NEUROBEHAVIOURAL MECHANISMS OF RESILIENCE AND VULNERABILITY IN ADDICTIVE DISORDERS

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NEUROBEHAVIOURAL MECHANISMS OF RESILIENCE AND VULNERABILITY IN ADDICTIVE DISORDERS

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EDITORIAL
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Editorial: Neurobehavioural Mechanisms of Resilience and Vulnerability in Addictive Disorders

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Editorial on the Research Topic

Neurobehavioural Mechanisms of Resilience and Vulnerability in Addictive Disorders

Substance use disorders (SUDs) and alcohol use disorder (AUD) entail serious global health, economic, and social problems. The efficacy of available addiction therapies is limited to subsets of responsive patients. A better understanding of the biological bases of transition from recreational drug use to drug abuse occurring in vulnerable drug users could help to tailor treatments and preventive interventions and would thus improve the quality of life of patients and consequently would reduce the negative impact of addiction on society. The aim of this Research Topic is to identify traits of resilience and vulnerability of SUDs and AUD and to unravel their neurobehavioral mechanisms. This Research Topic presents a collection of 16 contributions (three reviews, two min-review, nine original research papers, a brief research report, and one opinion paper) that provide evidence of critical individual-based differences modulating the response to different drugs of abuse (cocaine, alcohol, nicotine, opiates, and inhalants) and report preclinical findings on the efficacy of behavioral and pharmacological manipulations to prevent addiction-like behaviors.

The review by Kuhn et al. "*Understanding Addiction Using Animal Models*" discusses the advantages and disadvantages as well as the face validity of the most common preclinical models of addictive behavior and the attempts to better model individual vulnerability to drug abuse. These models have demonstrated the existence of individual genetic susceptibilities for traits such as sensation-seeking or impulsivity that contribute to vulnerability of addictive behavior.

Sex and age are two major variables that modulate the effects of drugs of abuse and the vulnerability of individuals to drug addiction. The review "*Sex Differences and the Role of Estradiol in Mesolimbic Reward Circuits and Vulnerability to Cocaine and Opiate Addiction*" by Kokane and Perrotti addresses the enhanced vulnerability of women to drug addiction and revises evidence that ovarian hormones are associated with faster progression into addiction. In female rodents, estradiol influences dopamine activity within the mesolimbic reward system that could explain why the rewarding effects of drugs and the response of animals to drug-associated cues are higher in females. The importance of age is highlighted by Guirado et al. in the mini-review "*A Critical Period for Prefrontal Network Configurations Underlying Psychiatric Disorders and Addiction.*" The authors analyze how early life and adolescence are critical periods in which exposure to drugs of abuse might lead to the formation of a particular prefrontal network configuration predisposing an individual to addition in adulthood. This network configuration is characterized by a predominance of inputs from the basolateral amygdala to the medial prefrontal cortex, the brain region responsible for higher cognitive functions and decision-making. A multiscale cerebral neurochemical connectome

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of the rat brain shows that the infra- and prelimbic cortices that comprise prefrontal cortex have very high out-going and in-going projections (Noori et al., 2017). This corresponds with the key role of prefrontal cortex in regulating cognitive functions. Traumatic events in early life and adolescence also contribute to shape a prefrontal network configuration predisposing to SUD. The review of María-Ríos and Morrow "*Mechanisms of Shared Vulnerability to Post-traumatic Stress Disorder and Substance Use Disorders*" focusses on this issue. SUD and post-traumatic stress disorder (PTSD) are often comorbid and could have a common etiology. Clinical and animal data suggest that some individuals have an intrinsic vulnerability that predisposes them to both PTSD and SUD. Dopaminergic, adrenocorticotropic, GABAergic, and glutamatergic neurobehavioral mechanisms, which underlie different emotional learning styles, may be involved in the etiological link between SUD and PTSD.

The opinion paper by Asensio et al. "*What Is the "Trigger" of Addiction?*" comments on the role of frustration in triggering a negative perception of the reality, an element shared in both, stress and addiction disorders. As relapse is frequently caused by intrapersonal determinants related to frustration, a dedicated therapeutic intervention could lead to increased relapse prevention.

The majority of original research papers in this Research Topic focuses on individual differences related with resilience or vulnerability to the effects of drugs of abuse in behavioral paradigms of preclinical research. The paper of Leite-Ferreira et al. "*Individual Differences in Hatching Time Predict Alcohol Response in Zebrafish*" reports that fishes classified as early or late emerging according to their egg emergence time showed different sensitivity to the effect of alcohol on locomotion and freezing. The work by Stafford et al. "*Individual Vulnerability to Stress Is Associated With Increased Demand for Intravenous Heroin Self-administration in Rats*" shows that rats with greater vulnerability to the behavioral and biological effects of an inescapable intermittent swim stress predicted the magnitude of individual demand of self-administered heroin weeks after the stress episode. In the same line, the research report by Calpe-López et al. "*Behavioral Traits Associated With Resilience to the Effects of Repeated Social Defeat on Cocaine-Induced Conditioned Place Preference in Mice*" identifies several behavioral traits that determine the long-term effects of social defeat stress on the rewarding properties of cocaine. Social defeat increased cocaine-conditioned place preference (CPP) but some mice are resilient to this effect of stress. In particular, mice showing less submission during defeat episodes, low novelty-seeking, high social interaction, and fewer depressive-like symptoms did not develop cocaine-induced CPP. Furthermore, the paper of Arenas et al. "*Prepulse Inhibition of the Startle Reflex as a Predictor of Vulnerability to Develop Locomotor Sensitization to Cocaine*" demonstrates that the baseline prepulse inhibition (PPI) levels of mice can predict their sensitivity to the locomotor effects of cocaine. Low-PPI mice presented low sensitivity to the motor effects of an acute dose of cocaine, but a higher increase of activity after repeated administration of the drug (behavioral sensitization) than High-PPI mice. Based on these and previous results - Low PPI presented a more persistent cocaine-induced place preference than High-PPI mice (Arenas et al., 2018) - it is suggested that a PPI deficit may be an endophenotype for cocaine use disorder. Finally, the research of Takahashi et al. "*Pavlovian to Instrumental Transfer Responses Do Not Correlate With Addiction-Like Behavior in Rats*" highlights the distinction between individual differences in the motivational impact of drug-associated cues and individual differences in the risk for addiction. Cocaine self-administration correlates with the level of Pavlovian-to-instrumental transfer (PIT), thus, a stronger PIT predicted improved learning of drug-cue association. However, in rats previously screened with the 0/3 criteria cocaine addiction model (Deroche-Gamonet et al., 2014), there are no differences in the PIT paradigm between addict-like and non-addict-like rats. This data suggests that stronger PIT may predict higher motivational impact of conditioned stimuli on drug self-administration and improved learning of drug-cue association rather than the risk to develop addiction as such.

Two research papers report on biological markers predicting the response to drug of abuse in humans. In the paper entitled "*Methylation Patterns of the HTR2A Associate With Relapse-Related Behaviors in Cocaine-Dependent Participants*" Land et al. show that the degree of methylation at several cytosine residues within the HTR2A promoter is positively correlated with impulsivity and attentional bias toward cocaine-associated cues in cocaine-dependent subjects. These results suggest that DNA methylation of the HTR2A gene may contribute to individual differences in behavioral effects that contribute to relapse. In the paper entitled, "*Default Mode Network Efficiency Is Correlated With Deficits in Inhibition in Adolescents With Inhalant Use Disorder*" Hernández-Álvarez et al. describe deficits in communication among brain regions involved in executive cognitive functions, mainly in the default mode network, of inhalant-consuming adolescents. These deficits may contribute to reduced inhibitory control and sequential planning seen with chronic inhalant abuse.

As commented before, animal models of drug addiction contribute to the development of therapies for SUD. Four papers in this Research Topic focus on the potential pharmacological and behavioral strategies to treat addiction to several drugs of abuse. In the paper "*Dopamine D3 Receptor Antagonism Reverses the Escalation of Oxycodone Self-administration and Decreases Withdrawal-Induced Hyperalgesia and Irritability-Like Behavior in Oxycodone-Dependent Heterogeneous Stock Rats*" de Guglielmo et al. report that the D3 dopamine antagonist VK4-116 is effective to prevent the motivation for oxycodone in a model of extended access and the negative emotional states associated with its withdrawal. The brief report by Garcia-Rivas et al., "*Varenicline Targets the Reinforcing-Enhancing Effect of Nicotine on Its Associated Salient Cue During Nicotine Self-administration in the Rat*" emphasized the individual differences in the capacity of varenicline to reduce the enhancement induced by nicotine in the rewarding properties of salient environmental stimuli associated with this drug. These results suggest that varenicline might be more beneficial in those smokers who are more sensitive to environmental stimuli associated with nicotine consumption. The original research of Khoo et al. "*Comparing ABA, AAB, and ABC Renewal*

of Appetitive Pavlovian Conditioned Responding in Alcohol- and Sucrose-Trained Male Rats" also addresses the importance of drug-associated cues in the treatment of addiction. From a treatment perspective, the data of this study suggests that exposure-based treatments for SUDs might benefit from implementation in real-world, drug-use contexts. Finally, the mini-review by Kuijer et al. "*Retrieval-Extinction and Relapse Prevention: Rewriting Maladaptive Drug Memories*?" focuses on the application memory reconsolidation to disrupt maladaptive drug-memories that trigger relapse. After retrieval, maladaptive drug memories are destabilized (by an amnestic agent or by an extinction training) and reconsolidated in an updated form. It is suggested that retrieval-extinction protocols may have promising applications to help preventing relapse to drug-seeking and individual-based differences may influence the therapeutic outcomes.

We as Research Topic Editors are grateful for excellent contributions and are convinced that the here presented collection of papers summarize critical factors that contribute to a better understanding of the neurobehavioral mechanisms underlying resilience and vulnerability to addictive disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Pavlovian to Instrumental Transfer Responses Do Not Correlate With Addiction-Like Behavior in Rats

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Pavlovian learning plays a prominent role in the etiology of addiction. The influence of Pavlovian conditioning on the expression of an instrumental response can be studied using the Pavlovian-to-instrumental transfer (PIT) paradigm. This paradigm consists of independent Pavlovian conditioning and instrumental training prior to the combination of both during the test. During this test, the reward is not available, and an increase in the instrumental responding during conditioned stimuli presentation is a measure of PIT. Recent studies have reported a higher PIT in alcohol and nicotine dependent patients, suggesting that enhanced PIT might be a marker for dependence vulnerability. However, these studies did not use standard PIT procedures, and a clear correlation between an enhanced PIT and drug-related and addictive behaviors has so far not been demonstrated. For a systematic evaluation rats were trained in a cocaine addiction model. Addicted-like and non-addicted-like rats were subsequently assessed in the PIT paradigm. In a further experiment, rats were first tested in the PIT paradigm and thereafter subjected to cocaine self-administration (CSA) training. Our results revealed that addicted-like rats did not differ from non-addicted-like in their performance in the PIT test. However, CSA behavior showed a positive correlation with PIT. This data suggests that stronger PIT may predict higher motivational impact of conditioned stimuli on drug self-administration and improved learning of drug-cue association rather than the risk to develop addiction as such.

Keywords: cocaine self-administration, 0/3-criteria rat model of cocaine addiction, outcome-specific PIT, general PIT, relapse

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INTRODUCTION

Addiction theories postulate that Pavlovian learning plays a key role in the development of drug addiction and maintenance of drug use. Pavlovian learning involves transfer of the motivational value of the primary reward to the conditioned stimulus (CS) associated with drug availability during the course of drug use (Berridge and Robinson, 2003; Sanchis-Segura and Spanagel, 2006). Such a CS can impact on ongoing instrumental behavior, even if the instrumental behavior is acquired independently of Pavlovian conditioning. This process is called

Pavlovian-to-Instrumental transfer (PIT; Colwill and Rescorla, 1988; Holmes et al., 2010; Cartoni et al., 2013, 2016). In PIT, positively valued Pavlovian cues promote instrumental responses and approach (e.g., enhance the frequency of pressing a button in order to obtain a drug; Cartoni et al., 2016). Pavlovian conditioned cues can thus bias instrumental behavior towards drug seeking and intake in both drug abusers and animals trained to self-administer drugs (Everitt et al., 2001; Glasner et al., 2005; Weiss, 2005; Hogarth et al., 2007; LeBlanc et al., 2012). Although it is well established that drug conditioned cues play a critical role in drug addiction the role of PIT on drug-related and addictive behaviors is less clear (Hogarth et al., 2018).

In the PIT paradigm, subjects are first trained in Pavlovian stimulus-outcome sessions, separately from the instrumental response-outcome sessions, to prevent development of the association between the CS and instrumental action. During the PIT test, presentation of the CS increases the instrumental response rate, demonstrating the "energizing" properties of the CS on the expression of the instrumental behavior. In outcome-specific PIT, presenting a particular reward-predicting cue can selectively elevate instrumental responses that are associated with the same unique reward, while in general PIT, a reward predicting cue can generally modify instrumental responses towards any outcome (Holmes et al., 2010; Cartoni et al., 2016).

PIT have been widely studied in animals, also in the context of drugs of abuse, demonstrating that drug-experienced (cocaine and alcohol) animals exhibit an enhanced PIT (Corbit and Janak, 2007; Holmes et al., 2010; Cartoni et al., 2016; Lamb et al., 2016). However, it has also been suggested that specific PIT effects are abolished following drug exposure (Shiflett, 2012; Hogarth et al., 2013). Studies in young adult smokers (Hogarth and Chase, 2011, 2012; Hogarth, 2012; Hogarth et al., 2015), young adult drinkers (Martinovic et al., 2014; Hardy et al., 2017) and treatment-engaged addicts (Hogarth et al., 2018) did not demonstrate a link between specific PIT effects and dependence or dependence severity. However, Garbusow et al. (2014) and Schad et al. (2019) showed that in alcohol-dependent patients both alcohol-related as well as non-alcohol-related PIT occurred more frequently than in healthy controls. Therefore, it is unclear whether general PIT might be different in addicts, and if this could be used as a marker of addictive behavior.

Here, we used a general PIT model for natural rewards and studied two research questions in a rat model of cocaine self-administration (CSA) and cocaine addiction: (i) given that cocaine-experienced rats may exhibit an enhanced PIT effect (Lamb et al., 2016) we asked if the performance in CSA correlates with the strength of PIT; and (ii) provided that general PIT seems to occur more frequently in addicted patients we asked if an enhanced PIT occurs in cocaine-addicted compared to non-addicted rats.

To evaluate if behavior during the PIT test correlates with addiction-like features, the DSM-based 0/3 criteria animal model of cocaine addiction was used. This animal model has a good face and construct validity (Deroche-Gamonet et al., 2004; Cannella et al., 2013, 2018; Spanagel, 2017). The model is based on long-term CSA that produces a ratio of cocaine addicted-like rats, equivalent to the addicted population of human cocaine users (Anthony et al., 1994). Following the establishment of an appropriate PIT paradigm (adapted from Holland, 2004) two experiments were performed: (i) rats were trained in the cocaine addiction model, and subsequently addicted-like and non-addicted-like rats were assessed in the PIT paradigm (ii) rats were first tested in the PIT paradigm and thereafter subjected to CSA training.

MATERIALS AND METHODS

Animals

Thirty-two-month-old male Wistar rats from Harlan Laboratories (Derby, United Kingdom) were used for the establishment of the PIT protocol. Other 68 2-month-old male Sprague-Dawley rats from Charles River Laboratories (Sulzfeld, Germany) was used for CSA and cocaine addiction model and PIT testing. All animals were acclimatized in the laboratory facilities for a week before catheter implantation or the initiation of behavioral experiments. Animals were housed individually in standard rat cages (Type-III; Ehret, Emmendingen, Germany) throughout the study, and maintained under reverse light/dark cycle (lights on at 5:00, lights off at 17:00). Temperature was controlled (22 ± 2°C), drinking water was provided *ad libitum* unless indicated otherwise. Twenty-grams of standard laboratory rat food (Sniff, Soest, Germany) was given daily for the rat group used in the cocaine addiction model, and rats used for the establishment of the PIT protocol were fed *ad libitum*. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe) and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 22 September 2010 (2010/63/EU).

Drugs

Cocaine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in sterile saline.

Experimental Design

Two groups of rats were used to establish a PIT paradigm: a group that received both Pavlovian conditioning and instrumental training (PIT group, $n = 24$), and a control group ($n = 8$) that was prevented from stimulus-reward pairings during Pavlovian conditioning but had identical instrumental training. The control group was included to measure any unconditioned effects of stimulus presentation on responding.

For Experiment 1, another group of rats was trained in the cocaine addiction model ($n = 48$). Cocaine addicted-like (3 criteria group, $n = 7$) and non-addicted-like (0 criteria group, $n = 8$) were subsequently assessed in the PIT paradigm.

To assess whether training of rats in the addiction model had no carryover effect on PIT performance, Experiment 2 employed a new group of naïve rats ($n = 20$), which were subjected first to the PIT paradigm and thereafter to CSA training.

PIT Paradigm

The training protocol used in the present study was adapted from Holland (2004) and followed several steps: habituation, Pavlovian conditioning, instrumental training and Pavlovian reconditioning. PIT test was done next after the last training session.

Operant Self-administration Apparatus for Pavlovian Instrumental Transfer

PIT paradigm was carried out in operant chambers (MED Associates Inc., St. Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. The chambers were equipped with two levers placed at opposite walls. Responses at the active lever activated a syringe pump that delivered 60 μl of 10% (w/v) sucrose solution into a liquid receptacle, which was placed on the left side of the lever. Responses at the inactive lever were recorded but had no programmed consequences. A cue-light stimulus was attached above both response levers of the operant chamber. Delivery of sucrose, presentation of CS and data recording were controlled by a computer with MED-PC software (MED Associates).

Magazine Training

Before the magazine training session, animals were water deprived for approximately 22 h to increase exploratory and consummatory behavior. Magazine training lasted for 1 h, and during this time sucrose was delivered at varying and unpredictable time intervals of 2 min on average (variable-interval, VI) but neither levers nor cue-light was presented.

Pavlovian Conditioning

Pavlovian conditioning was done in 60 min daily sessions for five consecutive days and levers were withdrawn throughout these sessions. Animals were not water deprived during this phase. During the conditioning session, a constant-cue-light, situated above the active lever, was presented for 2 min at random time intervals (inter-trial interval was 2–3 min) and served as the CS. Presentation of the CS was accompanied with an immediate delivery of 4–5 sucrose reinforcers [one reward corresponded to 60 μl of 10% (w/v) sucrose solution delivered into a liquid receptacle]. A total of nine pairings of CS and reward were given per session. The control group received the corresponding amount of sucrose (∼2.5 ml) in the receptacle at the beginning of the session to avoid association between the CS and reward.

