral and central nucleus of the amygdala based on the prediction of greater engagement of brain stress regions by contexts associated with WDL.

Quantitative c-fos immunohistochemistry

Following the completion of behavioural testing in Experiment 2, all rats were returned to the vivarium for 1 week. Rats then were re-exposed to the respective alcohol-predictive stimulus context without the presence of levers (and response-contingent CS). After exactly 90 min of exposure, rats were deeply anaesthetised by CO_2 and transcardially perfused with ice-cold saline, followed by 4% paraformaldehyde in 0.1-mM sodium tetraborate, pH 9.5. Brains then were postfixed in 4% paraformaldehyde overnight (12 h) and stored in a 30% (w/v) sucrose, 0/1% (w/v) sodium azide, and 0.01-mM potassium phosphate buffer saline (KPBS) solution.

Brains were sectioned coronally at 40 μm on a cryostat maintained at −20°C. The sections then were blocked for 1 h using 4% bovine serum albumin/3% normal donkey serum/0.3% Triton-X/PBS, followed by 24 h incubation at 4°C with anti-*c-fos* antibody (rabbit, 1:500, Millipore-Sigma). Tissue sections were processed for Fos detection by incubation with donkey anti-rabbit IgG secondary antibody (Alexa Fluor 647, 1:500, ThermoFisher Scientific) for 2 h at room temperature and counterstained with **DAPI** nuclear fluorescent stain (1:1000, ThermoFisher Scientific). Sections were mounted and imaged with a 20× lens (NA 1.4) using a Nikon A1 confocal microscope equipped with a robotic slide changer and custom software for automatic detection and imaging of entire sections. Anatomical partitioning was performed manually by drawing a region of interest around the central amygdala and basolateral amygdala. Fos^+ neurons were detected and counted using Image J software normalized to total DAPI per area for between group comparisons. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.5 | Statistical analysis

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

Reinstatement responses in the stimulus context associated with alcohol availability during withdrawal (SC-W) versus stimulus context associated with alcohol availability without prior dependence history (SC-"W") and stimulus context associated with alcohol availability in the post-dependent state (SC-POST-D) versus stimulus context associated with alcohol availability without prior dependence history (SC-POST-ND) groups were separately analysed by mixed-factorial ANOVA. Following confirmation of significant main effects in the overall ANOVAs, differences between groups were followed by post hoc Fisher's LSD tests. Sample sizes subjected to statistical analysis were at least 13 animals per group ($n = 13$) for the behavioural experiments and 4 animals per group ($N = 4$) for the immunohistochemical experiment.

Differences in tolerance of effort and "simple" reinstatement responses between dependent rats with WDL experience and their nondependent controls as well as between dependent rats without WDL experience and respective nondependent controls were separately analysed by mixed-factorial ANOVA comparing reinstatement to extinction responses. Significant interactions and main effects were followed by Tukey's post hoc tests. Differences in punished responses across ascending current intensities between dependent groups and their respective nondependent controls were separately analysed by within-subjects ANOVA for differences between reinstatement and extinction responses, followed by Sidak's multiple comparisons to ascertain differences among individual means.

Differences in the number of Fos^+ cells of dependent SC-W versus nondependent SC-"W" rats were analysed separately for each brain region by independent Student's *t* tests assuming equal variance among samples. Data are presented as mean ± SEM. $P<0.05$ is defined as the threshold for significance.

2.6 | Materials

Ethyl alcohol, purchased from Greenfield Global, was dissolved in tap water to a concentration of 20% w/v for 2-bottle choice and 10% w/v for self-administration. Glucose and saccharin were obtained from Sigma-Aldrich.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

2.8 | Results

Blood alcohol levels (BAL) in rats subjected to dependence induction by alcohol vapour reached daily peak values of 225–250 $mg{\cdot}dl^{-1}$. All rats showed significant withdrawal signs without differences between the SC-W and SC-POST-D groups (see Figure S3).