Instrumental Training

Instrumental training started on the next day following Pavlovian conditioning. Prior to the instrumental training, all animals were water deprived for 22 h to promote acquisition of lever responding. In this phase, cue-lights were switched-off and sucrose reinforcers were granted by pressing the active lever. Each daily session lasted 30 min and VI was increased progressively across the training days. In the first 3 days, animals were trained on a FR1 schedule of reinforcement for acquisition of instrumental response. On the subsequent 10 days, the average VI changed as follows: a training day on VI-10 (5 s, 10 s, 15 s), followed by a day on VI-20 (10 s, 20 s, 30 s) and finalizing with eight training days on VI-30 (10 s, 20 s, 30 s, 40 s, 50 s). Both active and inactive levers were available throughout the instrumental training; however, responses at the inactive lever did not result in reward delivery.

Pavlovian Reconditioning

All animals were subjected to a single conditioning session of 30 min the day prior to the PIT test. The conditions were the same as during the Pavlovian conditioning sessions described above.

Pavlovian Instrumental Transfer Test

Both active and inactive levers were available throughout the PIT test. CS was presented randomly four times for 2 min in a single 36-min session, and the intertrial interval was 4–10 min. A period of 2-min duration just before CS presentation was considered as the pre-CS period. Responding on both active and inactive levers was recorded throughout the PIT test and the number of active lever-presses prior (pre-CS) and during CS presentation was used to demonstrate the transfer effect. Neither active nor inactive lever-responding was reinforced by sucrose delivery.

Experiment 1: PIT in Cocaine Addicted-Like and Non-addicted-Like Rats

Surgery

A polyurethane catheter (internal diameter: 0.58 mm, external diameter: 0.94 mm) was implanted in the jugular vein under isoflurane anesthesia (induction: 5%; maintenance: ∼2.5%). The proximal end of the catheter was placed in the right atrium of the animal's heart, while the distal end was fixed in the mid scapular region. Rats were allowed to recover for 4–7 days after the surgery. Catheters were flushed daily with unfractionated heparin (100 IU/ml) solution containing enrofloxacin (Baytril®, 1 mg/ml).

Operant Cocaine Self-administration Apparatus

CSA trainings were carried out in nose-poke operant chambers (40 cm long × 30 cm width × 52 cm high; Imetronic, France) enclosed in ventilated sound-attenuating cubicles. Two nose-poke holes at opposite walls, 5 cm above the grid floor recorded the responses by the interruption of a photo-beam projected across the hole. Poking in the active hole resulted in the delivery of an infusion of 0.8 mg/kg of cocaine, whereas poking in the inactive hole had no programmed consequences. The chambers were also equipped with a white cue-light placed 9.5 cm above the grid floor, a green cue-light next to it, a blue cue-light located on the opposite wall 33 cm above the grid floor and a house light that illuminated the entire chamber. Data was collected using POLY software.

Cocaine Self-administration Training

CSA protocol was performed as initially described by Deroche-Gamonet et al. (2004) and in our previous work (Cannella et al., 2013, 2018; Vengeliene et al., 2018). Briefly, each CSA session consisted of alternated periods of drug availability (drug-ON, 40 min) and non-availability (NO-drug, 15 min). During drug-ON periods, a blue cue light signaled the availability of cocaine at FR5 schedule of reinforcement. If the schedule was completed within 40 s time, an infusion of cocaine

(0.8 mg/kg/infusion) was delivered paired with presentation of a white cue-light. During NO-drug periods, blue and white cue-lights were withdrawn and a house light indicated non-availability of cocaine. Nose-pokes had no scheduled consequences but recorded during NO-drug periods. Each CSA session lasted 2.5 h or session was ceased after 35 cocaine infusions a day.

Following 45 CSA training sessions, three addiction criteria were tested: (1) motivation to self-administer cocaine; (2) persistence of cocaine-seeking; and (3) resistance to punishment.

Motivation to Self-administer the Drug

Breakpoint test was used to assess animals' motivation to take cocaine. Test was based on the progressive-ratio schedule of reinforcement. Drug availability was signaled by the blue cue light and the ratio of responses was increased after each infusion according to the following progression: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1,025, 1,125, 1,235, 1,345, 1,465, and 1,585. The last completed ratio performed by the rat was used as a measurement of animals' motivation. The test elapsed either after 6 h or when the ratio was not completed within 1 h time.

Persistence of Cocaine-Seeking

Persistence of cocaine-seeking was measured as the number of active nose-pokes during the NO-drug periods averaged in the last four CSA training sessions prior to the BP test.

Resistance to Punishment

Test consisted of cocaine infusion paired with mild foot-shocks (0.2 mA, 1 s). Additionally to the blue and white cue lights, a green cue-light was lit immediately after the first active nose-poke to indicate the presence of shock. FR5 was used as the schedule of reinforcement; however, a foot-shock was delivered at the completion of the fourth as well as the fifth active nose-poke, which was then paired with a cocaine infusion (0.8 mg/kg/infusion). Percentage of cocaine infusions earned during the 40 min test in relation to the baseline infusions during the first drug-ON period in the last four CSA training sessions prior to the test was used as the measurement of resistance to punishment.

Addiction Criteria Classification

For each addiction-like behavioral criterion, a score of 0 or 1 was given to the animals depending on their performance. According to our previous studies (Cannella et al., 2018; Vengeliene et al., 2018) rats responding above 60th percentile of the population distribution received a score of 1, whereas animals responding below this threshold received a score of 0. The sum of scores from the three behavioral tests resulted in animals displaying 0–3 positive criteria. Rats positive for all three criteria were considered as addicted-like animals (3 criteria) rats, whereas animals negative for all criteria were considered as non-addicted-like (0 criteria) rats.

PIT Testing

Following characterization of addiction-like behaviors, 0 criteria and 3 criteria rats were subjected to further baseline CSA training, completing 55 CSA sessions. Thereafter, animals were left undisturbed for 1–2 weeks, and subsequently trained in the PIT paradigm as described above.

Experiment 2: Cocaine Self-administration in PIT Tested Rats

After testing an additional group of naïve rats in the PIT paradigm, all rats underwent catheter implantation surgery as described above. Following the recovery, 20 rats were subjected to 33 CSA training sessions under the same conditions as in the Experiment 1, which was enough to establish stable responding.

Data Analysis

Animal performance during the PIT test was analyzed using either a three-way analysis of variance (ANOVA) with repeated measures [factors were: condition (pre-CS vs. CS), group (either control vs. PIT or 0 criteria vs. 3 criteria) and lever (active vs. inactive)] or two-way ANOVA with repeated measures in case of a rat group used for CSA (factors were: condition and lever). For analysis of responses during the instrumental training in the PIT paradigm and CSA in 0 criteria vs. 3 criteria rats, a three-way ANOVA with repeated measures was used, and the factors were lever (active vs. inactive), group (0 criteria vs. 3 criteria) and training sessions. Reinforcers earned during instrumental training in the PIT paradigm and CSA was analyzed by two-way ANOVA with repeated measures [factors: group (0 criteria vs. 3 criteria) and training sessions]. Whenever significant differences were found, Newman-Keuls *post hoc* test was applied. Student *t*-test was used for the analysis of 0 criteria and 3 criteria rats in each addiction-like behavioral test. Pearson Correlation was used to assess linear relationship between performances in the CSA, addiction criteria and the PIT test. Performance during the last CSA sessions was calculated as [(number of active nose-pokes/number of total nose pokes)*100], and it demonstrated how well the animal learned to discriminate between reinforced (active) and non-reinforced (inactive) responding, and Performance in the PIT test was calculated as [number of active lever presses during CS presentations/(total number of active lever presses during both CS and pre-CS periods)*100], and it demonstrated whether the animal discriminates between CS and pre-CS periods. The chosen level of significance was $p < 0.05$.

RESULTS

PIT Paradigm

Our results show that animals used for the establishment of the PIT paradigm increased lever responding during the CS presentations of the PIT test (factor condition: $F_{(1,120)} = 16.2$, $p < 0.001$) compared to the pre-CS condition. Three-way ANOVA revealed that lever-responding was different during the CS presentation between animals which received Pavlovian conditioning sessions (PIT group) and animals that were prevented from learning stimulus-reward association during

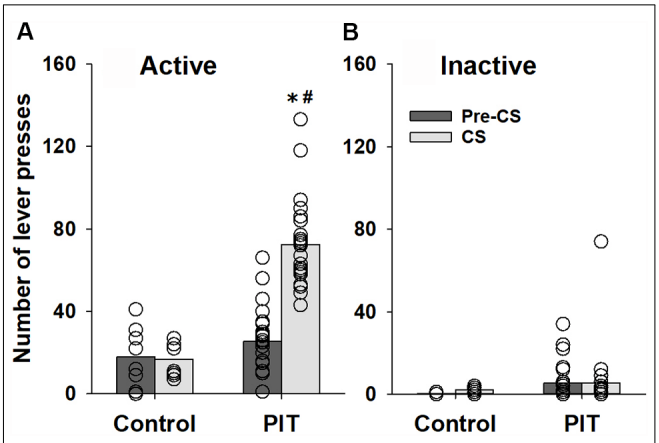

FIGURE 1 | Establishing a Pavlovian instrumental transfer (PIT) test. Performance during the PIT test by control rats (n = 8) that were prevented from learning stimulus-reward association during Pavlovian conditioning sessions and rats that were subjected to five Pavlovian conditioning sessions prior to instrumental training (PIT, n = 24). The total number of **(A)** active and **(B)** inactive lever presses during four random 2-min conditioned stimulus (CS) presentations and during the 2-min periods just before CS presentations (pre-CS) are shown. Data are presented as means (bars) and individual values (open circles, please note that identical values are overlapping). *Indicates significant difference from pre-CS and #indicates significant difference from the control group, $p < 0.05$.

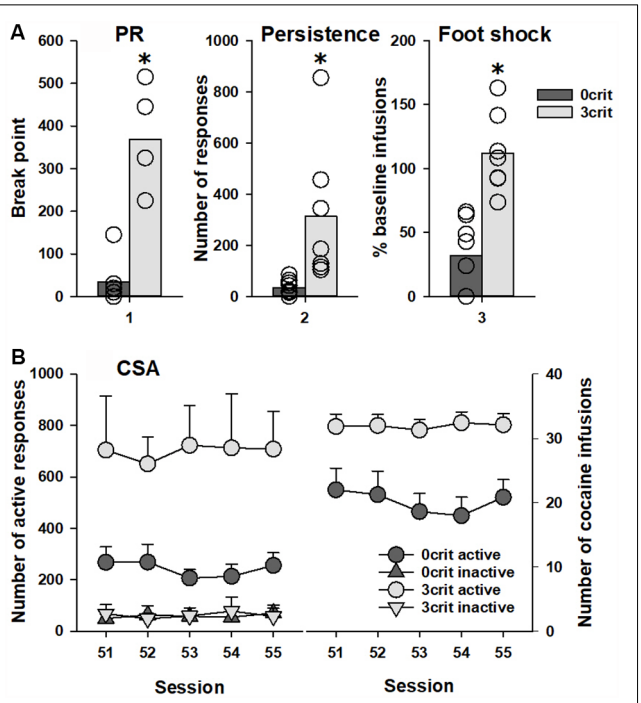

FIGURE 2 | Behavioral characterization of 0 criteria and 3 criteria rats. Rats characterized by addicted-like phenotype (3 crit; n = 7) showed higher response than non-addicted-like rats (0 crit; n = 8) in each criterion for addiction-like behavior **(A)**: (1) motivation to self-administer cocaine measured in the breakpoint test as the last completed ratio in the progressive ratio schedule (PR); (2) persistence of drug-seeking measured as the number of active nose-pokes during the no-drug periods; and (3) resistance to punishment measured as the number of cocaine infusions earned in association with a foot-shock delivery in relation to the last four cocaine self-administration (CSA) training sessions prior to the test (%). Panel **(B)** shows the last five CSA sessions (51–55) as (left) number of responses in both the active and inactive hole and (right) the number of cocaine reinforcers earned during these sessions. Data are presented as **(A)** means (bars) and individual values (open circles, please note that identical values are overlapping) and **(B)** means ± SEM. Groups were different in number of both active responses and cocaine reinforcers, *Indicates significant difference from the 0 criteria group, $p < 0.05$.

Pavlovian conditioning sessions (control group; factor group: $F_{(1,120)} = 37.6$, $p < 0.001$, group × condition interaction: $F_{(1,120)} = 15.5$, $p < 0.001$ and group × condition × lever interaction: $F_{(1,120)} = 18.0$, $p < 0.001$). The subsequent *post hoc* analysis demonstrated that these differences were caused by changes in responding on the active lever (**Figure 1A**). CS presentation had no significant effect on inactive lever-responding (**Figure 1B**).

Characterization of Addiction-Like Behavior in the 0/3 Criteria Model

Out of 48 rats that initiated the CSA training, 36 rats successfully completed it. This reduction in animal numbers occurred due to either loss of catheter potency or death of animals during training. A total of seven rats were characterized as positive for all addiction-like criteria (3 criteria), and classified as addicted-like rats, and 11 rats were negative for all criteria (0 criteria), and classified as non-addicted-like rats, eight of which were tested in the PIT paradigm. Student t-test showed significant difference between 0 criteria and 3 criteria rats in each of the addiction-like behaviors/criteria: motivation to take cocaine ($t_{(13)} = 7.0$, $p < 0.001$), persistence of drug-seeking ($t_{(13)} = 2.8$, $p < 0.05$) and resistance to punishment ($t_{(13)} = 5.3$, $p < 0.001$; **Figure 2A**). Analysis of data during the last CSA sessions demonstrated that number of active nose-pokes as well as number of cocaine infusions was significantly lower in 0 criteria rats compared to 3 criteria animals (factor group: $F_{(1,26)} = 9.0$, $p < 0.01$ and $F_{(1,13)} = 11.8$, $p < 0.01$ for the number of pokes and the number of cocaine reinforcers, respectively; **Figure 2B**). In conclusion, 0 criteria and 3 criteria rats shared identical experimental conditions but differed pronouncedly in their addictive-like behavior and could therefore be ideally used for further PIT testing.

No Difference in PIT Testing in Cocaine Addicted-Like and Non-addicted-Like Rats

Analysis of the data obtained during the PIT training procedure demonstrated that behavior of 0 criteria and 3 criteria rats during the instrumental training did not differ ($p = 0.21$ and $p = 0.63$ for the number of responses and reinforcers earned, respectively), indicating that 0 criteria and 3 criteria rats received equivalent number of sucrose reinforcers during the instrumental training (data not shown). During the transfer test, the number of active lever presses increased during CS presentation in both (0 criteria and 3 criteria groups; factor condition: $F_{(1,52)} = 23.0$, $p < 0.001$). However, no difference was found between two groups (factor group:

p = 0.74, group × condition interaction: p = 0.96 and group × condition × lever interaction: p = 0.91; **Figure 3A**). Responding on the inactive lever was not significantly affected by CS presentation (**Figure 3B**).

Given that cocaine-experienced rats exhibit an enhanced PIT (Lamb et al., 2016) we asked if the performance in CSA in the 0/3 criteria model correlates with the strength of PIT. Our analysis shows a positive correlation between rat performance during the last 5 CSA sessions (i.e., ability of a rat to discriminate between the active and inactive nose-hole, %) and the transfer in the Pavlovian to instrumental response (r = 0.60, $p < 0.05$; **Figure 4A**).

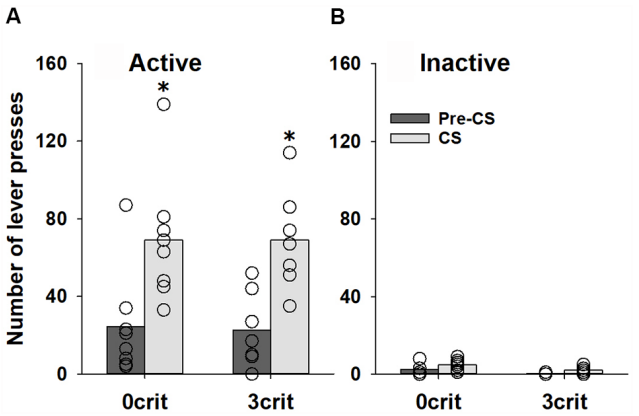

FIGURE 3 | No difference in PIT between cocaine addicted and non-addicted rats. Performance during the PIT test in non-addicted-like (0 crit, n = 8) and addicted-like (3 crit, n = 7) rats subjected to five Pavlovian conditioning sessions prior to instrumental training. The total number of **(A)** active and **(B)** inactive lever presses during four random 2-min CS presentations and during the 2-min periods just before CS presentations (pre-CS) are shown. Data are presented as means (bars) and individual values (open circles, please note that identical values are overlapping). *Indicates significant difference from pre-CS, $p < 0.05$.

PIT Correlates With the Performance in Cocaine Self-administration

To confirm the robustness of the correlation analysis and to ensure that there were no carryover effects from the prior experience with cocaine operant conditioning on PIT performance, a group of rats (n = 20) was subjected to a reversed experimental order. The rats were initially trained in the PIT paradigm and thereafter they were subjected to the cocaine-conditioned self-administration procedure. Similar to the previous experiment, there was a positive correlation between performance in the Pavlovian to instrumental response and the last five CSA training sessions (r = 0.61, $p < 0.01$; **Figure 4B**), demonstrating that the behavior during PIT may predict cocaine-taking behavior.

DISCUSSION

In the present study, rats subjected to Pavlovian stimulus-outcome conditioning increased instrumental lever responding upon CS presentation, which indicates the occurrence of a transfer effect. On the contrary, rats that did not receive CS pairing with the reward delivery during the Pavlovian conditioning phase did not show a transfer effect during the test. Addicted-like rats did not differ from non-addicted-like in their performance in the PIT test. However, there was a

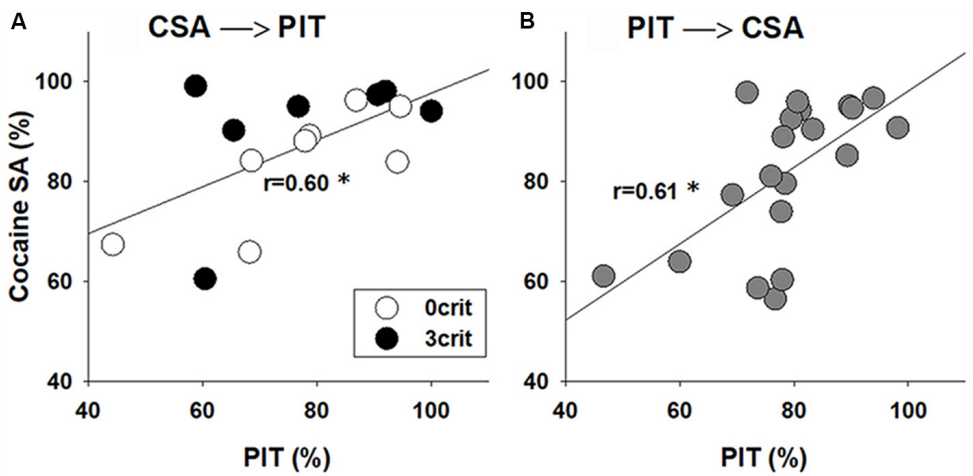

FIGURE 4 | PIT correlates with the performance in CSA. Correlation analysis between performance during the last CSA sessions and the PIT test in rats that were first subjected to **(A)** 55 sessions of CSA and addiction-like behavioral testing that yielded addicted-like (3 crit) and non-addicted-like (0 crit) rats and then to PIT testing or **(B)** first were tested in the PIT paradigm and then subjected to CSA training. Performance during CSA demonstrates (Y-axis) how well the animal discriminate between reinforced (active) and non-reinforced (inactive) responding, and it was calculated as [(number of active nose-pokes/number of total nose pokes)*100]. Performance in the PIT test (X-axis) was calculated as [number of active lever presses during CS presentations/(total number of active lever presses during both CS and pre-CS periods)*100], and it demonstrates whether the animal discriminates between CS and pre-CS periods. *Indicates a significant correlation between performance in PIT and CSA, $p < 0.05$.

positive correlation between the performance in CSA behavior and PIT. Thus, animals that showed a high accuracy and preference for the cocaine reinforced responding also showed a higher PIT than animals that performed less well in the self-administration paradigm. The latter finding was confirmed in a reversed experimental approach where PIT was first assessed in cocaine-naïve rats; then rats were trained in the CSA paradigm, and after stable responding, their self-administration performance was measured. Again, a positive correlation between the strength of PIT and self-administration behavior was found. This is in line with previous PIT experiments in rats showing that drug-experienced animals exhibit an enhanced PIT (Holmes et al., 2010; Cartoni et al., 2016; Lamb et al., 2016) and extend this conclusion by the finding that an enhanced PIT in drug-naïve rats can predict higher and more accurate rates of drug-self-administration.

The PIT paradigm has been used to understand the motivational influence of cues on instrumental performance, and it was demonstrated that alcohol addicted patients performed better in the PIT test than healthy controls (Garbusow et al., 2014; Schad et al., 2019). These two human studies suggest that a more pronounced PIT could be a marker for addictive behavior. In our study, however, contrary to these human findings, the transfer of the Pavlovian to instrumental response was not different between addicted-like and non-addicted-like rats. It has been previously demonstrated that prior cocaine experience may potentiate PIT (Saddoris et al., 2011), suggesting that enhanced performance during the PIT test in addicted patients may be caused by more frequent drug use than that of healthy controls. We could not confirm this finding since in this study cocaine addicted-rats have higher number of cocaine-associated responses and earn more cocaine infusions during CSA training.

Higher cocaine intake in addicted-like rats may have caused a stronger impairment of executive control, which could have led to more pronounced compulsive behavior and impacted instrumental learning and PIT (Jentsch and Taylor, 1999; Jentsch et al., 2002). However, analysis of the acquisition of instrumental learning in the PIT paradigm showed that addicted- and non-addicted-like animal groups were similar in their performance, ruling out the possibility of different learning capabilities in these rats. Nevertheless, to confirm that prior drug use did not affect animal performance during PIT, a group of drug naïve rats were tested in the PIT paradigm and then subjected to CSA. Similarly to the first experiment, a clear correlation was found between PIT and CSA. These results confirmed that PIT may be used to predict animal performance during drug cue-conditioned self-administration in rats.

Our experiments suggest that PIT responses do not correlate with addictive behavior but correlate with the extent of drug self-administration. Thus, the extent to which rats self-administer drugs (in the present study cocaine) and learn cue-drug associations is positively related to how strongly their instrumental behavior is under the influence of conditioned stimuli. The effect that the Pavlovian cue exerts on instrumental responding might be attributable to a general enhancement of motivation and/or to triggering an expectation of the instrumental outcome. In this respect, it is important to note that PIT effects correlate with the expectation that stimuli play a discriminative stimulus role in signaling the effectiveness of the instrumental response (Hogarth et al., 2014; Seabrooke et al., 2016; Hardy et al., 2017). Thus, it is possible that the correlation seen in our study between PIT responses and drug self-administration behavior reflect individual differences in awareness/attention to the discriminative function of stimuli. In conclusion, our data confirm that PIT can be used to predict individual performance in a drug self-administration paradigm but PIT alone is insufficient to predict or to be used as a marker of severity of substance use disorder. Furthermore, since two recent human studies indicate that PIT effects can predict relapse behavior in addicted patients (Garbusow et al., 2016; Sommer et al., 2018) further studies are needed to measure PIT effects and subsequent relapse behavior in the 0/3 crit model.

ETHICS STATEMENT

All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe) and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 22 September 2010 (2010/63/EU).

AUTHOR CONTRIBUTIONS

TT contributed to designing experiments and analyzing the data, performed CSA and PIT experiments, and wrote the first draft of the manuscript. VV contributed to designing experiments, analyzing the data and writing the manuscript. TE established the PIT protocol. SR performed the experiment 2. RS contributed to designing experiments and writing the manuscript.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Individual Vulnerability to Stress Is Associated With Increased Demand for Intravenous Heroin Self-administration in Rats

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Opioid use is a widespread epidemic, and traumatic stress exposure is a critical risk factor in opioid use and relapse. There is a significant gap in our understanding of how stress contributes to heroin use, and there are limited studies investigating individual differences underlying stress reactivity and subsequent stress-induced heroin self-administration. We hypothesized that greater individual vulnerability to stress would predict higher demand for heroin self-administration in a within-subjects rodent model of stress and heroin use comorbidity. Male rats were exposed to inescapable intermittent swim stress (ISS) and individual biological (corticosterone) or behavioral [open field, social exploration, and forced swim tests (FSTs)] measures were assessed before and after the stress episode. Individual demand for self-administered heroin (0.05 mg/kg/infusion; 12-h sessions) was assessed using a behavioral economics approach followed by extinction and reinstatement tests triggered by stress re-exposure, non-contingent cue presentations, and yohimbine (0, 1.0, or 2.5 mg/kg). We found that behavioral, biological, and a combination of behavioral and biological markers sampled prior to and after the stress episode that occurred weeks before the access to heroin self-administration predicted the magnitude of individual demand for heroin. Non-contingent presentation of cues, that were previously associated with heroin, reinstated heroin seeking in extinction. For the first time, we show that individual biological response to an ecologically relevant stressor in combination with associated behavioral markers can be used to predict subsequent economic demand for heroin.

Keywords: heroin, stress, swim-stress, post-traumatic stress disorder, heroin demand, economic demand, stress reinstatement, cue reinstatement

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INTRODUCTION

People subjected to traumatic experiences are especially susceptible to the abuse of depressants, including opioids, but there is evidence of individual vulnerability or resilience to such stresses. Traumatic psychological stress, often accompanied by physical trauma, can include harassment, sexual abuse, bullying, domestic violence, life-threatening events, and other individually subjective negative experiences. It is known that stress is an important factor in the development of addiction disorders, and it is generally understood what critical neurobiological substrates underlie this

mechanism (Sofuoglu et al., 2014). What is not known is how individual vulnerability or resilience to stress relates to susceptibility to use and abuse of opioids. Heroin use in the US is on the rise and without effective treatment options to combat this change (Jiang et al., 2017). Understanding individual underlying factors linking stress vulnerability to opioid use may lead to the development of more efficacious individualized treatment approaches than what is currently available.

Substance use and addiction are strongly associated with risk factors like stress and mental trauma (Perkonigg et al., 2000; Breslau et al., 2003; Zywiak et al., 2003; Dewart et al., 2006; Mills et al., 2006). The evidence for stress and heroin use comorbidity is less extensive as for other drugs of abuse, but the existing findings from epidemiological research, and few preclinical studies, strongly link stress with opioid use. For example, in one epidemiological study (Cottler et al., 1992) 43% of polydrug or cocaine/opiate users reported experiencing traumatic stress in the past and met DSM-III criteria for posttraumatic stress disorder (PTSD; odds ratio = 5.06; nearly twice the rate for other drugs). In another epidemiological study, 88% of people that abused opioids had been exposed to traumatic stress and the highest prevalence of PTSD was among individuals with an opioid use disorder (Mills et al., 2006). Importantly, these studies show that not every individual with such adverse experiences transitions to abuse opioids.

There is limited evidence from preclinical studies implicating stress in increased opioid self-administration. For example, rodent studies show that daily immobilization stress increased oral consumption of both heroin and fentanyl (Shaham, 1993), while daily footshock stress increased lever-press responding for liquid fentanyl in an operant self-administration paradigm (Shaham et al., 1992). Subsequent work by Shaham and Stewart (1994) demonstrated that intermittent footshock stress also increased intravenous self-administration of heroin. The study by Shaham and Stewart (1994) provided direct evidence of stress-induced enhancement of heroin reinforcement obtained using a preclinical model of intravenous self-administration. Additional preclinical studies also found that physical and pharmacological (yohimbine) stress can also reinstate heroin-seeking behavior in extinction (Shaham and Stewart, 1995; Shaham et al., 1996; Banna et al., 2010). Furthermore, there are a number of studies that have investigated the role of individual differences in preclinical animal models of addiction (Deroche-Gamonet et al., 2004; McNamara et al., 2010; Belin and Deroche-Gamonet, 2012; Dilleen et al., 2012; Koffarnus and Woods, 2013; for review see Belin et al., 2016). For example, Deroche-Gamonet et al. (2004) showed that only a small percentage of rats show addiction-like behaviors when assessed using extended cocaine self-administration paradigm, while Dilleen et al. (2012) showed that only high-anxious rats exhibited a higher pattern of escalation of cocaine but not heroin self-administration in comparison to less anxious rats. Although there is a strong body of preclinical evidence demonstrating that stress can contribute to heroin taking or seeking behaviors and that these effects may vary on the individual level, there is a significant gap in our understanding of how individual sensitivity to stress interacts with these behaviors.

Many individuals are exposed to traumatic stress in their lifetime; however, only a small proportion eventually transition to use and abuse opioids (Cottler et al., 1992; Breslau et al., 2003; Dewart et al., 2006; Mills et al., 2006). Based on this premise, we conceptualized a hypothetical human model where vulnerability to stress mediates stress-induced progression to heroin use and abuse. In that theoretical model, many individuals are exposed to traumatic stress, but only those that are vulnerable to traumatic stress have a higher risk of using or abusing heroin after a traumatic stress experience. In humans, vulnerability to traumatic stress is often defined as exhibiting long-lasting symptoms after the stress episode and may include hyperarousal, hypervigilance, social withdrawal, and cognitive alterations, to name a few. We further hypothesized that we could simulate some of these individual effects in a preclinical animal model of stress and heroin use comorbidity. In that preclinical rodent model of stress and heroin use comorbidity, all rats are first exposed to a stress episode and then, after a period of time that allows for the development of long-term stress effects, assessed for heroin consumption using behavioral economics approach. Importantly, rather than investigating the effects of stress on rates of heroin self-administration, the approach that often requires additional non-stress controls, this model uses a within-subjects design and focuses on understanding how individual variability in reactivity to stress relates to heroin taking. Thus, in this preclinical model of stress and heroin use comorbidity, all rats are exposed to a stressor, and all subsequent individual effects are assessed as they unfold over time using mixed-effects linear modeling that is especially suitable for these types of designs (Glass and Mackey, 1988; O'Connor, 1990).

In the present study, we used intermittent swim stress (ISS) to induce long-lasting effects of stress. The ISS protocol is derived from both the learned helplessness and forced swim test (FST) models (Brown et al., 2001; Drugan et al., 2013). ISS is an effective stressor that induces long-lasting behavioral symptoms analogous to cognitive deficits (Christianson and Drugan, 2005; Levay et al., 2006; Drugan et al., 2009, 2014), behavioral despair (Christianson and Drugan, 2005; Drugan et al., 2014), social anxiety (Stafford et al., 2015), altered drug reactivity (Brown et al., 2001; Drugan et al., 2007), and is sensitive to pharmacological treatments like selective norepinephrine reuptake inhibitors (Drugan et al., 2010; Warner and Drugan, 2012). It is important to note that cold water is a natural stressor for a rat that they can encounter in the environment and thus it is a stressor with high ethological relevance inducing relevant behavioral and neurobiological responses (for review see Drugan et al., 2016). Individual reactivity to ISS-induced stress episode can be assessed by measuring biological or behavioral stress that can be then correlated with subsequent economic demand for heroin. We hypothesized that rats that are more sensitive to the effects of stress will have higher demand for heroin after the stress exposure. We here show that: (a) rats vary in their biological response to stress and the behavioral responses sampled before and after the stress episode; (b) that variation can be conceptualized as a continuous phenotype ranging from vulnerability to resilience; and (c) vulnerability

to stress, in this model, predicts higher economic demand for self-administered heroin.

MATERIALS

Animals

A total of 24 male adult (PD 70–90) and 12 juvenile (PD 28–32) Sprague Dawley rats (SAS Derived, Charles River Labs, Kingston, NY, USA) were used in the study. Juvenile Sprague Dawley males served as social exploration stimuli in the social exploration tests. Upon arrival at the vivarium, adults were single housed and acclimated to a colony for at least 1 week prior to experimentation. The vivarium was maintained on a 12 h light/dark cycle with lights on at 07:00. For all rats, food and water were available *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, USA, 2011) and were reviewed and approved by the University of New Hampshire Institutional Animal Care and Use Committee.

Apparatus

Social Exploration

Social exploration pretest and posttest were conducted in identical test chambers, which consisted of a plastic tub cage (40.6 cm × 20.3 cm × 20.3 cm; l × w × h), wire lid, and 3 cm of wood shaving bedding free of food and water. The room was lit by cool fluorescent bulbs and light penetration into the test chamber was 200–300 lx. Juvenile stimuli were used for a maximum of four tests and adults were never exposed to the same juvenile more than once. A camera that was mounted above the apparatus recorded behavior during each test session (Stafford et al., 2015).

Open Field

Open-field tests were conducted in an open-top square plywood box (120 cm × 120 cm × 25 cm; l × w × h) painted with flat black enamel. Test sessions were video recorded from a camera mounted above the apparatus and processed using Ethovision XT software (version 8.5; Noldus Information Technology, Wageningen, Netherlands).

Intermittent Swim Stress

ISS was conducted in two acrylic cylinders (21 cm × 42 cm; d × h) with a 6.35 mm galvanized wire mesh at the bottom of each cylinder. Cylinders were suspended over a tank (80.6 cm × 45.7 × 28.6 cm; l × w × h) filled with water maintained at 15 ± 1°C. The apparatus was controlled by a Med-PC interface and software (Med Associates Inc., St. Albans, VT, USA). Space heaters, above and in front of each cylinder, circulated warm air (∼36°C) in and around the cylinders to limit the effects of hypothermia during the inter-trial intervals.

Forced Swim Test

The FST was conducted in acrylic cylinders (20 cm × 46 cm; d × h). The water was filled to 30 cm height and was kept at 24°C. Test sessions were video recorded and quantified as described above.

Self-administration Chambers

Self-administration chambers (ENV-008CT; Med Associates Inc., St. Albans, VT, USA) measuring 30 cm × 20 cm × 20 cm (l × w × h), were enclosed in a sound- and light-attenuating cubicle equipped with an exhaust fan. Each chamber was equipped with two retractable levers, two stimulus lights, and a house light. The infusion pump, that was located outside of the sound-attenuating cubicle, was equipped with a syringe that was connected to a swivel inside the chamber and that was further extended through a spring leash suspended over the ceiling of the chamber on a balanced metal arm. Lighting in the test room was maintained at 400–500 lx and exposure to light was minimized during the transfer from the colony.

Drugs

Diamorphine hydrochloride (generously provided by NIDA Drug Supply Program) and yohimbine hydrochloride (Sigma, St. Louis, MO, USA) were mixed in a 0.9% sterile saline solution.

METHODS

Experimental progression is shown in **Figure 1**. At the start of experimentation, all rats received twice daily handling for 1 week by all experimenters with the last 3 days including towel restraint habituation for blood collection. Rats were first subjected to a series of pretests consisting of social exploration and open field. On the following day, rats were subjected to the ISS, an ethologically relevant stressor with high ecological validity (Brown et al., 2001; Drugan et al., 2005, 2010, 2016). Twenty-four hours after the ISS, rats were subjected to a series of post-tests that included social exploration, open field, and FST. In order to assess stress-induced changes in behavior and corticosterone, stress reactivity was tested during the light phase when basal glucocorticoid levels nadir. Eighteen to 22 days after the initial ISS-induced stress episode rats started heroin self-administration that consisted of multiple phases including the acquisition of self-administration, assessment of individual demand for heroin, and reacquisition of self-administration. After that, rats underwent extinction and reinstatement testing with heroin no longer available.

Social Exploration

Social exploration consisted of a 1 h acclimation to the test chamber after which a juvenile was placed into the cage for 3 min. Social exploration tests were analyzed in real time by two experimenters (interrater reliability: $r = 0.93$). The following behaviors of the adult directed toward the juvenile were quantified: sniffing (direct snout contact against any portion of the juvenile and primarily directed at the anogenital region), pinning (minimum of two fore-paws against juvenile), allogrooming, and chasing. A total sum of these measures comprised the reported social exploration score.

Open Field

Rats were placed individually into the center of the open field apparatus for 10 min, after which they were returned to the vivarium. Locomotor activity, defined as the distance

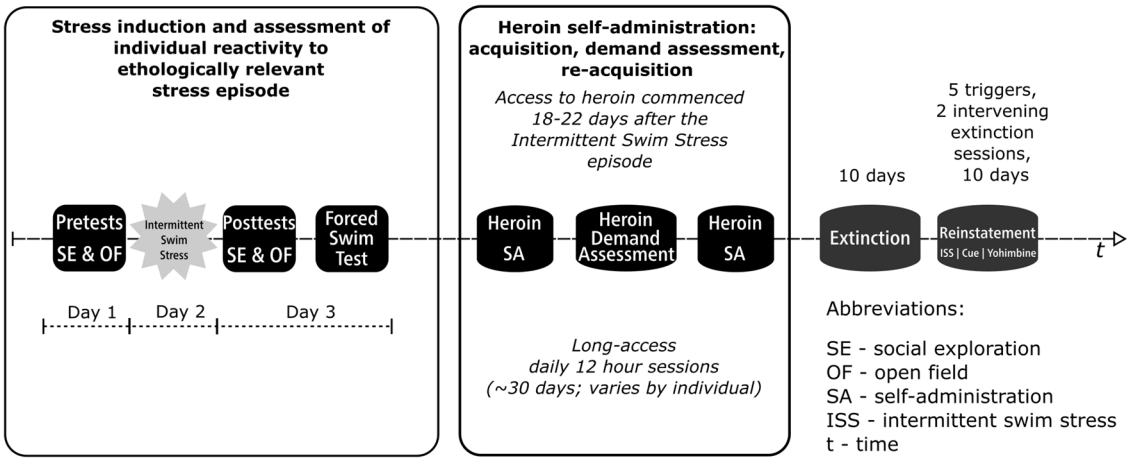

FIGURE 1 | Experimental progression. The study used a within-subjects design (final *N* = 16; eight rats removed from the study due to patency loss) with several distinct experimental phases occurring sequentially as outlined in the figure. The first phase modeled exposure to a stress episode. During that phase, rats were subjected to Intermittent swim stress (ISS) and their individual reactivity to stress was assessed using a series of pretests and posttests that included social exploration, open field, and forced swim test (FST; see first four blocks of the timeline). The second phase modeled drug taking (see Heroin SA, Heroin Demand Assessment, and Heroin SA blocks). In the second phase, rats were allowed to self-administer heroin (0.05 mg/kg/infusion; 12 h/day) and their demand for heroin was assessed using behavioral economics model; rats were trained to self-administer heroin before this assessment and retrained after to ensure a stable level of responding. The third phase modeled abstinence (see Extinction block). Extinction phase was identical to self-administration phase except that heroin and cues that were previously paired with heroin infusions were no longer available. Fourth and final phase modeled relapse (see Reinstatement block). In this final phase, the resurgence of active lever responding in extinction was triggered by an abbreviated version of the ISS, pharmacological stressor yohimbine (0, 1.25, and 2.5 mg/kg), or a non-contingent cue presentation.

traveled (path length), and time spent in the center vs. perimeter (thigmotaxis) were measured using Ethovision XT 8.5. Ethovision software was calibrated to identify and track the center point of the white subject against the black arena background using the static subtraction method (subject contour detection was set to erode first, then dilate, at one pixel for optimal subject detection) at the default five samples/s for the Open Field Template within the program. The following parameters were defined in Ethovision for the locomotor variables. The open-field apparatus was divided into two portions: the center consisted of a central 60 cm × 60 cm square (located 30 cm from the apparatus wall), while the remaining surrounding area of the apparatus consisted of the perimeter. The path length was calculated as total distance moved (in cm) of the center point of the subject throughout the entire arena and movement was defined when the center point changed position above a threshold of 1 cm/s. All dependent measures were divided into the first 5 min (habituation; 0–5 min) and last 5 min (test; 5–10 min) of the test. Behaviors during the second 5 min bin were used for data analyses.