2.8.1 | Experiment 1: Motivating effects of stimuli associated with alcohol availability in the nondependent state versus alcohol availability during withdrawal

Effects of withdrawal-related learning (SC-W) on reinstatement of alcohol seeking

The SC-W produced significant reinstatement of alcohol seeking in the SC-W group whereas the SC-PRE did not (Fisher's LSD tests following significant *stimulus context* [Figure 3a left panel]). Corresponding effects of the SC-PRE and SC-"W" on alcohol seeking in controls not subjected to dependence induction and tested in the presence of the SC-"W" produced significant alcohol seeking without differences as a result of physical properties of the stimulus combinations or recency of conditioning (i.e. SC-PRE vs. SC-"W"; Figure 3b right panel)

Effects of dependence history without WDL on reinstatement of alcohol seeking

The contexts conditioned to alcohol availability in the nondependent (SC-PRE) and post-dependent (SC-POST-D) states both produced significant alcohol seeking without differences as a result of pre- versus post-dependence conditioning or recency of conditioning (Figure 3c left panel) Presentation of the SC-PRE and

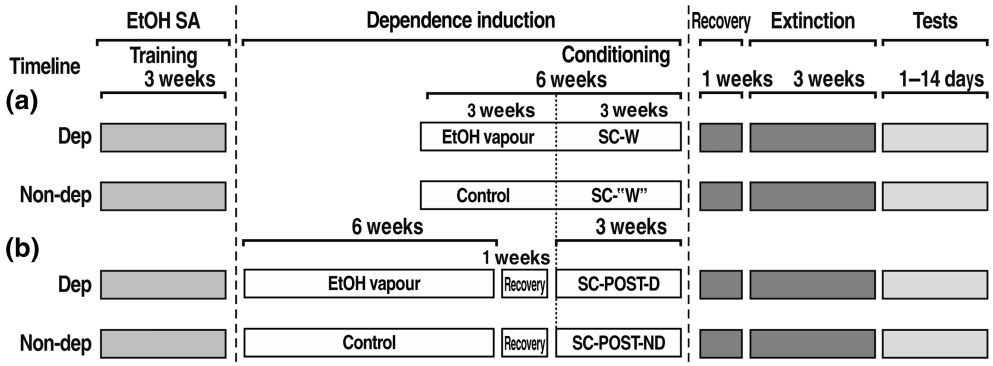

FIGURE 2 Experimental design. (a) Withdrawal-related learning (WDL). The purpose of this condition was to establish the effects of contextual stimuli (SC) conditioned to alcohol reinforcement during withdrawal (SC-W) on reinstatement involving motivational and environmental challenges. Following alcohol self-administration training, rats were subjected to alcohol vapour inhalation (Dep) or remained nondependent (Non-dep). After 3 weeks, rats were transiently removed from the vapour (or control) chambers and, after 8 h of withdrawal, given the opportunity to operantly self-administer alcohol in the presence of the SC and conditioned stimulus (CS) in 30-min sessions (SC-W). Training in this phase continued for nine sessions, separated by 1–2 days during which rats remained undisturbed in the vapour chambers (or room air). (b) No withdrawal-related learning (N-WDL). The purpose of this condition was to provide a control/comparison for the effects of a dependence history alone without alcohol reinforcement during withdrawal. Following dependence induction rats were withdrawn from alcohol for 1 week and then given the opportunity to operantly self-administer alcohol in the presence of SC and CS (SC-POST-D). Paralleling the procedures for the SC-W group (in "a" above), conditioning was conducted in nine sessions, separated by 1–2 days during which rats remained undisturbed in the home cages. After completion of procedures in the "dependence induction" phase, rats remained in their home cages for 1 week, followed by daily re-exposure to the self-administration chamber under extinction conditions. Subsequent tolerance of increased effort, resistance to punishment and "simple" reinstatement tests were conducted across 14 days