Exposure to a Stress Episode: Intermittent Stress Swim

ISS was administered 24 h after pretests between 07:00 and 12:30. Each swim trial consisted of a 5-s forced swim in which the cylinder was submerged to a depth of 25 cm. One-hundred trials were presented at a variable 60 s (10–110 s) inter-trial-interval. Immediately following ISS, rats were towel-dried and returned to the vivarium to heated cages (heating pad placed underneath cages) to promote vasodilation for blood collection that occurred 30 min post-ISS and was performed in a separate room.

Forced Swim Test

The FST was administered immediately following the post-ISS social exploration and open field tests. During the FST, rats were forced to swim for 5 min in 24 ± 1°C. Water depth was kept at 30 cm. This depth level forced rats to swim because it avoids the possibility of body support by the tail touching the bottom of the cylinder (i.e., tail-standing). FST videos were analyzed *via* a widely used and reliable serial time-sampling procedure in which behavior is scored as either immobile, swim or climb in 5 s intervals and reported as mean counts for each behavior (Detke et al., 1995; Drugan et al., 2010). Immobility was defined as only necessary movements to keep the head above water. Swimming was defined as active movements, including dives that did not involve struggling against the side of the cylinder. Climbing was defined as active struggling against the side wall of the cylinder in which the fore-paws broke the surface of the water. Inter-rater reliability was calculated for all measures (immobility, r = 0.96; climbing, r = 0.97; swimming, r = 0.94).

Blood Collection and Analysis

Twenty-four hours before initiation of behavioral tests, a baseline blood sample was collected from the lateral tail vein; all samples were collected between 09:00 and 13:00. Second blood collection occurred 30 min after ISS test. Additional blood collections were performed 30 min post-FST when circulating corticosterone

concentration peaks (Connor et al., 1997), the day after the last self-administration session, and 30 min after the abbreviated ISS exposure that was used as one of several reinstatement triggers. Rats were lightly restrained in a towel; the tail was placed into 46 ± 2°C water to promote vasodilation and approximately 300 microliters blood was collected *via* lateral tail vein incision with a #11 scalpel into a capillary tube. The first incision was made in the distal 2 cm of the tail with subsequent incisions made at least 1 cm rostral of the previous. All samples were collected within 3 min and rats were returned to a home cage within 5 min (Fluttert et al., 2000; Drugan et al., 2005). Samples were centrifuged at 4°C for 4 min at 1,300 rpm to separate red blood cells and extract plasma, which was stored at −80°C until assay. Samples were analyzed *via* enzyme-linked immunoabsorbent assay (Arbor Assays, Ann Arbor, MI, USA) and processed in duplicates. Corticosterone concentrations were read at 405 nm on a BioTek microplate reader using Gen5 software (BioTek, Winooski, VT, USA). The intra-assay and inter-assay coefficients were 6% and 8%, respectively.

Catheter Implantation Surgery

Subjects were anesthetized with 1 ml/kg ketamine (100 mg/ml) and xylazine (20 mg/ml) mixture (2:1 ratio; administered intramuscularly; Sigma, St. Louis, MO, USA). A polyurethane catheter (22 Ga; RJVR-23; Strategic Applications Inc., Lake Villa, IL, USA) with a rounded tip and double suture beads (one secured internally and other externally) was implanted into the right external jugular vein. The other end of the catheter was subcutaneously placed around the shoulder and exited below the scapula *via* subcutaneously implanted polycarbonate back-mount access port (313-000BM; Plastics One Inc., Roanoke, VA, USA). Immediately following the surgery, catheters were flushed with 0.2 mL mixture of 30 U/ml heparin and 50 mg/mL of cefazolin (antibiotic) diluted in sterile saline (0.9% NaCl). Atipamezole hydrochloride (0.5 mg/kg; IM; Sigma, St. Louis, MO, USA) diluted in saline was used to terminate anesthesia. To manage post-surgical pain, butorphanol tartrate (1 mg/kg; SC) was administered immediately after the surgery and daily for the next two recovery days. Starting from the day after surgery, catheters were flushed daily with 0.2 mL heparinized saline (30 U/ml) and cefazolin (50 mg/mL) mixture. Catheter patency was assessed when patency loss was suspected or upon completion of the self-administration phase with an infusion of 0.05 ml xylazine (20 mg/ml; IV). This xylazine concentration produces clear motor ataxia within 5–10 s (Charntikov et al., 2013, 2018). Rats that did not exhibit noticeable motor ataxia within 5–10 s following xylazine infusion were considered non-patent.

Preliminary Training

Rats were trained to lever press over three daily sessions. At the start of each session, the house-light was turned on and a randomly selected lever (right or left) was inserted. A lever press or lapse of 15 s resulted in sucrose delivery (100 μl of 30% liquid solution; 4-s access) *via* a raised dipper, lever retraction, and commencement of a timeout (average = 60 s; range = 30–89 s). Following the timeout, a randomly selected lever was inserted with the condition that the same lever could not be presented more than twice in a row. This protocol was repeated for 60 sucrose deliveries. Sessions lasted 65–80 min depending on individual performance. Training continued until a lever press was made on at least 80% of the lever insertions for two consecutive days (i.e., 3–5 sessions). After rats met this criterion, they were surgically implanted with an intravenous catheter as described earlier. Following 7 days of recovery, lever press training continued as described above, but the response contingency was changed to a variable ratio (VR3) schedule of reinforcement where on average every third response was followed by a sucrose delivery (range = 1–6 presses). At least 80% of the 60 available sucrose deliveries had to be earned to move to the self-administration phase; this occurred after 3–5 sessions. This protocol ensures high rates of responding, yet both levers have similar reinforcement history to avoid any potential bias of differential lever press training in later phases.

Heroin Self-administration and Assessment of Individual Demand for Heroin

After recovery from surgery, all rats were retrained to lever press on both levers using a variable schedule of reinforcement (VR3) and after meeting a criterion transitioned to daily 12 h heroin self-administration (0.05 mg/kg/infusion; VR3). Each session began with a termination of the house light, insertion of both levers, and a 0.9 s infusion to flush approximately 90% of catheter volume. Completion of the required response resulted in a ∼1 s infusion of heroin, retraction of both levers, and illumination of both cue lights above each lever for a 20 s timeout. Additional 5 min timeout with levers retracted and house light illuminated was instituted every 55 min to mitigate possible overdose-related deaths. All rats self-administered the exact dose of heroin using a variation in infusion duration that was automatically controlled by the program based on their pre-session weight. To closely simulate drug taking conditions observed in humans, all self-administration sessions were conducted during the night cycle which corresponds to rodents' active phase (19:00–07:00). Access to heroin self-administration commenced 18–22 days after the initial ISS-induced stress episode. After 8 days of heroin self-administration on VR3 as described above, heroin was earned on fixed ratio (FR) schedule of reinforcement that was escalated daily using the following sequence: 1, 3, 5, 8, 12, 18, 26, 38, 58, 86, 130, 195, 292, 438, and 657. Subsequently, rats were allowed to self-administer heroin on VR3 as described above for an additional 5 days.

Extinction and Reinstatement

Extinction training was identical to self-administration sessions except that active lever responding had no programmed consequences; no heroin, cues, or lever retractions. Reinstatement tests commenced on the day after last (10th) extinction session at the usual time of self-administration or extinction sessions (19:00). There were a total of five 1-h reinstatement tests with two intervening daily extinction sessions

between these tests. Reinstatement triggers included abbreviated ISS re-exposure (20 trials), non-contingent cue presentations, and yohimbine (0, 1.0, or 2.5 mg/kg; IP). Abbreviated ISS and yohimbine were administered 30 min before the beginning of each test. Non-contingent cue presentations were identical to the cues that were associated with heroin infusions and consisted of both cue lights turned on, and both levers retracted for 20 s. These cue triggers were presented at the start of the session and every 5 min from the beginning of the session thereafter. The order of reinstatement tests for each rat was assigned using a Latin square design.

Analytical Approach

Data from eight rats were removed from the study due to intravenous catheter patency loss (final $N = 16$). Some individual corticosterone datum was not available because of failed sampling attempts or not enough serum for the analysis (two samples after the FST, one sample after extinction, and three samples after stress-induced reinstatement). Statistical analyses involving corticosterone data were restricted to subjects with available data. Individual demand for heroin was derived from the amount of heroin consumed (mg/kg) over each FR schedule of reinforcement (Hursh and Silberberg, 2008). Essential value from the demand model was used to estimate individual demand for a reinforcer and was calculated from the nonlinear least squares regression model fit to the individual consumption data from each schedule of reinforcement using the following formula: $log\ Q = log\ Q_0 + k(e^{-\alpha Q_0^C} - 1)$ where Q represents reinforcer consumption, Q_0 is a consumption when price is zero or free, κ is a constant for the range of demand, e is the base of the natural logarithm, C is the varying cost of each reinforcer, and α is the rate of decline in relative log consumption with increases in price. The essential value was calculated from the demand model using the following formula: $EV = 1/(100 \times a \times k^{1.5})$. The main advantage of using essential value is that it is a unifying measure based on several critical parameters forming exponential-demand equation. For example, essential value takes into consideration consumption when the price of a reinforcer is low (e.g., FR1), when the price of the reinforcer is high (e.g., higher or terminal FR schedules), and the slope of the demand curve—also referred to as elasticity. Using this approach that we previously demonstrated in nicotine self-administration study, it is possible to plot individual demand curves for each subject, and most importantly, derive a single value of individual demand for heroin that is based on performance over a range of schedules of reinforcement (Kazan and Charntikov, 2019).

The difference between pre- and post-stress behaviors were assessed using t-tests (performed using R 3.4.2; stats package). Responding on active and inactive levers was analyzed using ANOVAs (performed using GraphPad Prism). Individual effects were analyzed using linear mixed-effects modeling with maximum likelihood fit (performed using R 3.4.2; lme package). Individual effects were analyzed by building a model with a maximum likelihood fit from a baseline that does not include any predictors other than an intercept. The model was then built by adding one predictor or a combination of predictors and comparing it to a baseline. The model fit was declared significant when its addition improved the model by accounting for significantly more variance (the fit was examined using the Likelihood Ratio test of fixed effects; $p < 0.05$). The proportion of variance explained by the factors is reported as the marginal R^2. Additional model fitting criteria like AIC and BIC are presented in **Tables 1–3**. Effect sizes were estimated using G*Power 3.1.9.2. Reinstatement tests evoked by abbreviated ISS or non-contingent cue presentation were assessed using paired t-tests by comparing the responding on the active lever during reinstatement to the average of active lever presses on the last two extinction sessions. Reinstatement tests involved yohimbine were assessed using linear mixed-effects modeling followed by ANOVA.

RESULTS

The Effect of Intermittent Swim Stress on Social Exploration and Open Field Behaviors

ISS did not affect social exploration behavior ($t_{(15)} = 0.42$, $p = 0.68$; **Figure 2A**). ISS significantly decreased time spent in the center of open field ($t_{(15)} = 4.48$, $p < 0.001$), significantly increased time spent in the perimeter of open field ($t_{(15)} = 4.85$, $p < 0.001$), and significantly decreased total distance traveled during the open field (pathlength; $t_{(15)} = 6.42$, $p < 0.0001$; **Figures 2B–D**).

Heroin Self-administration and Extinction

During the first eight sessions of heroin self-administration responding on levers varied by Session ($F_{(7,240)} = 10.13$, $p < 0.0001$), Lever ($F_{(1,240)} = 212.6$, $p < 0.0001$), and there was no interaction ($F_{(7,240)} = 1.205$, $p = 0.30$). Active lever responding was consistently higher over the first eight sessions of heroin self-administration (Bonferroni comparisons; **Figure 3A**). The lowest individual demand (essential value) for heroin was 1.13 and the highest was 13.08 with the mean of 5.15, the standard deviation of 3.57, and coefficient of variation equal to 69.48% (**Figure 3B**). Active lever responding was consistently higher over the five sessions of self-administration following demand acquisition phase ($F_{(1,150)} = 409.7$, $p < 0.0001$; Bonferroni comparisons; **Figure 3C**). Lever responding over the five sessions of self-administration following demand acquisition phase did not varied by Session ($F_{(4,150)} = 0.5653$, $p = 0.68$) and there was not Session by Lever interaction ($F_{(4,150)} = 0.8616$, $p = 0.4886$). During extinction active lever responding decreased from 315.19 (SD = 209) on session 1–44.44 (SD = 35.72) on last session 10 (**Figure 3D**).

Behavioral and Biological Markers Predict Demand for Heroin

Individual Behavioral Markers Are Associated With Increased Demand for Heroin

The detailed statistical output from the tests below is provided in **Table 1**. In the open field, neither the difference score in

TABLE 1 | The statistical output from analyses testing the relation between behavioral markers and the demand for heroin.

	Model	Test	*df*	AIC	BIC	logLik	L.Ratio	*p*-value	Marginal R^2
Baseline Model (DV = EV)	1		3	91.16	93.48	−42.58			
Adding OF Center Diff	**2**	**2 vs. 1**	**4**	**84.54**	**87.63**	**−38.27**	**8.63**	**<0.01**	**0.43**
Adding OF Path Diff	3	3 vs. 1	4	91.98	95.07	−41.99	1.18	0.28	0.07
Adding OF Perimeter Diff	4	4 vs. 1	4	90.52	93.61	−41.26	2.65	0.10	0.16
Adding FS Climbing	**5**	**5 vs. 1**	**4**	**82.40**	**85.49**	**−37.20**	**10.76**	**<0.01**	**0.50**
Adding FS Immobility	**6**	**6 vs. 1**	**4**	**82.84**	**85.93**	**−37.42**	**10.32**	**<0.01**	**0.49**
Adding FS Swimming	7	7 vs. 1	4	92.97	96.06	−42.49	0.19	0.66	0.01
Adding OF Center Diff and FS Climbing together	**8**	**8 vs. 1**	**5**	**75.27**	**79.14**	**−32.63**	**19.88**	**<0.001**	**0.72**

Significant effects are marked in bold.

TABLE 2 | The statistical output from analyses assessing the relation between a combination of behavioral and biological markers and the demand for heroin.

	Model	Test	*df*	AIC	BIC	logLik	L.Ratio	*p*-value	Marginal R^2
Baseline Model (DV = Cort post ISS)	1		3	255.71	257.62	−124.85			
Adding Cort post FST	**2**	**2 vs. 1**	**4**	**251.52**	**254.07**	**−121.76**	**6.19**	**<0.01**	**0.37**
Baseline Model (DV = EV)	1		3	81.78	83.7	−37.89			
Adding Post-ISS Cort	2	2 vs. 1	4	90.49	93.58	−41.25	2.67	0.10	0.16
Adding Post-FST Cort	3	3 vs. 1	4	93.21	96.30	−42.61	0.47	0.83	0.03
Adding Cort ISS/FST	**4**	**4 vs. 1**	**4**	**79.18**	**81.74**	**−35.59**	**4.6**	**0.03**	**0.29**
Baseline Model (DV = EV)	1		3	81.78	83.7	−37.89			
Adding OF Center Diff and Cort post ISS together	**2**	**2 vs. 1**	**5**	**74.64**	**77.84**	**−32.32**	**11.14**	**<0.01**	**0.56↑**
Baseline Model (DV = EV)	1		3	81.78	83.7	−37.89			
Adding FS Climbing and Cort post FST together	**2**	**2 vs. 1**	**5**	**70.73**	**73.93**	**−30.36**	**15.05**	**<0.001**	**0.67↑**
Baseline Model (DV = EV)	1		3	81.78	83.7	−37.89			
Adding OF Center Diff, FS Climbing, and Cort ISS/FST together	**2**	**2 vs. 1**	**6**	**65.36**	**69.19**	**−26.68**	**22.43**	**<0.001**	**0.81↑**

↑-indicates an increase in R^2 when comparing to a behavioral predictor or predictors used in a corresponding model. Significant effects are marked in bold.

TABLE 3 | The statistical output from tests assessing the relation between behavioral markers and the reinstatement behavior.

	Model	Test	*df*	AIC	BIC	logLik	L.Ratio	*p*-value	Marginal R^2
Baseline Model (DV = Cue)	1		3	149.83	152.14	−71.91			
Adding Essential Value	2	2 vs. 1	4	149.71	152.80	−70.85	2.12	0.15	0.13
Adding OF Center Diff	3	3 vs. 1	4	151.15	154.24	−71.57	0.68	0.41	0.04
Adding FS Climbing	**4**	**4 vs. 1**	**4**	**143.19**	**146.28**	**−67.59**	**8.64**	**<0.01**	**0.43**

Significant effects are marked in bold.

distance traveled nor the difference score in time spent in the perimeter of the open field before and after the ISS significantly related to the individual demand for heroin. Swimming during the FST did not correlate with the demand for heroin. On the other hand, time spent in the center of the open field after the stress minus the time spent in the center of the open field before the stress (difference score) significantly predicted individual demand for heroin ($\chi^2_{(1)} = 8.63$, $p < 0.01$). The difference in the open field center activity explained approximately 43% of the variance in demand for heroin with the effect size (f^2) equal to 0.75 ($R^2 = 0.43$; **Figure 4A**). Climbing ($\chi^2_{(1)} = 10.76$, $p < 0.01$) and Immobility ($\chi^2_{(1)} = 10.32$, $p < 0.01$) during the 5 min FST, administered a day after the ISS exposure, also significantly predicted an increased demand for heroin. Because climbing and immobility measures had an almost perfect negative relation with each other ($R^2 = 0.97$), we are only showing and discussing climbing activity to avoid redundancy. Climbing activity during the FST explained approximately 50% of the variance in demand for heroin ($R^2 = 0.50$; $f^2 = 1$, **Figure 4B**). When both open field center difference score and the climbing during FST were added to the linear model at the same time, they improved the fit of the model by explaining a larger proportion of variance in demand for heroin then each predictor alone ($\chi^2_{(2)} = 19.88$, $p < 0.001$; $R^2 = 0.72$; $f^2 = 2.57$). The fact that a combination of these measures improves the predictive ability of the linear model indicates that these measures explain a different portion of the variance in heroin demand and complement each other in their predictive qualities. Predicted values from the linear model that included open-field center difference score and climbing measure from FST as predictors for heroin demand are visualized in **Figure 4C**. Overall, these results indicate that difference in open field activity and climbing during the FST that followed the ISS are strong behavioral predictors of heroin demand that was assessed in a later phase of the study.