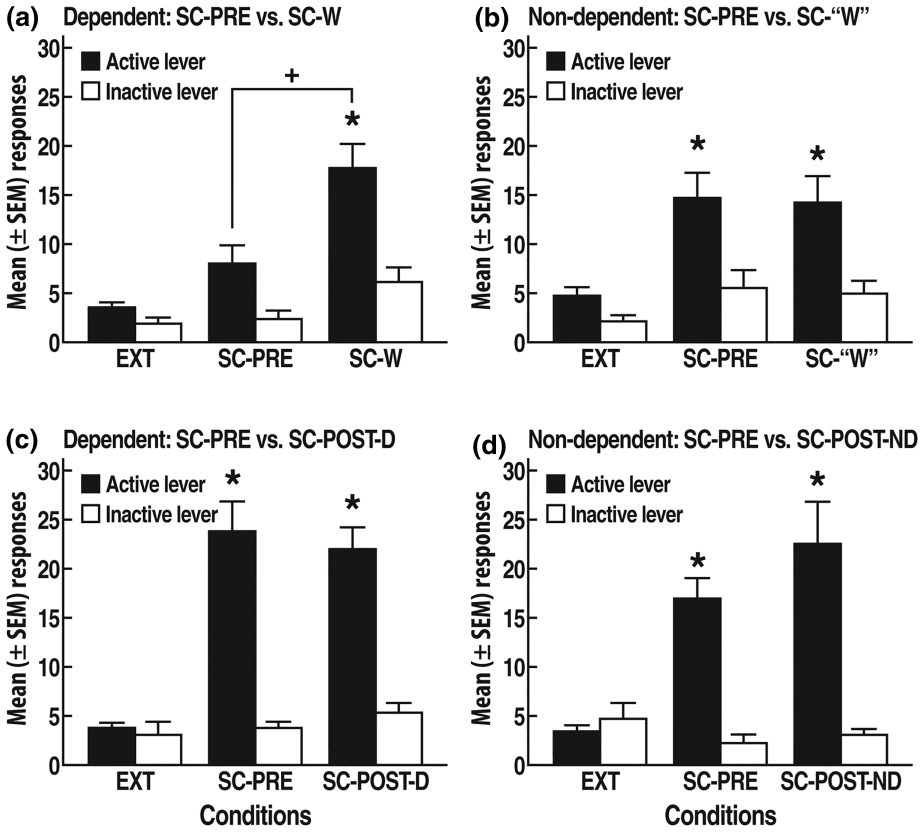

FIGURE 3 Effects of withdrawal-related learning on reinstatement of alcohol seeking. (a) Extinction (EXT) and reinstatement (RST) responses induced by contextual stimuli conditioned selectively to either alcohol availability/self-administration before alcohol dependence induction (SC-PRE) or availability/self-administration during alcohol withdrawal (SC-W). *$P < 0.05$ versus EXT; $^{+}P < 0.05$ versus SC-PRE. (b) Corresponding effects of SC-PRE and SC-"W" in controls not subjected to alcohol dependence induction. *$P < 0.05$ versus EXT. (c) Extinction and reinstatement responses induced by contextual stimuli conditioned selectively to either alcohol availability/self-administration before alcohol dependence induction (SC-PRE) or alcohol availability/self-administration after completion of alcohol withdrawal (SC-POST-D). *$P < 0.05$ versus EXT. (d) Corresponding effects of SC-PRE and SC-POST-ND in controls not subjected to alcohol dependence induction. *$P < 0.05$ versus EXT.

SC-POST-ND in controls not subjected to alcohol dependence and having received conditioning with both sets of alcohol-predictive contexts (SC-PRE and SC-POST-ND) only in the nondependent state, also produced significant reinstatement, again without differences related to stimulus combination or recency of conditioning (Figure 3d right panel).

2.8.2 | Experiment 2: Effects of withdrawal-related learning (WDL) on compulsive-like alcohol seeking

Blood alcohol levels during vapour exposure ranged between 250–300 mg%. All rats showed significant withdrawal during the last week of alcohol dependence induction without differences between the SC-W and SC-POST-D groups (see Figure S4).

2.8.3 | Experiment 2a: Withdrawal-related learning and alcohol seeking under conditions of motivational and environmental challenges

Reinstatement—Tolerance of increased effort requirements

Reinstatement induced by the respective alcohol-associated stimulus contexts and maintained by the alcohol CS increased significantly over extinction performance both in rats with a WDL history (SC-W) and rats with a history of only positive reinforcement by alcohol (SC-"W"). However, alcohol seeking in rats with a WDL history was significantly greater than in rats without this history and, thus, more resistant to increased effort demands (Figure 4a).

Reinstatement—Resistance to punishment

Rats in the WDL history (dependent; SC-W) group showed significant reinstatement at low current intensities (Figure 4b). Moreover, rats with a WDL history showed significantly greater punished responding across current intensities (i.e. substantial resistance to punishment) than rats without this history (*alcohol history* × *current intensity* interaction, Figure 4b).

Simple reinstatement

Following exposure to the highest current intensity in the punished responding tests, rats in both alcohol history groups showed extinction. When re-exposed to the respective alcohol-associated contexts after 2 days in the home cage, rats in the WDL history group resumed FR3 responding for presentation of the alcohol CS at levels significantly different from extinction, whereas responding in rats with only a positive alcohol reinforcement history remained at extinction levels (Figure 4c).

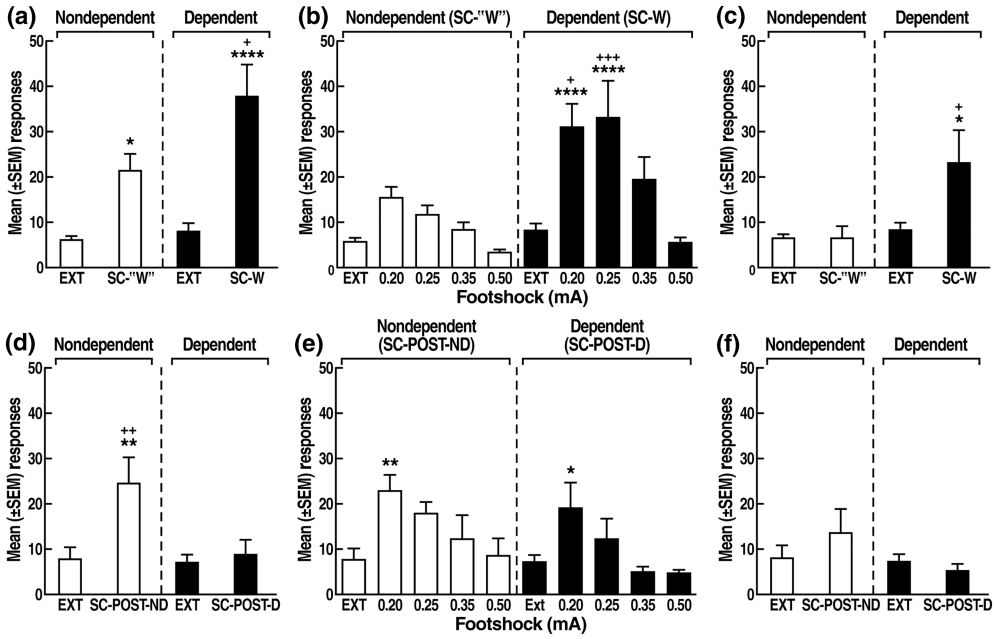

FIGURE 4 Reinstatement in rats with and without a WDL history (SC-W and SC-POST) over sequential sessions testing for the effects of effort challenge, punishment of reinstatement (RST) responses and subsequent "simple" reinstatement without punishment/effort challenge. (a) Tolerance of increased effort requirements. Response requirements for presentation of an alcohol-associated CS were arithmetically increased by one step after every second response (FR3 to FR6) and then maintained at FR6. WDL-experienced rats (SC-W, $n = 15$) maintained significantly greater responding than nondependent rats without WDL experience (SC-"W," $n = 15$ [pooled data across FR ratios]). $^{*}P < 0.05$ versus respective extinction (EXT) performance; $^{+}P < 0.05$ versus RSTin SC-"W" rats. (b) Resistance to punishment. Alcohol-associated stimuli maintained greater responding across current intensities in the SC-W ($n= 15$) than SC-"W" ($n = 15$) condition. $^{*}P < 0.05$ versus respective EXT levels; $^{+}P < 0.05$ versus reinstatement in SC-"W" rats. (c) Simple reinstatement. WDL-experienced rats show greater reinstatement following extinction of alcohol seeking during punished reinstatement tests ($n = 15$), while nondependent rats continued responding at extinction levels ($n= 15$). $^{*}P < 0.05$ versus EXT; $^{+}P < 0.05$ versus RST in SC-"W" rats. (d) Tolerance of increased effort requirements. The alcohol-associated CS did not convey resistance to effort challenge in dependent rats without WDL experience (SC-POST-D, $n = 15$; SC-POST-ND, $n= 16$), but instead decreased it. $^{*}P < 0.05$versus EXT; $^{+}P < 0.005$ versus reinstatement in SC-POST-D rats. (e) Resistance to punishment. Alcohol-associated stimuli did not sustain greater punished responding in the postdependent SC-POST group ($n = 15$) compared to nondependent SC-POST rats ($n = 16$). $^{*}P < 0.05$ versus respective EXT levels. (f) Simple reinstatement. In contrast to WDL-experienced rats, dependent rats without WDL experience (SC-POST-D, $n= 15$) no longer showed reinstatement