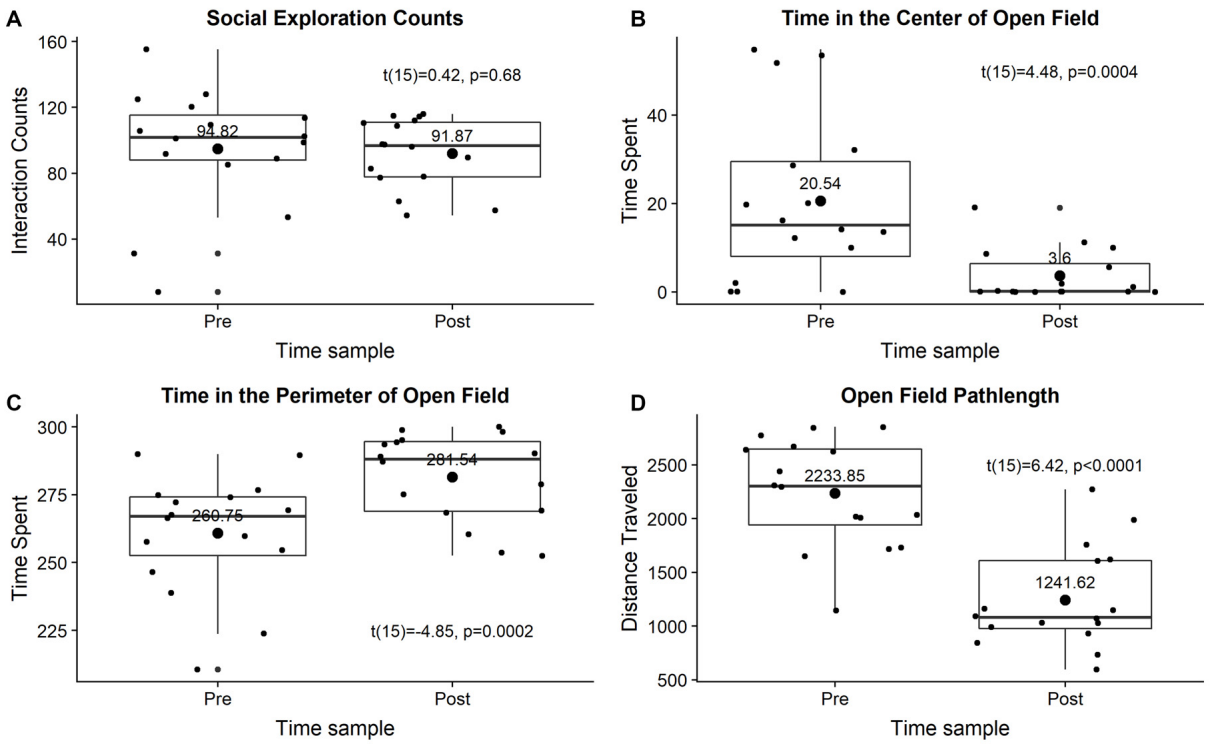

FIGURE 2 | The effect of ISS on social exploration **(A)** time spent in the center of the open field **(B)**, time spent in the perimeter of the open field **(C)**, or distance traveled during the open field test **(D)**. All data were visualized as grouped pretest and posttest measures using box plots with small points showing individual datum and large points showing means with mean values above.

Individual Biological Response to Stress Predicts Demand for Heroin

The detailed statistical output from the model fitting tests below is provided in **Table 2**. Plasma corticosterone levels were increased from baseline post-ISS and post-FST ($F_{(3,12)} = 3.89$, $p < 0.05$; Dunnett's tests; **Figure 5A**). Individually, post-ISS or post-FST corticosterone responses did not predict demand for heroin; however, there was a positive linear relationship between these measures ($\chi^2_{(1)} = 6.19$, $p < 0.01$; $R^2 = 0.37$; $f^2 = 0.58$; **Figure 5B**). To assess whether combined individual corticosterone response to ISS and FST related to the demand for heroin we created a corticosterone ISS/FST composite score by centering (z-score) both of these variables and summating them for each subject. With that in mind, combined corticosterone response to ISS and FST positively predicted demand for heroin ($\chi^2_{(1)} = 4.60$, $p = 0.032$; $R^2 = 0.29$; $f^2 = 0.41$; **Figure 5C**) indicating that individual biological response to stress alone can be used to predict demand for heroin in the later phase of the study.

Combination of Behavioral and Biological Markers Improves the Ability to Predict Demand for Heroin

We have shown above that behavioral and biological markers that were strategically sampled at time points relevant to a stress response induced by the ISS predict the demand for heroin in the later phase of the study. We then assessed how a combination of behavioral and corresponding biological markers relate to a demand for heroin. Open field center difference score and corticosterone response after the ISS together explained 55% of the variance in demand for heroin ($\chi^2_{(2)} = 11.14$, $p < 0.01$; $R^2 = 0.56$; $f^2 = 1.27$; **Figure 5D**); R^2 change = +0.13 when compared to a corresponding behavioral marker alone. Climbing during FST and corticosterone after the FST together explain 67% of the variance in demand for heroin ($\chi^2_{(2)} = 15.05$, $p < 0.001$; $R^2 = 0.67$; $f^2 = 2.03$; **Figure 5E**); R^2 change = +0.17 when compared to a corresponding behavioral marker alone. Finally, a combination of open field center difference score, climbing during the FST, and corticosterone ISS/FST composite together explain 81% of variance in demand for heroin ($\chi^2_{(3)} = 22.42$, $p < 0.001$; $R^2 = 0.81$; $f^2 = 4.26$; **Figure 5F**); R^2 change = +0.09 when compared to a model that includes both corresponding behavioral markers. These findings indicate that behavioral and biological markers are complimentary in their nature and together explain a large proportion of variance in individual demand for self-administered heroin.

Reinstatement

Stress- and Cue-triggered Reinstatement

Abbreviated intermittent stress swim re-exposure (20 swim trials), administered before the 60 min extinction test, did

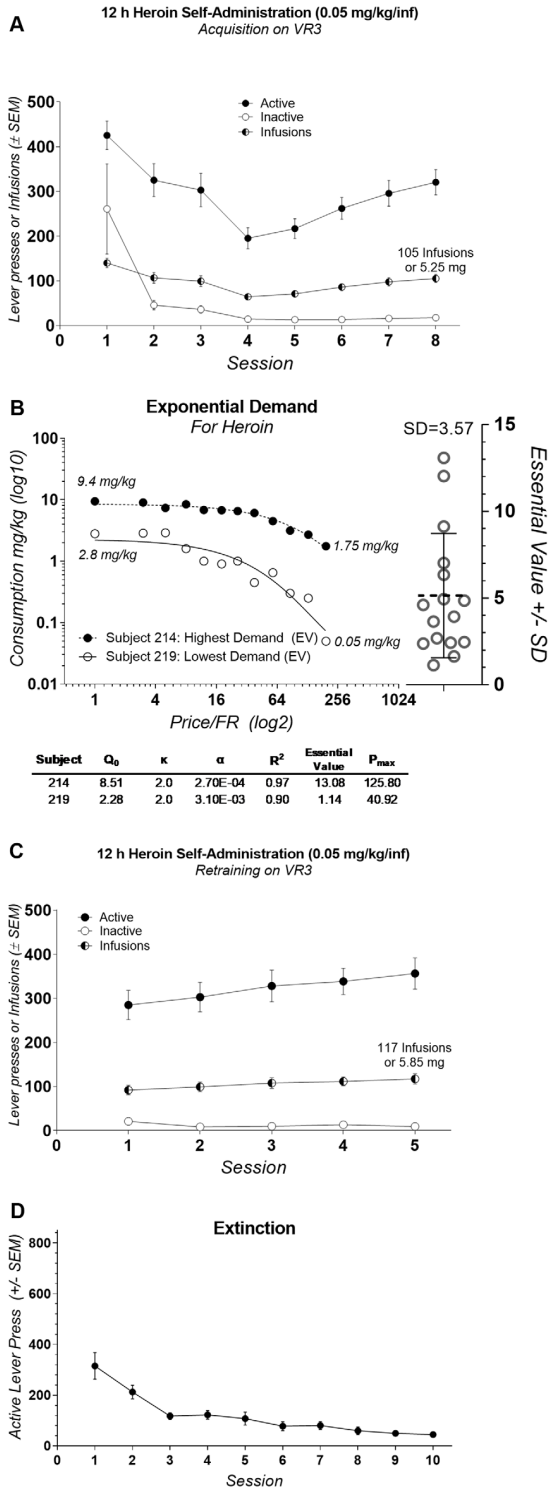

FIGURE 3 | (A) Mean (±SEM) number of lever presses and infusions earned during the acquisition of heroin self-administration. **(B)** Exponential demand curves from rats with the highest and lowest essential value (left panel; critical demand values are shown below) and Essential Value scatterplot from all subjects (right panel). **(C)** Mean (±SEM) number of lever presses and infusions earned during reacquisition of heroin self-administration. **(D)** Mean (±SEM) number active lever presses during extinction.

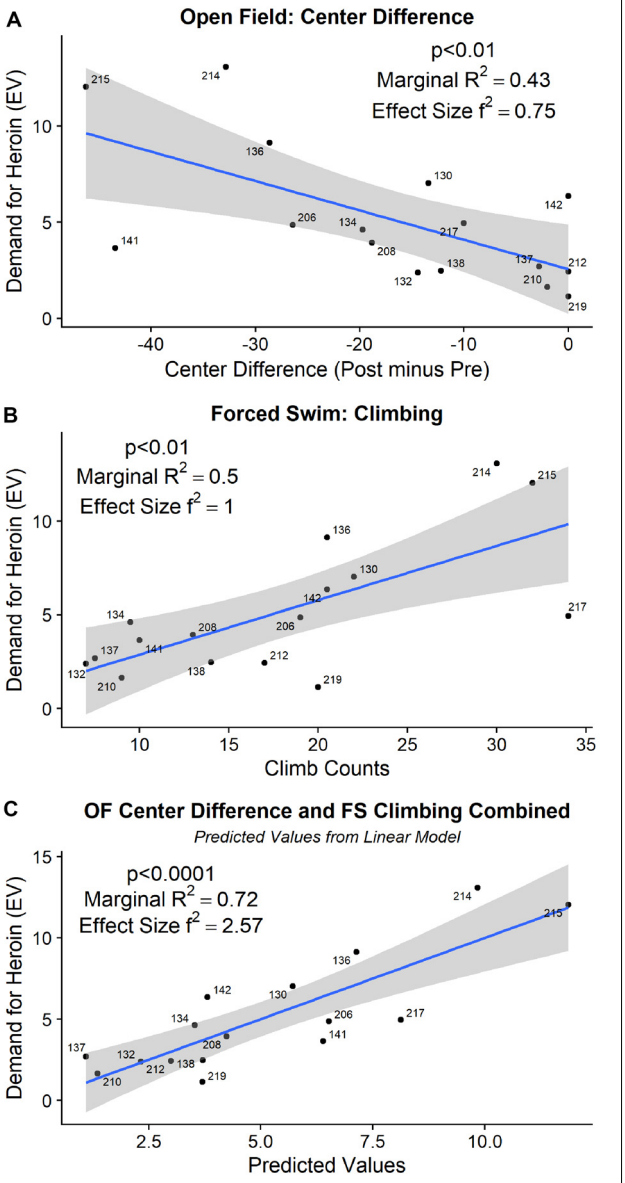

FIGURE 4 | (A) The difference in time spent in the center of the open field, before and after the ISS, predicted subsequent demand for heroin. **(B)** Increased climbing during the 5 min of FST, administered the day after ISS, predicted subsequent demand for heroin. **(C)** Together, open field difference score and forced swim climbing (visualized as predicted values from a linear model) explain 72% of the variance (R^2) in subsequent demand for heroin.

not reinstate active lever responding when compared to the average of responding on the last two extinction sessions ($t_{(15)} = 1.52, p = 0.14$; **Figure 6A**; compare ISS bar with Extinction bar). In contrast, non-contingent cue presentations during the extinction test significantly increased active lever responding when compared to the responding on the last two extinction sessions ($t_{(15)} = -3.68$, $p < 0.01$; **Figure 6A**; compare Cue bar with Extinction bar). Followup analysis also indicated a significant increase in inactive lever responding from an average

of 2.4 lever presses in extinction (average of last two extinction sessions) to an average of 7.06 during the non-contingent cue presentation test ($t_{(5)} = -3.73$, $p < 0.01$; data not shown). Importantly, this is the first demonstration of heroin-associated cues presented non-contingently during extinction reinstating heroin seeking in rats with a history of stress exposure.

Yohimbine-Triggered Reinstatement

There was trending but not significant effect of yohimbine dose on active lever responding in extinction ($F_{(2,30)} = 2.625$; $p = 0.089$; **Figure 6A**; compare bars labeled Y-0 to Y-2.5).

Assessing the Relationship Between the Demand for Heroin, Stress Markers, and Reinstatement Behavior

Individual responding during non-contingent cue-triggered reinstatement was positively related to enhanced individual climbing during FST administered after the initial ISS exposure ($\chi^2_{(1)} = 8.64$, $p < 0.01$; **Figure 6B**). Neither relevant behavioral markers (demand for heroin, open field center difference activity) nor relevant biological markers (corticosterone response to ISS/FST) predicted the magnitude of cue-triggered reinstatement.

DISCUSSION

We hypothesized that individuals vulnerable to the effects of stress have a higher risk of abusing heroin after the exposure to stress. We further hypothesized that we could simulate these individual effects in a preclinical model of stress and heroin use comorbidity. In this model, all rats are initially exposed to a stress episode and then their individual biological response to stress in the form of corticosterone response and individual behavioral responses that are strategically sampled around the stress exposure are used to predict individual levels of heroin self-administration that are derived using a behavioral economics model. Importantly, in this experimental design the time between the stress exposure and the initiation of heroin self-administration models clinically relevant time interval between stress and drug use. Using this approach, we show that: (a) corticosterone response after ISS and FST was higher than at baseline; (b) the magnitude of the corticosterone response after ISS and FST combined predicted the magnitude of the demand for heroin; (c) behavioral markers strategically sampled around the time of stress exposure predicted the demand for heroin; and (d) a combination of biological and behavioral markers greatly improved the ability to predict individual demand for heroin, when compared to biological or behavioral markers alone. Furthermore, for the first time, we show that heroin-associated cues, which were non-contingently presented during extinction, reinstated heroin seeking in extinction in rats with a history of stress exposure. Importantly, higher climb counts from FST, that was administered the day after ISS, predicted a higher magnitude of cue-triggered reinstatement. Overall, these findings show that the individual biological response to stress and individual behaviors sampled around the time of stress exposure can be used as predictors for the magnitude of stress-induced heroin self-administration and cue-triggered reinstatement of drug seeking in extinction.

The protocol employed in the current study was designed to simulate progression from stress exposure to stress-induced opioid use. To induce long-lasting effects of stress, with a high degree of ecological validity, we used the ISS paradigm (Brown et al., 2001; Christianson and Drugan, 2005; Drugan et al., 2014; Stafford et al., 2015). The ISS paradigm was developed as a hybrid model combining the strengths from two other animal models: (1) the use of the ecologically valid stressor of inescapable swim stress (Porsolt et al., 1977); and (2) the unpredictable and inescapable intermittent stress exposure from the learned helplessness paradigm (Maier and Seligman, 1976). The severity of the stressor is furthermore controlled *a priori* by the experimenter so that each subject receives the identical amount of stress and therefore allows direct comparison across groups or individual subjects (Christianson and Drugan, 2005; Drugan et al., 2014, 2010; Stafford et al., 2015). In this study, we show that ISS evoked significantly higher corticosterone response when compared to the no-stress baseline. This finding is consistent with our previous results demonstrating that the ISS in 20°C or 25°C water evokes comparable serum levels of corticosterone (∼3,500–4,000 pg/ml; Drugan et al., 2005). Although individual corticosterone response after the ISS did not predict the demand for heroin in our study, possibly demonstrating the lack of statistical power, we do show that the magnitude of combined corticosterone response after the ISS and the FST positively related to a demand for heroin assessed weeks after the stress episode. Importantly, the corticosterone response after the FST was also significantly higher than no-stress baseline levels and was positively correlated with the corticosterone response after the ISS. This positive relationship between the biological response to these two swim stressors indicates that rats that are vulnerable to the effects of ISS also exhibit higher stress response after the FST that was administered the day after the ISS and was designed to assess the short-term effects of ISS. These results indicate that biological response to stress alone in the form of serum levels of corticosterone can be used to predict subsequent demand for heroin and that a combination of ISS and FST may serve as a reliable model to study individual long-term effects of stress and its interaction with other pathologies (e.g., substance use disorder).

To assess various modalities of stress-induced deficits on the individual level and relate them to heroin self-administration behavior, we administered three widely validated tests that model anxiety and helpless behaviors. Rats underwent social exploration and open field tests 24 h before ISS and the same tests followed by a 5 min FST 24 h after stress exposure. We found that greater decrease in time spent in the center of the open field and greater bouts of climbing during the FST predicted higher demand for heroin. Reduced time spent in the center area of the open field is widely validated as an analog of anxiety and stress induces long-term changes in this behavior (van Dijken et al., 1992; Prut and Belzung, 2003; Hale et al., 2008). Likewise, the FST has been widely used to assess the effects of stress in preclinical models

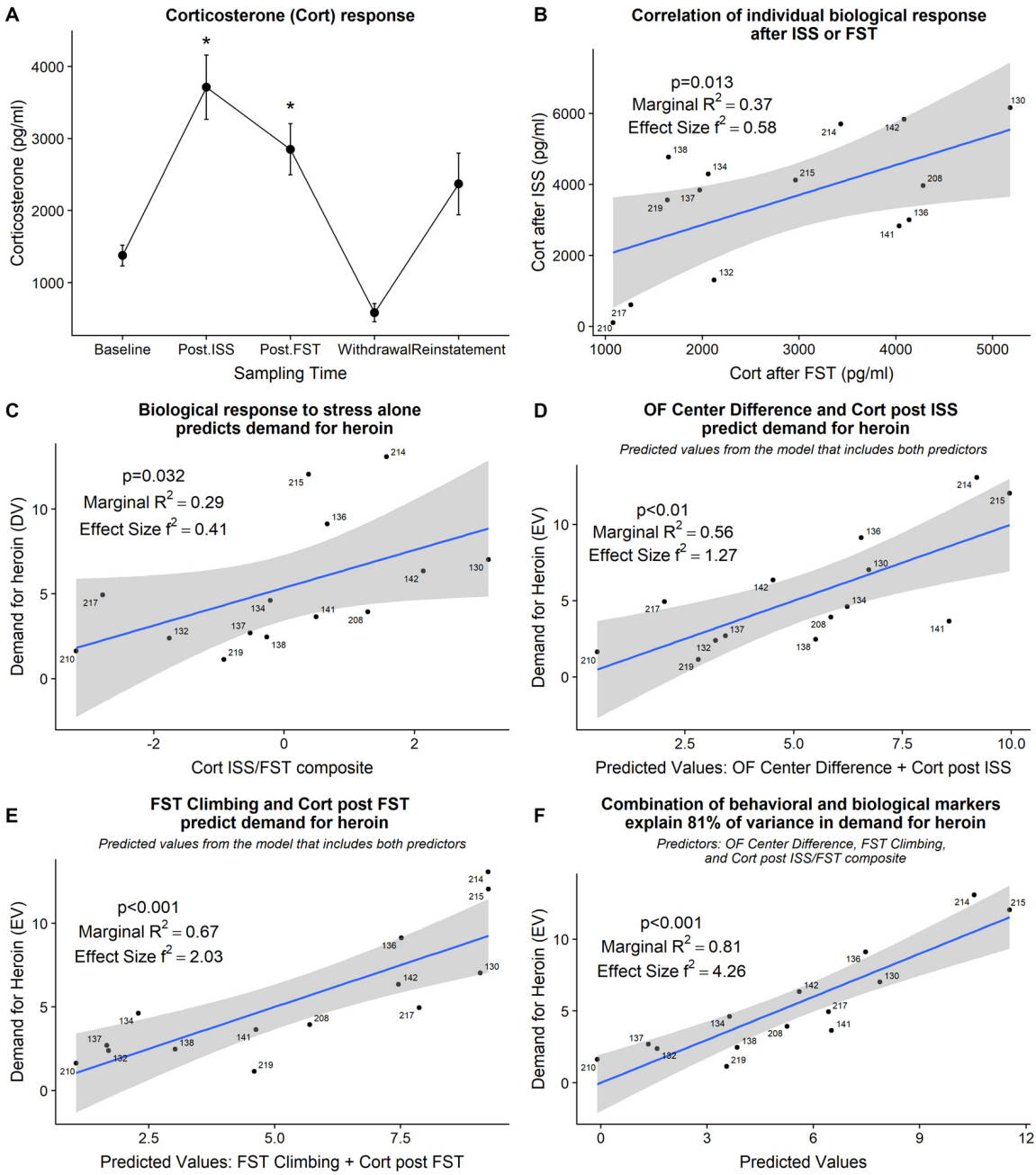

FIGURE 5 | (A) Grouped corticosterone response at various points of the study. *Significant difference from baseline ($p < 0.05$). *Post.ISS*—blood collected after ISS test. *Post.FST*—blood collected after FST. **(B)** Corticosterone response after ISS positively relates to corticosterone response after FST. **(C)** Corticosterone response after ISS and FST, combined into a composite score, predicts demand for heroin. **(D)** Individual open field center difference score and individual corticosterone response after ISS combined explain 56% of variance in demand for heroin. **(E)** Increased climbing during FST and increased corticosterone response after FST combined explain 67% of variance in demand for heroin. **(F)** Combination of behavioral and biological markers explain 81% of variance in demand for heroin. ISS, intermittent swim stress; FST, forced swim test; OF, open field; Cort, corticosterone.

including the effects of ISS. For example, exposure to ISS has been shown to increase immobility in a subsequent FST in comparison to non-stressed controls, however, the individual variability has not been previously assessed (Christianson and Drugan, 2005; Drugan et al., 2010). Interestingly, our study shows that the increased climbing during FST that was administered the day after ISS predicted greater heroin demand. Forced swim climbing behavior is usually decreased following prior swim exposure in the typical 2-day behavioral despair paradigm (Detke et al., 1995; Drugan et al., 2010).

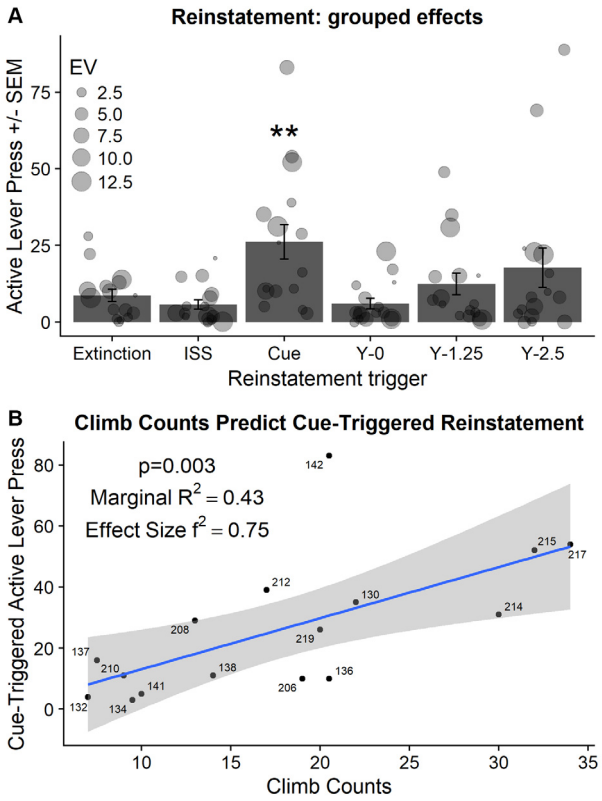

FIGURE 6 | (A) Active lever presses (±SEM) during reinstatement tests compared to active lever presses in extinction (average of last two extinction sessions). EV-indicates the essential value or demand for heroin during self-administration phase of the experiment and is visualized using the size of the individual point. ISS—indicates abbreviated Intermittent Swim Stress that was used as a trigger. Y—indicates yohimbine pretreatment followed by the dose in mg/kg. **Significant difference from responding in extinction ($p < 0.01$). **(B)** Active lever responding in extinction triggered by non-contingent cue presentations was predicted by climbing during the FST that was administered the day after the exposure to ISS.

However, the interpretation of forced swim behaviors has been debated for decades (Nishimura et al., 1988; Commons et al., 2017). The alternative argument contends that passive behaviors during subsequent swim exposures are an adaptive coping strategy, whereas climbing movements may reflect susceptibility (Commons et al., 2017). It is important to note that our FST followed an initial stress experience with water as an aversive stimulus; by that time rats likely developed conditioned response to water submersion—conditioned avoidance. Also, hyperarousal or hyperreactivity is commonly associated with long-term effects of stress (Pynoos et al., 1996; Strekalova et al., 2004; Pibiri et al., 2008; Schöner et al., 2017). Our statistical analysis shows that the elevated climbing is a complementary measure to the deficits in open field activity and both measures significantly improve the predictive ability of the mixed-effects linear model to explain variability in demand for heroin. Also, adding climb counts during the FST and corticosterone response after the FST together into one predictive model significantly improved the ability to predict the demand for heroin when compared to each of those predictors alone. With all this in mind, we argue that elevated climbing during the FST test, which followed a stress episode, likely represents "conditioned hyperreactivity" and is consistent with signs of stress vulnerability.

To closely approximate human drug-taking conditions and to study relevant behavioral and neurobiological processes we used a long-access (12 h) preclinical model of drug self-administration in conjunction with a well-validated economic demand approach to study individual differences in stress-induced heroin drug taking. Early preclinical models of drug self-administration used unlimited access, often resulting in overdose-related deaths (e.g., Johanson et al., 1976), prompting adaptation of short-access protocols that are more economic, allow higher throughput, but lacking translational relevance (for more see Gawin and Kleber, 1988; Gawin, 1991). Importantly, using this protocol and VR3 schedule of reinforcement, rats self-administer large amounts of heroin per session (5.25 mg/kg during 8th self-administration session; 0.05 mg/kg/inf) when compared to previous reports. For example, Kenny et al. (2006) used 23 h access protocol (0.02 mg/kg/inf) on FR schedule of reinforcement (FR1) and showed total consumption after first eight sessions of heroin self-administration to be approximately 0.8 mg/kg and after 24 consecutive sessions to be approximately 1.8 mg/kg. In addition, Lynch and Carroll (1999) used 6 h access (0.015 mg/kg/inf) on FR1 and showed total consumption after five sessions to be approximately 0.75 mg/kg. Thus, using our 12 h access approach and a variable schedule of reinforcement we are able to ensure high heroin intake, robust lever discrimination, and no overdose-related deaths. Using this approach, we show that individual reactivity to stress was a strong predictor for increased heroin demand. Because this is a first study to show these individual effects in male rats, additional studies will be required to further confirm and extend these findings.

Relapse is a critical factor contributing to substance use and abuse (Hendershot et al., 2011). Clinical and preclinical studies show that relapse can be precipitated by stress and cues that have been previously associated with the drug (Sinha et al., 2011; Mantsch et al., 2016). For example, intermittent footshock, or pharmacological stressors like metyrapone or yohimbine, trigger higher rates of heroin seeking in extinction (Shaham and Stewart, 1995; Shaham et al., 1996, 1997, 1998; Banna et al., 2010). Cues that have been previously paired with heroin infusions and that are presented contingently during extinction, those that require a response to earn a cue presentation, also reliably reinstate drug seeking in extinction (Banna et al., 2010; Doherty and Frantz, 2012). In comparison, previous studies show that non-contingent cue presentations during extinction, that is when cues are presented by the experimenter and do not require a response, do not reinstate cocaine or heroin seeking (Alderson et al., 2000; Grimm et al., 2000). In contrast with the previous reports (Banna et al., 2010), we show that yohimbine, a pharmacological stressor with high affinity for the α_2-adrenergic receptor that induces noradrenergic tone, did not reinstate heroin seeking in rats with the previous history of stress although the grouped effect was trending towards

significance (p = 0.089). Importantly, the variance observed at the highest doses indicates that some rats had a much greater response to yohimbine that others (observe individual response evoked by yohimbine that is visualized in **Figure 6A**). In addition, we show that brief ISS exposure also did not reinstate heroin seeking. This lack of effect following brief ISS exposure may be partially explained by the temporal dissociation from the reinstatement test, which was administered 30 min later, or by the limited stress effects induced by 20 trials rather than the prolonged 100 trials administered during the initial stress episode. Finally, one of the most interesting findings in our study is the reinstatement of heroin seeking by non-contingent cue presentations during extinction in rats with a history of stress exposure. In our study, non-contingent cue presentations consisted of lever retractions and illumination of both cue lights for 20 s, a sequence that was repeated every 5 min from the beginning of the session. The magnitude of cue-induced responding was predicted by the climbing behavior during the FST administered the day after the initial ISS stress induction.

Clinical and preclinical studies show that stress is an important factor in substance use disorders but there is also evidence of individual variability across various phases of stress and substance use comorbidity. These individual effects involved in stress and substance use comorbidity are not well studied and presently are not well understood. To advance towards more effective and individualized prevention and treatment strategies targeting substance use disorder there is a need to better understand how individual vulnerability or resilience to stress interacts with drug use. The study presented here starts filling this gap by outlining a framework for predicting individual economic demand for self-administered heroin based on biological and behavioral markers strategically sampled around the stress episode. To this end, our findings show that behavioral, biological, and a combination of behavioral and biological markers sampled prior and after the stress episode that occurred weeks before the access to heroin self-administration can predict individual demand for heroin. We also show that the individual biological response to stress can be measured by assessing individual corticosterone response evoked either by ISS or FST and then this biological response can be used to predict subsequent demand for heroin. For example, rats with higher corticosterone response to stress and higher demand for heroin can be conceptualized as vulnerable to stress and heroin use comorbidity phenotype. On the other hand, it is unclear that the behavioral responses following the stress episode were directly affected by the exposure to stress. Because our study does not include a no-stress control condition required to make such an assessment, we are not able to make a claim that a change in behavioral responses after the ISS is stress induced. However, because we show that behavioral responses sampled before and after the stress episode complement biological markers in their ability to predict the demand for heroin it is likely that they are related to the effects of stress although additional studies with appropriate controls are necessary to confirm this speculation. Furthermore, the fact that there was no correlation between behavioral and biological markers associated with stress response suggests that these measures explain different proportion of variance in demand for heroin and that these effect should be further investigated in future studies. With all this in mind, our demonstration that a combination of biological stress markers and behavioral responses sampled before and after the stress episode can explain most variance (81%) in subsequent demand for heroin provides a framework for a variety of future studies that can further investigate behavioral, biological, or mediating factors underlying this effect.

ETHICS STATEMENT

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, USA, 2011) and were reviewed and approved by the University of New Hampshire Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

NS, TK, RD and SC contributed to the conception and design of the study. NS, TK, CD and EH conducted the study. NS, RD and SC contributed to writing the manuscript.

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frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
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Varenicline Targets the Reinforcing-Enhancing Effect of Nicotine on Its Associated Salient Cue During Nicotine Self-administration in the Rat

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Nicotine is acknowledged as the key addictive compound of tobacco. Varenicline (Champix® or Chantix®), mainly acting as a partial agonist at the α4β2 nicotinic receptor, is an approved smoking cessation pharmacotherapy, although with efficacy limited to a portion of smokers. Smokers differ in the motives that drive their drug seeking and Varenicline might be more efficient in some groups more than others. Studies in rodents revealed that nicotine-seeking is strongly supported by complex interactions between nicotine and environmental cues, and notably the ability of nicotine to enhance the reinforcing properties of salient environmental stimuli. It is not yet understood whether the decrease of nicotine-seeking by acute Varenicline in rats results from antagonism of the primary reinforcing effects of nicotine, of the reinforcement-enhancing effect of nicotine on cues, or of a combination of both. Thanks to a protocol that allows assessment of the reinforcement-enhancing effect of nicotine on cues during self-administration in rats, we showed that Varenicline targets both nicotine reinforcing effects and reinforcement-enhancing effect of nicotine on cues. Importantly, individual variations in the latter determined the amplitude of acute Varenicline-induced decrease in seeking. These results suggest that Varenicline might be more beneficial in smokers who are more sensitive to nicotine effects on surrounding stimuli.

Keywords: intravenous self-administration, nicotine, cues, individual differences, varenicline, rat

INTRODUCTION

Tobacco dependence continues to be a worldwide health burden, being responsible for as many as 7 million deaths per year (WHO, 2017). More than 70% of smokers wish to quit (U.S. Department of Health and Human Services, 2012), but less than 10% succeed without medical support (Rigotti, 2012). Even so, a major obstacle in ceasing to smoke is the limited efficacy of available treatments against tobacco dependence (Schuit et al., 2017). For instance, from all

patients treated with Varenicline (Champix® or Chantix®), one of the most effective approved pharmacotherapies in supporting smoking cessation (Cahill et al., 2013; Hartmann-Boyce et al., 2014), only 40% remain abstinent at the end of a 12-week-long treatment, while post-treatment abstinence rates drop to 20% in the following months after treatment cessation (Oncken et al., 2006; Niaura et al., 2008; Jordan and Xi, 2018).

Varenicline is a full agonist at the α7-, and a partial agonist at the α4β2-containing nicotinic cholinergic receptors (Coe et al., 2005; Rollema et al., 2007a,b), which mediate the primary reinforcement properties of nicotine, the major psychoactive compound of tobacco (Benowitz, 1992). However, the relatively weak primary reinforcement of nicotine cannot explain the pervasiveness of tobacco abuse alone (Caggiula et al., 2001; Rose, 2006). Recent studies have highlighted that nicotine can increase the reinforcing value of environmental cues that are primary reinforcers by themselves, or that have acquired reinforcing value through pairing with another reinforcer (Caggiula et al., 2009; Rupprecht et al., 2015). The interplay between nicotine and environmental cues is complex and difficult to disentangle, but plenty of evidence suggests it is a determinant factor in tobacco seeking (Caggiula et al., 2001, 2002; Garcia-Rivas and Deroche-Gamonet, 2019). Importantly, newer evidence suggests that smokers differ in the psychobiological mechanisms that drive their nicotine-seeking (for review, see Garcia-Rivas and Deroche-Gamonet, 2019). In this regard, understanding the psychopharmacological dimensions of nicotine-seeking that are being affected by Varenicline could clarify its limited efficacy. However, the numerous studies that have shown that Varenicline can acutely decrease nicotine self-administration in rodents (Rollema et al., 2007b; O'Connor et al., 2010; Le Foll et al., 2012; Funk et al., 2016), have done so in experimental conditions that do not clearly allow the disentangling of the psychopharmacology of Varenicline against nicotine and nicotine-cue interactions.

Furthermore, even though the effects of Varenicline on nicotine-cue interactions have also been subject to extensive studies (Levin et al., 2012; Schassburger et al., 2015; Barrett et al., 2018), they have been studied in conditions under which nicotine is not self-administered. Varenicline has been shown to dose-dependently antagonize the reinforcement-enhancing effect caused by nicotine (Levin et al., 2012). Consistent with its nature as a partial agonist, it has also been shown that Varenicline can enhance responding for a visual cue in a dose-dependent manner, although with a much weaker effect than nicotine (Barrett et al., 2018). This last result is consistent with a previous study, which used self-administration of Varenicline and a visual cue self-administered through two different levers, to reveal such reinforcement-enhancing effect of Varenicline (Schassburger et al., 2015). However, since the psychopharmacological actions of Varenicline in humans are of therapeutic relevance when nicotine intake is volitional, the testing of Varenicline effects on passive nicotine administration has weaker face validity when compared to the classical approach of drug self-administration (Panlilio and Goldberg, 2007).

Thus, the precise psychopharmacological mechanisms through which Varenicline opposes nicotine self-administration in rodents is still not well understood, but warrant further investigation. Because a key determinant of the synergistic interaction between nicotine and a salient cue is the primary reinforcing effects of the cue (Chaudhri et al., 2006; Caggiula et al., 2009), we developed an experimental procedure that allows for increasing these primary reinforcing effects during self-administration and tested the effect of Varenicline while contingently manipulating the reinforcing-enhancing effect of nicotine on the cue.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (Charles River, France), weighing 280–300 g at the beginning of the experiments, were single housed under a 12 h reverse dark/light cycle. In the animal house, temperature (22 ± 1°C) and humidity (60 ± 5%) were controlled. Rats were habituated to environmental conditions and experimental handling for 15 days before surgery. Standard chow food and water were provided *ad libitum*. All procedures involving animal experimentation and experimental protocols were approved by the Animal Care Committee of Bordeaux (CEEA50, N° 50120168-A) and were conducted in accordance with the guidelines of the European Union Directive 2010/63/EU regulating animal research.