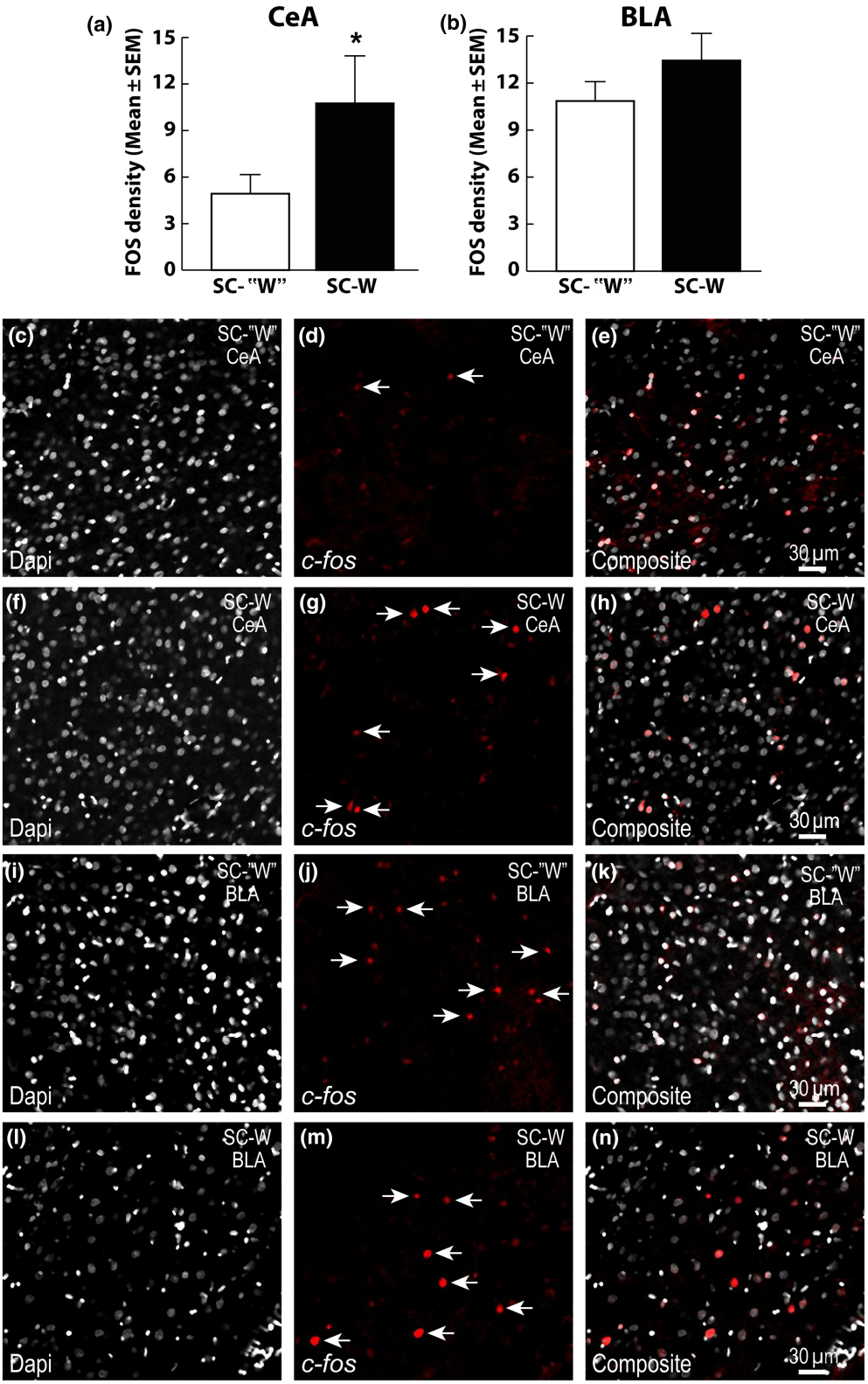

FIGURE 5 Differential *c-fos* activation in rats with and without WDL experience. (a) Exposure to the respective stimulus contexts produced significantly greater *c-fos* activation in the central amygdala (CeA) of rats with WDL experience (SC-W) compared with nondependent rats without WDL experience (*$P < 0.05$ vs. SC-"W"). (b) Differential *c-fos* activation was not observed in the basolateral amygdala (BLA). ($n = 5$ per group). (c–n) Representative brain sections showing DAPI (grey), *c-fos* (red) and merged. White arrows represent typical *c-fos* positive nuclei

2.8.4 | Experiment 2b: History of alcohol dependence alone without withdrawal-related learning does not result in alcohol seeking resistant to motivational and environmental challenges

The stimulus context and CS associated with alcohol availability following completion of withdrawal did not elicit or maintain responding in dependent rats without WDL experience (SC-POST-D, Figure 4d). Thus, not only was no tolerance of increased effort observed, but responding remained at extinction levels. In nondependent controls (SC-POST-ND), tolerance of increased effort was identical to that in the nondependent SC-"W" group in Experiment 2.1a (Figure 4d]). As well, the alcohol associated stimulus context did not sustain greater punished responding in the dependent SC-POST-D group compared with the nondependent SC-POST-ND group (Figure 4e). Thus, resistance to punishment was not observed under these alcohol dependence and conditioning histories and the current-intensity function in the Dependent SC-POST-D group was indistinguishable not only from that in nondependent SC-POST-ND controls, but from the respective function in the nondependent (SC-"W") group in Experiment 2.1a. In contrast to rats with a WDL history (SC-W; Figure 4c), dependent rats without WDL experience (SC-POST-D) no longer showed alcohol seeking when tested for "simple" reinstatement after completion of punished reinstatement tests (Figure 4f).

2.9 | Experiment 3: Neuronal recruitment in rats with and without alcohol withdrawal-related learning

Neuronal recruitment in rats with and without alcohol withdrawal-related learning: Exposure to the WDL-associated context produced significantly greater *c-fos* activation in the central amygdala compared to stimulus context not associated with withdrawal in nondependent rats (Figure 5a, central amygdala: SC-"W" 4.92 ± 1.24, SC-W 10.74 ± 3.07). No differential neuronal activation was observed in the basolateral amygdala (Figure 5b, basolateral amygdala).

3 | DISCUSSION AND CONCLUSION

The results suggest that, as consequence of withdrawal-related learning (WDL), environmental stimuli conditioned specifically to amelioration of withdrawal (i.e., the negative reinforcing effects of alcohol) exert more powerful control over alcohol-directed behavior than stimuli conditioned to the positive reinforcing effects alone as implied by the four major findings: (1) Stimuli conditioned to alcohol consumption during withdrawal elicited significant reinstatement. (2) Stimuli conditioned to alcohol in the nondependent state lost efficacy for inducing reinstatement in rats with subsequent WDL experience. (3) WDL experience rendered alcohol seeking impervious both to punishment and increased effort requirements for presentation of WDL-related stimuli thus producing compulsive-like alcohol seeking, whereas stimuli conditioned to alcohol in the nondependent state (SC-"W" and SC-POST-ND) did not produce these effects. (4) A dependence history alone (without WDL) was not associated with punishment- and effort- resistant alcohol seeking. These findings implicate WDL as a major factor in vulnerability to relapse and compulsive alcohol seeking.

Consistent with the hypothesis that withdrawal-related learning (SC-W) represents a major factor in alcohol seeking and relapse, stimuli associated with WDL produced significant reinstatement in Experiment 1. More importantly, stimuli conditioned to alcohol in the nondependent state lost substantially in efficacy to induce reinstatement in rats with a subsequent WDL history. Yet, the number of reinstatement responses induced by the WDL-associated context was not greater than that produced by stimuli conditioned to alcohol in rats without subsequent WDL history. This finding did not appear consistent with the hypothesis that WDL increases the incentive salience of alcohol-related environmental stimuli. However, absolute reinstatement responses may not provide a sensitive measure of the motivating valence of these stimuli. For, example, alcohol dependent and nondependent rats (or rats differing in genetic alcohol preference) often do not differ in spontaneous anxiety but show significant differences in anxiety after stress challenges (Hansson et al., 2018; Valdez et al., 2003; Zhao et al., 2007). Consequently, WDL-related stimuli may have "latent" motivating valence that can be unmasked by behavioral and environmental challenges. Confirming this hypothesis, the introduction of such challenges (i.e., punishment and effort manipulations) in Experiment 2 revealed that alcohol seeking-behaviour in WDL-experienced rats was impervious to punishment and increased effort requirements, whereas such behaviour when induced by stimuli conditioned to alcohol in the nondependent state was not.