Surgeries

A silastic catheter (internal diameter = 0.28 mm; external diameter = 0.61 mm; dead volume = 12 μl) was implanted in the right jugular vein under ketamine (80 mg/kg)/xylazine (16 mg/kg) anesthesia. The proximal end reached the right atrium through the right jugular vein, whereas the back-mount passed under the skin and protruded from the mid-scapular region. Rats were given 5–7 days recovery before nicotine self-administration training began.

Drugs

Ketamine hydrochloride (80 mg/kg; Imalgène 1000; Rhône Mérieux, Lyon, France) and xylazine hydrochloride (16 mg/kg; Rompun; Rhône Mérieux, Lyon, France) were mixed with saline and administered intraperitoneally in a volume of 2 ml/kg of body weight. (-)-Nicotine-Hydrogen-Tartrate (Glentham, UK) was dissolved in sterile 0.9% physiological saline for a final dose of 0.04 mg/kg free base. Nicotine, as well as sterile 0.9% physiological saline in control groups, was self-administered by the rats *via* intravenous (i.v.) route in a volume of 40 μl per self-infusion. Nicotine solution was adjusted to a pH of 7.

Varenicline or 7,8,9,10-Tetrahydro-6, 10-methano-6H-pyrazino[2,3-h] [3]benzazepine tartrate (Tocris, UK) was dissolved in sterile 0.9% physiological saline for a final dose of 1 mg/kg free base. Varenicline was administered intraperitoneally (i.p.) 30 min prior to self-administration, in a volume of 2.5 ml/kg.

Intravenous Self-administration

Self-administration Apparatus

The self-administration setup consisted of 48 self-administration chambers made of plexiglas and metal (Imetronic, France), and equipped with holes as operant manipulanda. Each chamber (40 cm long × 30 cm width × 36 cm high) was located in an opaque sound-attenuating cubicle equipped with an exhaust fan to assure air renewal and mask background noise (**Supplementary Figure S1**). For self-administration sessions, each rat was placed in one chamber where its chronically implanted intravenous catheter was connected to a pump-driven syringe (infusion speed: 20 μl/s). Two holes, located at opposite sides of the chamber at 5.5 cm from the grid floor, were used to record instrumental responding. In given experimental groups and experiments, a common white light (white LED, Seoul Semiconductor, South Korea), 1.8 cm in diameter, located 11.5 cm above the active hole, was used as nicotine (or saline) delivery-associated discrete visual cue, and is named thereafter "cue light" or "cue." It produced 5 Lux. As well, in given experimental groups and experiments, a blue light (blue LED, Sloan Precision Optoelectronics, Switzerland), 1.8 cm in diameter, located on the opposite wall at 17 cm of the floor on the left side, was used as, and is named thereafter, "Ambient light" and abbreviated *AL*. It produced 15 Lux at a wavelength of 470 nm, which is known to not affect vision in Sprague–Dawley rats in a similar exposure pattern as in our experimental approach (Tosini et al., 2016). LED intensities were both measured in the middle of the cage with a Lux-meter (Moineau Instruments, France). Experimental contingencies were controlled and data were collected with a PC-windows-compatible SK_AA software (Imetronic, France).

Self-administration Procedures

In the three experiments presented below, self-administration testing began 2 h after the onset of the dark phase. Nose-poke in the active hole under a fixed ratio three schedule of reinforcement (FR3) produced the activation of the infusion pump (40 μl over 2 s). Nose-pokes at the inactive hole were recorded but had no scheduled consequences. Rats in all protocols of self-administration described in this study were placed under an FR3 schedule of reinforcement from the first session onwards, with the reinforcer varying according to the experimental group in which they were allocated (**Figure 1**). Neither food-training nor FR-1 transition period was used. Whatever the reinforcer, rats were trained 2 h daily, 5 days per week, from Monday to Friday, except for the first session of Experiments 1 and 3 that took place on a Tuesday. To maintain catheter patency, catheters were flushed with ∼10 μl of heparinized saline (30 IU/ml) after each self-administration session and before the self-administration sessions run on Monday.

In Experiment 1, to define a significant self-administration behavior at the individual level, we used a discrimination index between active and inactive holes [(active nose-pokes/total nose-pokes)*100] strictly superior to 50%, together with a minimal number of at least six self-infusions per session over three consecutive sessions and with stability in the number of self-infusions (±10%) over the last two sessions.

Experimental Procedures

Effect of Varenicline on Self-administration Behavior Reinforced by Either a Discrete Cue Light, a Nicotine Infusion or a Combination of Both Nicotine and Cue Light (Experiment 1)

Nose-poking in the active hole at FR3 was reinforced either by an infusion of 0.04 mg/kg nicotine free base (*nicotine*, $n = 25$), a nicotine 0.04mg/kg infusion plus a discrete cue light (*nicotine + cue*, $n = 8$), or a saline infusion plus a discrete cue light (*saline + cue*, $n = 10$; **Figure 1A**). For the *nicotine* group, following nose-poking in the active hole at FR3 the infusion pump was activated for 2 s. For the *nicotine + cue* and *saline + cue* groups, nose-poking in the active hole at FR3 turned on the cue light located above the hole, simultaneous to the activation of the infusion pump. The cue light remained on for 4 s in total. Since it is known that nicotine alone is poorly self-administered in the absence of other salient stimuli (Caggiula et al., 2002), the *nicotine* group was substantially larger than the other two experimental groups, as it was expected based on preliminary data that only 40%–50% of animals in this group would meet the desired self-administration criteria.

After 27 daily basal sessions (**Figure 1A**), rats showing significant self-administration behavior were administered with Varenicline (1 mg/kg, ip) 30 min prior to a basal self-administration session. The average number of infusions over training sessions 26–27 was used as the baseline. The Varenicline dose was chosen based on previous literature (e.g., O'Connor et al., 2010).

Effect of Varenicline on the Reinforcement-Enhancing Effect of Nicotine During *Nicotine + Cue* Self-administration

A Procedure to Alter the Primary Reinforcing Effects of the Cue Light (Experiment 2)

A key determinant of the interaction between nicotine infusion and an associated discrete cue light relies on the primary reinforcing effect of the cue. A key issue is then to be able to manipulate the reinforcing effect of the cue during nicotine self-administration. The goal of Experiment 2 was to establish a protocol where the reinforcing effects of the cue can be altered. Therefore, we tested in rats self-administering *saline + cue* whether we could decrease or increase the primary reinforcing effects of the cue by altering its visual salience, by either adding or removing an interfering ambient light, respectively.

Two groups of rats were trained for *saline + cue* self-administration, as described in Experiment 1 except that for one group (*AL*, $n = 15$), the *Ambient light* (*AL*) was on throughout the first seven acquisition sessions. For the other group (*No Ambient light*, *No AL*, $n = 15$) the *AL* was off during the same period (**Figure 1B**). On the eighth session of self-administration, the *Ambient light* conditions were switched; turned off for the *AL* group and on for the *No AL* one. On sessions 9 and 10,

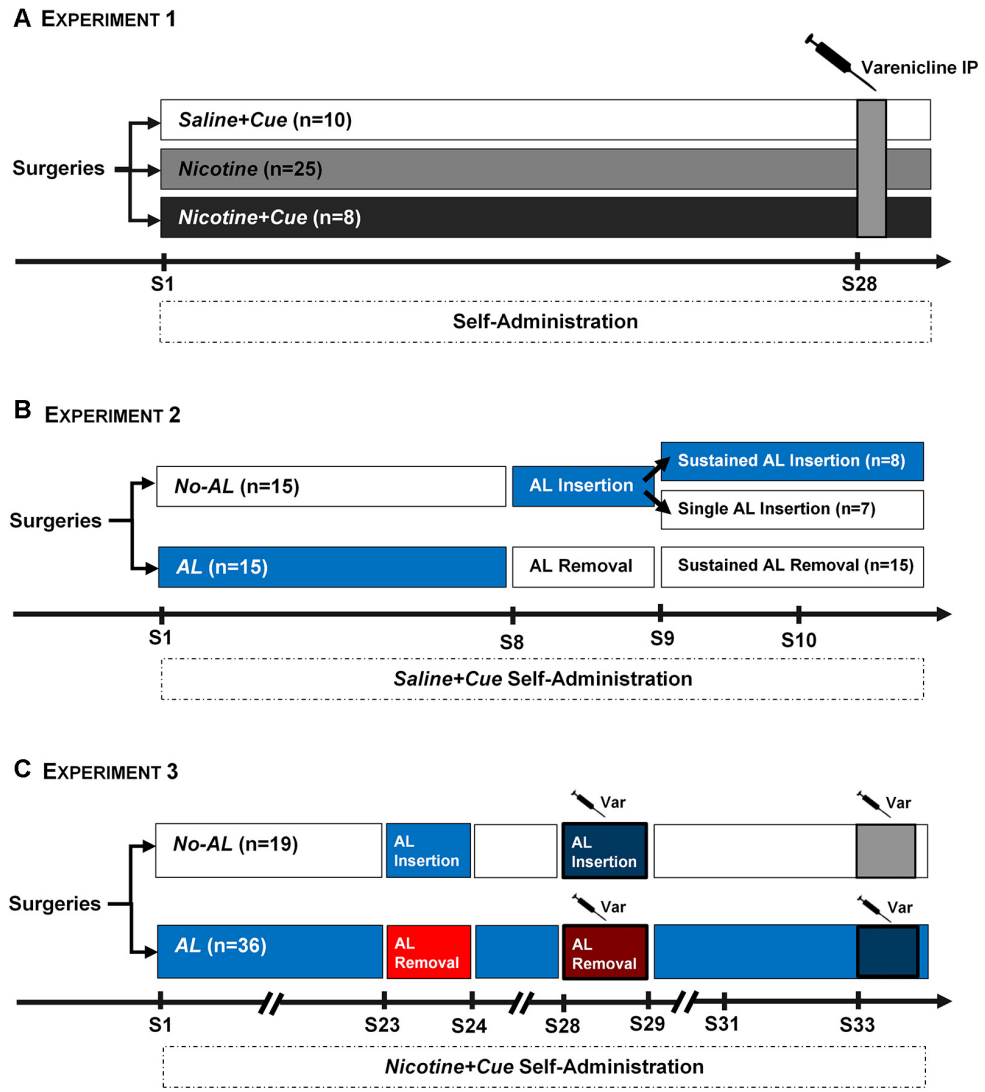

FIGURE 1 | Experimental Protocols. **(A)** Experiment 1. Three groups of rats (*saline + cue*, $n = 10$; *nicotine*, $n = 25$; *nicotine + cue*, $n = 8$) were trained for self-administration for 27 sessions. The *Nicotine* group was substantially larger than the other two experimental groups, as it was expected that only around 40%–50% of animals in this group would acquire self-administration criteria. An acute IP injection of Varenicline was applied 30 min before session 28 of self-administration. **(B)** Experiment 2. Two groups of rats were trained for *saline + cue* self-administration. For one group (*AL*, $n = 15$), the Ambient light was on throughout the first seven sessions. For the other group (*No AL*, $n = 15$) the Ambient light was off during the same period. On the eighth session of self-administration, the Ambient light conditions were switched; removed for the *AL* group and inserted for the *No AL* group. On sessions 9 and 10, the *No AL* group was split into two, with half of the rats switched back to their original *No AL* condition (Single AL insertion, $n = 7$), while the other half remaining under the new *AL* condition (*Sustained AL insertion*, $n = 8$). All rats from the *AL* group remained without the Ambient light for sessions 9 and 10 (*Sustained AL removal*). **(C)** Experiment 3. Two groups of rats were trained for *nicotine + cue* self-administration, using the same *AL* and *No AL* conditions as in Experiment 2. The *AL* group was substantially larger ($n = 36$) than the control *No AL* condition ($n = 19$), as it was expected that *AL* could delay acquisition of *nicotine + cue* self-administration. Similar to Experiment 2, the *AL* conditions were switched in Session 23, after which rats were returned to basal conditions. On session 28, the switch of *AL* conditions was re-applied, with the addition of a Varenicline IP injection 30 min before session. Rats were then allowed to return to a stable baseline before a final test using a single Varenicline injection on a basal self-administration.

the *No AL* group was split into two, with half of the rats switched back to their original *No AL* condition (*Single AL Insertion* subgroup, $n = 7$), while the other half remaining under the new *AL* condition (*Sustained AL Insertion* subgroup, $n = 8$). All rats from the *AL* group remained without the *AL* for sessions 9 and 10.

Effect of Varenicline on the Reinforcement-Enhancing Effect of Nicotine During Nicotine + Cue Self-administration (Experiment 3)

Based on the results of Experiment 2, two groups of rats were trained for *nicotine + cue* self-administration, as described in Experiment 1. As in Experiment 2 the *AL* was on throughout

the basal training self-administration sessions, for one group (*AL*, $n = 36$), and was off for the other one (*No AL*, $n = 19$; **Figure 1C**). The *AL* group was substantially larger than the control *No AL* condition, as it was expected that the *AL* could delay acquisition of *nicotine + cue* self-administration. On session 23, we tested the effect of: (1) suppressing; and (2) adding, the *AL* on self-administration in the *AL* and *No AL* groups, respectively. Rats were then brought back to the respective basal conditions until session 28, when we tested the effect of Varenicline (1 mg/kg, i.p.) administered 30 min prior to session during which the *AL* was manipulated, i.e., suppressed in the *AL* group and inserted in the *No AL* group. Rats were then returned to basal conditions, and once responding was stable over two consecutive sessions and had returned to the level of infusions of sessions 21–22, we tested the effect of Varenicline (1 mg/kg, i.p.) administered 30 min prior to a basal session.

Data Analyses

Self-administration

Total responses in the active and inactive holes and total number of infusions per self-administration session were considered.

Effect of Varenicline and/or *AL* Manipulation

To evaluate Varenicline and/or *AL* manipulation (*AL* removal or *AL* insertion), delta infusions from baseline (infusions at test − infusions at baseline) were calculated. Baseline infusions correspond to the mean infusions over the two sessions preceding a test.

Statistical Analyses

Self-administration behavior was analyzed using repeated measures ANOVA with Time (number of sessions), Hole (active vs. inactive), Test (Baseline vs. Test), Condition (*AL*On to *AL*Off, *AL*Off to *AL*On, *AL*On to *AL*Off+Var, *AL*Off to *AL*On+Var), as within-subject factor, and experimental group (*saline + cue*/*nicotine + cue*/*nicotine*, *AL*/*No AL*) as between-subject factor.

Significant main effects or interactions were explored by pairwise comparisons of means using the Newman Keuls *post hoc* test. Pearson's correlation analyses were used to investigate the correlation between variables of interest. A *t*-test was used to compare the *AL* Removal effects (or of *AL* Insertion effects) on *saline + cue* and *nicotine + cue* self-administration.

The results are presented as mean ± SEM. Differences were considered significant at $p < 0.05$.

The statistical analyses were performed using the STATISTICA 13.3.0 (2017) data analysis software system (TIBCO Software Inc., Palo Alto, CA, USA).

RESULTS

Nicotine and a Cue Light Contribute Synergistically to Self-administration (Experiment 1)

Over the first 15 self-administration sessions, *saline + cue, nicotine + cue* and *nicotine* rats differed significantly regarding number (Group, $F_{(2,42)} = 10.77$, $p < 0.001$) and pattern (Group × Session, $F_{(28,588)} = 6.7$, $p < 0.0001$) of reinforcers earned (**Figure 2A**), as well as number and discrimination in responses (**Figure 2B**).

Nicotine first tended to compromise, but secondarily amplified, the reinforcing effects of a discrete cue light. Thus, *nicotine + cue* rats increased self-infusions from session 1 to session 6 ($p < 0.0001$) while the *saline + cue* rats showed the opposite profile ($p < 0.0001$) when the *nicotine* rats remained stable over the same sessions ($p = 0.87$). The compared self-administration patterns of the three groups suggest that nicotine and cue interact synergistically.

Nicotine and Saline + Cue Are Mild but Different Reinforcers (Experiment 1)

The behavior of the *saline + cue* and the *nicotine* groups stabilize at a similar level from session 6 (**Figure 2A**). Observations exclude, however, that the behavior is just driven by the stimulus that is common to the two groups, i.e., intravenous infusion. Indeed, up to session 6, the *saline + cue* group produced a higher number of self-infusions than the *nicotine* one (Group, $F_{(1,36)} = 8.5$, $p < 0.01$) and the two profile of self-infusions differ with decrease, and progressive increase, up to stabilization, respectively (Group × Session, $F_{(5,180)} = 5.7$, $p < 0.0005$). Also, in a preliminary experiment, eight rats were trained for *saline + cue* for 13 sessions in conditions similar to the ones described in Experiment 1. Omission of the cue on session 14 produced a significant decrease in self-administration (**Supplementary Figure S2**) supporting that the cue contributes to the reinforcing effects in *saline + cue* rats.

The mild reinforcing effects in *nicotine* and *saline + cue* rats, as compared to *nicotine + cue* rats, were further confirmed when using threshold criteria for discrimination, i.e., number of infusion and stability in behavior (see "Materials and Methods" section), to define a significant self-administration behavior at the individual level. By session 15, only 40% of the *nicotine* rats (10/25) had acquired self-administration, compared to 100% of the *nicotine + cue* rats (8/8), and 50% of the *saline + cue* rats (5/10; **Supplementary Figure S3A**).

Distribution of the individual scores of self-infusions in the rats showing self-administration based on these criteria (**Supplementary Figure S3B**) also further supports the difference in nature of the reinforcers acting in the *nicotine* and the *saline + cue* groups. **Supplementary Figures S3C–F** show the self-infusions and hole responses in rats, which either reached (**Supplementary Figures S3C,D**) or did not reach (**Supplementary Figures S3E,F**) these criteria.

Varenicline Decreases *Nicotine + Cue* and Nicotine Self-administration (Experiment 1)

After 27 sessions, the effect of Varenicline on self-administration was tested in the *saline + cue* ($n = 5$), *nicotine + cue* ($n = 8$) and *nicotine* ($n = 11$) rats that met self-administration criteria evaluated on behavior during sessions 26 and 27. Varenicline decreased self-administration as measured by the number of self-infusions earned (Test effect, $F_{(1,24)} = 30.6$, $p < 0.0001$). This effect was function of the experimental group (Test × Group, $F_{(2,24)} = 4.71$, $p < 0.05$) with a significant effect in rats self-administering *nicotine + cue*

($p < 0.0001$) and *nicotine* ($p < 0.05$; **Figure 2C**). According to the effect on self-infusions, Varenicline decreased nose-poking in a group-dependent (Test effect, $F_{(1,24)} = 22.49$, $p < 0.0001$; Test × Group, $F_{(2,24)} = 4.55$, $p < 0.05$) and hole-dependent manner (Test × Hole, $F_{(1,24)} = 28.4$, $p < 0.0001$), exclusively targeting the active hole (**Supplementary Figure S4**).