The findings across the two behavioural experiments then establish that environmental stimuli conditioned specifically to amelioration of withdrawal (i.e. the negative reinforcing effects of alcohol) come to exert more powerful control over alcohol seeking than stimuli conditioned to the positive reinforcing effects of alcohol. First, the findings reveal that once conditioning occurs to the effects of alcohol during withdrawal states, associations between environmental stimuli and subjective effects of alcohol established earlier in the nondependent state diminish in relevance for alcohol-seeking whereas WDL-motivated behaviour becomes dominant. A recency effect did not account for this finding because in all three comparison groups that did not experience WDL, stimuli established during the initial conditioning occasion, produced the same degree of reinstatement as those established during the second occasion (Figure 3b–d). Second, stimuli conditioned to WDL not only acquired dominance in controlling alcohol seeking (Experiment 1), but their effects were characterized by significant resistance to punishment and increased response demands (Experiment 2). It is evident that the compulsive-like profile of alcohol seeking produced by stimuli conditioned to alcohol consumption during WDL is contingent specifically on the WDL experience and is not a function of an alcohol dependence history per se. This is because animals made dependent on alcohol, but a conditioning experience to alcohol consumption in the post-dependent

state (i.e. after completion of withdrawal) only, did not sustain greater resistance to punishment or effort challenges compared with nondependent controls (Figure 4d,e). Compulsive alcohol seeking—a hallmark of substance dependence on alcohol—is characterized by maintenance of drug-directed behaviour despite adverse consequences as well as willingness to expend inordinate effort or time to obtain the drug (DSM-IV, 1994). The findings here confirm that WDL-related stimuli elicit these manifestations of compulsive-like alcohol seeking, whereas stimuli associated with alcohol in the nondependent state do not produce this behavioural profile. Lastly, and most importantly, a dependence history alone without WDL experience did not diminish the effects of stimuli conditioned to alcohol in the pre-dependent state (Figure 2c; SC-PRE vs. SC-POST-D), as seen in the SC-W group (Figure 2a; SC-PRE vs SC-W). Similarly, a dependence history per se without WDL experience was not associated with significant effort- and punishment-resistant reinstatement. These findings establish that exacerbated punishment- and effort-resistant alcohol seeking does not occur in dependent rats without WDL history. Consequently, exacerbated alcohol seeking cannot be explained by a dependence history per se but depends on learning of the negative contingency between alcohol consumption and reversal of aversive withdrawal effects.

There is a dearth of literature on the significance of WDL-related conditioning factors in the perpetuation of drug seeking. However, ample evidence confirms that alcohol is a more potent reinforcer in dependent than non-dependent animals (e.g. Hunter et al., 1974; Roberts et al., 2000; for review, Vendruscolo & Roberts, 2014). Similarly, experience with heroin in the withdrawal state enhances subsequent heroin-seeking (Hutcheson et al., 2001). These findings suggest that drug experiences during withdrawal render the drug a qualitatively different and more potent reinforcer that eventually dominates an individual's behavioural repertoire. Extending this hypothesis, the present findings suggest that the effects of environmental stimuli associated with alcohol consumption during withdrawal states exert more powerful control over drug-directed behaviour than stimuli conditioned to the positive reinforcing effects alone, and thereby play a central role in the perpetuation of compulsive alcohol seeking.

An important behavioural observation was that a history merely of dependence, rather than conditioning effects associated with negative reinforcement by alcohol during withdrawal, does not alter the motivational impact of alcohol-related stimuli. This finding suggested that the neural regulation of alcohol seeking differs in subjects with and without a WDL history. Consistent with this hypothesis, WDL-related stimuli produced significantly greater neuronal activation in the central amygdala than stimuli conditioned to alcohol in the nondependent state, implicating the central amygdala as a site mediating the behavioural consequences of alcohol withdrawal-related learning. This interpretation is supported by recent related findings that inactivation of a neuronal ensemble in the central amygdala significantly reduces the motivation to consume alcohol rats as well as somatic withdrawal signs in dependent (de Guglielmo et al., 2016). Consistent with the present findings as well, alcohol dependent rats show increased extracellular levels of CRF in the central amygdala during withdrawal, linking this site to the aversive effects of alcohol withdrawal (Merlo Pich et al., 1995). Exposure to WDL-related stimuli, however, did not produce differential neuronal activation in the basolateral amygdala, confirming that the behavioural effects of WDL are neuronally regulated differently from the effects of alcohol-related learning that occurs in the nondependent state. It will be important to identify more systematically the neurocircuitry mediating WDL-associated alcohol seeking in the future.

The behavioural findings experimentally confirm clinical reports suggesting that stimuli conditioned specifically to the effects of alcohol consumed during withdrawal states represent a major factor in alcohol craving and relapse (Gauggel et al., 2010; Kreusch et al., 2017; Myrick et al., 2004; Sjoerds et al., 2014; Streeter et al., 2002; Witteman et al., 2015). Withdrawal-related conditioning in the model employed here was conducted during full alcohol withdrawal. However, WDL experiences in both man and animals do not necessarily have to include reversal of full physical withdrawal because major adverse subjective effects occur already very early into withdrawal. For example, dependent rats generalize to the interoceptive effects of the anxiogenic agent pentylenetetrazol and show elevated brain reward thresholds (i.e. reward deficits, dysphoria) very shortly after removal from alcohol (Lal et al., 1988; Schulteis et al., 1995). Therefore, it seems likely that, whether linked to reversal of full physical withdrawal or reversal of early adverse anxiogenic and dysphoric effects, WDL increases incentive salience of alcohol-related environmental stimuli over those associated with the positive reinforcing effects of alcohol alone. Nonetheless, it will remain for future research to conclusively establish whether the motivating impact of WDL linked to early versus full withdrawal experiences is identical or not.

CONCLUSION

In summary, the findings document that once conditioning occurs to the effects of alcohol consumption during withdrawal states, associations between environmental stimuli and subjective effects of alcohol established in the nondependent state diminish in relevance for alcohol-seeking. Moreover, stimuli conditioned specifically to negative reinforcement by alcohol during withdrawal acquire conditioned incentive value of their own, with effects impervious to punishment and elevated effort requirements such that these stimuli come to play a dominant role in perpetuating compulsive alcohol seeking. As such withdrawal-related learning (WDL) is likely to represent a major factor in alcohol seeking and relapse. Here, it should be noted that the findings are limited to male rats such that interrogation of possible sex differences in the behavioural and neuronal effects of WDL will remain for future research. Historically, withdrawal-related conditioning has been studied in terms of behaviour produced by stimuli conditioned directly to the aversive effects of drug withdrawal (conditioned withdrawal). These stimuli typically produce drug opposite anxiogenic and aversive effects although very recently olfactory cues conditioned to

naloxone-precipitated heroin withdrawal have been shown to increase heroin intake (Carmack et al., 2019). In contrast, the significance of environmental conditioning to alleviation of withdrawal symptoms by alcohol (and other drugs of abuse) in initiating and maintaining alcohol seeking under conditions modelling relapse-like behaviour that are documented here, to our knowledge, have not received previous attention. Therefore, better understanding of the control and neurobiological basis of alcohol-related conditioning that includes the reinforcing dimensions of this drug associated with the experience of withdrawal states seems essential for generating translationally relevant insight to advance the treatment of alcohol addiction.

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AUTHOR CONTRIBUTIONS

F.W., O.O.K., and P.R.K. designed the studies. O.O.K and P.K. conducted the experiments. M.M. provided the confocal microscope system designed for whole brain slice imaging and assisted with neural mapping. H.N. generated the *c-fos* photomicrographs and assisted with *c-fos* data analysis and preparation of figures. O.O.K., F.W., and P.R.K. wrote the manuscript. H.N. proofread the manuscript and provided constructive comments.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available upon request from the corresponding author.

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SUPPORTING INFORMATION

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