The effect of Varenicline, as measured by the delta-infusions from baseline (Group effect, $F_{(2,24)} = 3.29$, $p < 0.05$), was higher in the *nicotine + cue* group than in the *saline + cue* ($p < 0.05$) and *nicotine* groups ($p < 0.05$), in which the delta-infusions were similar (**Figure 2D**). However, the effect of Varenicline was different from zero in the *nicotine* group ($p < 0.0001$), but not in the *saline + cue* group. Notably, in the nicotine group, the Varenicline effect, as measured by delta-infusions from baseline, did not correlate with basal self-infusions (**data not shown**).

Varenicline Targets the Reinforcing-Enhancing Effect of Nicotine on Its Associated Salient Cue

Results of Experiment 1 supported that nicotine and the cue interact to produce reinforcing effects, and that Varenicline significantly decreased the *nicotine + cue* combined reinforcer. However, it did not allow concluding whether Varenicline was specifically targeting this interaction. To further explore this hypothesis, we aimed at testing the effect of Varenicline while manipulating this nicotine-cue interaction in the same individuals. As a first step, we aimed at developing a procedure that would allow promoting (vs. compromise) the nicotine-induced enhancement of the reinforcing properties of its associated cue. As this enhancement is depending on the primary reinforcing effects of the cue, we initially worked on a procedure allowing to increase (vs. decrease) these reinforcing effects.

An Interfering Ambient Light (AL) Appears to Alter the Primary Reinforcing Effects of the Discrete Cue Light (Experiment 2)

As in Experiment 1 rats self-administered *saline + cue*, as shown by a significant discrimination between active and inactive holes over the seven sessions of self-administration (Hole effect, $F_{(1,28)} = 28.7$, $p < 0.0001$; **Supplementary Figures S5A,B**). However, this discrimination was a function of the experimental

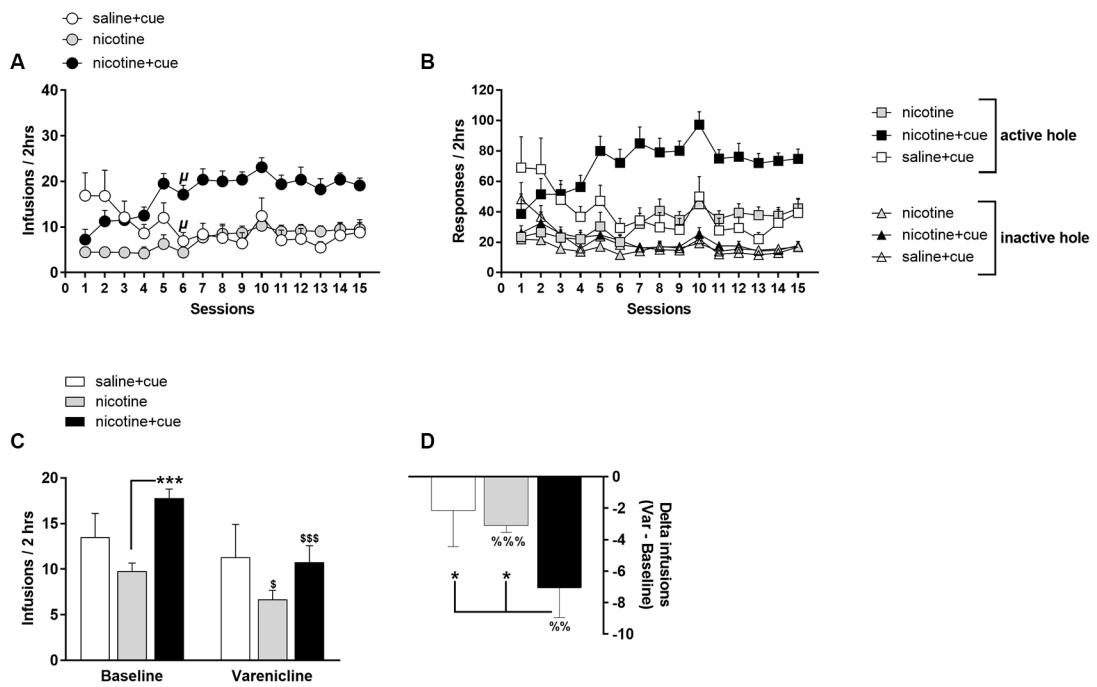

FIGURE 2 | Nicotine and infusion-associated discrete cue light contribute synergistically to self-administration behavior. Operant nose-poking at FR3 in active hole was reinforced by the delivery of an intravenous infusion of saline associated with the lighting of a salient visual cue above the active hole (*saline + cue*), of a nicotine intravenous infusion associated with the lighting of a salient visual cue above the active hole (*nicotine + cue*) or of the sole delivery of a nicotine intravenous infusion (*nicotine*). **(A)** Infusions earned per session over the 15 first behavioral sessions. **(B)** Responses in the active and inactive holes per session over the 15 first behavioral sessions. Symbols denote group mean and error bars denote SEM. **(C)** Mean infusions earned in basal conditions (*Baseline*) and after Varenicline administration (1 mg/kg i.p., 30 min prior to session) in rats self-administering *saline + cue*, *nicotine + cue* or *nicotine*. For *Baseline*, infusions are averaged over the two last sessions prior to Varenicline test. **(D)** Effect of Varenicline as calculated by the delta between infusions earned in baseline and infusions earned under Varenicline effect, in rats self-administering *saline + cue*, *nicotine + cue* or *nicotine*. Symbols and bars denote group mean and error bars denote SEM. $^{\mu}p < 0.0001$ as compared to respective session 1. $^{*}p<0.05$, $^{***}p < 0.001$. $^{\$}p < 0.05$ and $^{\$\$\$}p < 0.001$ as compared to respective baseline. $^{\%\%}p < 0.01$, $^{\%\%\%}p < 0.001$, as compared to zero.

group. The *AL* appears to compromise the expression of the reinforcing effects of the discrete cue light (Group effect, $F_{(1,28)} = 10.4$, $p < 0.01$). In standard conditions (*No AL*), *saline + cue* induced self-administration behavior, while in the *AL* condition, with the same *saline + cue* reinforcer, rats did not discriminate significantly between active and inactive holes (Group × Hole, $F_{(1,28)} = 18.7$, $p < 0.0001$). In the standard *No AL* condition, although behavior decreased over sessions, discrimination remained significant, up to the last session ($p < 0.005$).

Not only *No AL* rats discriminated between the inactive control hole and the active hole associated with *saline + cue* delivery (**Supplementary Figures S5A,B**), but they also earned significantly more reinforcers than the *AL* rats (Group effect, $F_{(2,44)} = 8$, $p < 0.01$; **Figure 3A**).

It is unlikely that the absence of discrimination and the reduced number of reinforcers in *AL* rats was due to a non-specific stress-like or aversive effect. First, the number of inactive nose-poking was not affected (**Supplementary Figure S5B**), suggesting that the *AL* effect may be targeting the reinforcement of the cue light. Second, the switch of the *AL* conditions on session 8 further attested that the *AL* compromises the cue light reinforcing effects. While *AL Insertion* decreased self-administration, *AL removal* increased it (Condition × Group, $F_{(1,28)} = 7.7$, $p < 0.01$; **Figure 3B**).

To better understand the effect of *AL* Removal and Insertion, *No AL* rats were split into two groups for the following two sessions (9 and 10): one group (*Sustained AL Insertion*, $n = 8$), maintained the newly acquired *AL* condition, while the other (*Single AL Insertion*, $n = 7$) returned to their *No AL* condition (**Figure 1B**). *Sustained AL Insertion* further diminished self-administration in sessions 9 and 10, compared to sessions 6 and 7, while rats in the *Single AL Insertion* group appeared to compensate by increasing their mean infusions, when back to the initial *No AL* condition (**Supplementary Figure S6**). In the case of the *Sustained AL Removal* rats, the removal of the *AL* was maintained for sessions 9 and 10, further increasing self-administration in comparison to sessions 6 and 7 (**Supplementary Figure S6**).

The Interfering AL Procedure Appears to Reveal the Reinforcement-Enhancing Effect of Nicotine on Its Associated Salient Cue During Nicotine Self-administration (Experiment 3)

Having revealed that it was possible to increase the reinforcing effects of the cue by *AL* Removal, we tested its effect on *nicotine + cue* self-administration, both on acquisition and once behavior was established.

During acquisition under the *No AL* condition, the number of *nicotine + cue* self-infusions was higher than under the *AL* condition (Group effect, $F_{(1,49)} = 5.36$, $p < 0.05$), but the difference decreased over the 20 self-administration sessions (Group × Session, $F_{(19,331)} = 4.14$, $p < 0.0001$) and the *AL* group reached and maintained the level of self-infusions of the *No AL* group by session 15 (**Figure 3C**).

Rats in the *AL* condition did not discriminate between active and inactive holes in the first session, contrary to *No AL* condition (**Supplementary Figure S5C**). Even though inactive nose-poking was similar in the *AL* and *No AL* conditions from session 2, in a manner similar to *saline + cue* self-administration, active responding in the *AL* condition remained low compared to *No AL* conditions up to session 5.

Once stabilized, removal of the *AL* increased self-administration behavior by the *AL* group (Test effect, $F_{(1,35)} = 47.9$, $p < 0.0001$), while insertion of the *AL* decreased self-administration behavior by the *No AL* group (Test effect, $F_{(1,18)} = 24.46$, $p < 0.001$; **Figure 3D**).

As for *saline + cue*, it is unlikely that *AL* compromised *nicotine + cue* self-administration due to a non-specific stress-like or aversive effect. Notably, during the first self-administration session (**Supplementary Figure S5C**), total responses were not lower in *AL* rats, and absence of discrimination between active and inactive holes resulted from equal high responses in inactive and active holes, and not reduced responses in the active hole.

Critically, as summarized in **Figure 3E**, the effect of the *AL* removal was much more pronounced in *nicotine + cue* conditions compared to *saline + cue* conditions (*t*-test, $p < 0.01$), suggesting that any increase in visual salience of the cue is magnified by nicotine. By comparison, introduction of the *AL* had the same effect in both *nicotine + cue* and *saline + cue* conditions, suggesting a non-specific effect on visual perception, which is not potentiated by nicotine.

Varenicline Targets the Reinforcement-Enhancing Effect of Nicotine on Its Associated Salient Cue (Experiment 3)

Once stabilized, self-administration behavior by the *AL* group was altered by removal of the *AL*, by Varenicline or a combination of both (Test effect, $F_{(2,70)} = 64.8$, $p < 0.0001$). According to the condition tested, the test effect was different however (Test × Condition, $F_{(2,70)} = 76.3$, $p < 0.0001$). *AL* removal alone produced an increase (**Figure 4A**, red bar) in *nicotine + cue* self-administration ($p < 0.001$). When *AL* removal was combined with Varenicline administration, Varenicline abolished completely the effect of *AL Removal* and decreased *nicotine + cue* self-administration below *AL* Baseline (**Figure 4A**, dashed red bar, $p < 0.01$ vs. *AL Baseline*). However, this latter effect was of a lower extent than when Varenicline was applied in the basal self-administration conditions, i.e., with maintenance of the *AL* ($p < 0.001$; **Figure 4A**, gray bar). Critically, Varenicline and *AL* Removal effects were not simply additive. When evaluating the effect of *AL Remov + Var* to the effect of *AL Remov* alone, one yields an effect which is much higher than the one of Varenicline alone on basal self-administration, suggesting that Varenicline specifically abolishes the enhancing effects of the *AL* Removal (**Figure 4B**). Noteworthy, this interpretation is supported by the correlation analysis (**Figure 4C**) showing a strong inverse correlation between the effect of *Increased Cue Salience* by *AL* Removal (ΔALRemov = *AL*Remov − *AL* baseline) and the calculated Var effect during *Increased Cue Salience* by *AL* Removal (ΔALRemov + var − ΔALRemov). Varenicline treatment during Increased Cue Salience by *AL* Removal appears to reduce infusions from an amount equivalent to the increase produced by the Increased Cue Salience. In other

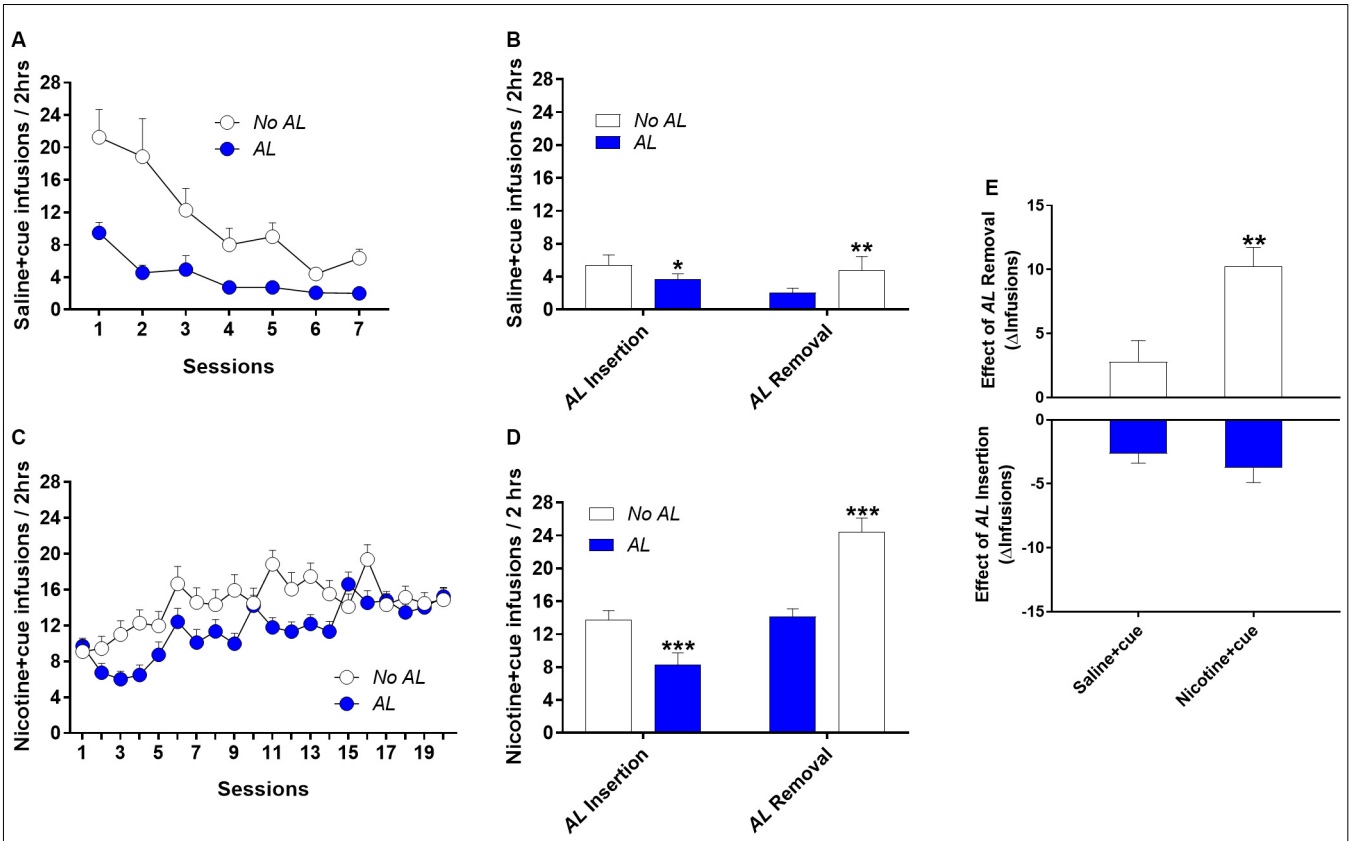

FIGURE 3 | An interfering ambient light (AL) appears to alter the primary reinforcing effects of a salient discrete cue light. **(A)** Infusions earned per session over seven behavioral sessions during which operant nose-poking in the active hole was reinforced at FR3 by the delivery of an intravenous infusion of saline associated with the lighting of a cue light above the active hole. The presence of a 15 Lux *Ambient light* (*AL*) reduced self-administration behavior as compared to the control condition (*No AL*). **(B)** Effect on infusions earned of *AL Removal* and *AL Insertion* in rats trained for *saline + cue* self-administration over seven sessions in the *AL* and *No AL* conditions, respectively. Basal infusions are averaged over the two last sessions prior to *AL Insertion (or Removal)* test. The interfering AL delays acquisition of nicotine + cue self-administration. **(C)** Infusions earned per session over the first 19 behavioral sessions during which operant nose-poking in the active hole was reinforced at FR3 by the delivery of an intravenous infusion of nicotine associated with the lighting of a cue light above the active hole. **(D)** Effect on infusions earned of *AL Removal* and *AL Insertion* in rats trained for *nicotine + cue* self-administration in the *AL* and *No AL* conditions, respectively. The interfering AL procedure allows revealing the reinforcement-enhancing effect of nicotine on its associated salient cue. **(E)** Comparison of *AL Removal* and *AL Insertion* effects in rats trained for *saline + cue* or *nicotine + cue* self-administration. While *AL Insertion* in *No AL* rats produced a similar decrease in *saline + cue* and *nicotine + cue* rats (bottom), *AL Removal* produced a stronger increase in *nicotine + cue* rats (top). Symbols and bars denote group mean and error bars denote SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

words, in these *AL* Removal conditions, Varenicline appears to decrease specifically the individual increase produced by *AL* Removal, i.e., the individual potentiation of *nicotine + cue* self-administration produced by the Increased Cue Salience.

Self-administration behavior by the *No AL* group was decreased by insertion of the *AL*, by Varenicline or a combination of both (Test effect, $F_{(2,36)} = 4.4$, $p < 0.05$; **Figure 4D**). According to the condition tested, the test effect was different however (Test × Condition, $F_{(2,36)} = 9.3$, $p < 0.001$). Insertion of the *AL*, in rats trained in absence of it, produces a significant decrease in *nicotine + cue* self-administration (**Figure 4D**, blue bar), which was similar in amplitude to the effect of Varenicline (**Figure 4D**, gray bar). When combined with *AL* Insertion, Varenicline amplified the effect of the *AL* Insertion (**Figure 4D**, dashed gray bar). Notably, the combined effect of *AL* Insertion and Varenicline were not synergistic but additive as shown in **Figure 4E**. When subtracting the *AL Insert* effect from the *AL insert + Var* effect, to get the *Var effect on decreased cue salience*, the result was similar to the effect of Varenicline alone (*Var effect alone*; **Figure 4E**). Although less strong, similarly to the effect of Varenicline on *Increased Cue Salience* by *AL* Removal, there was a correlation between the decreased effect of *AL* Insertion on self-administration and the effect of Varenicline on this *AL* Insertion effect (**Figure 4F**), supporting that Varenicline had a bi-directional effect on the nicotine-induced increase cue reinforcement, depending on how the AL manipulation altered said cue reinforcement.

DISCUSSION

Varenicline is acknowledged as one of the most efficient therapeutic tools for tobacco dependence. However, its efficacy is limited both in time and to a portion of patients (Oncken et al., 2006; Niaura et al., 2008; Jordan and Xi, 2018). Even though

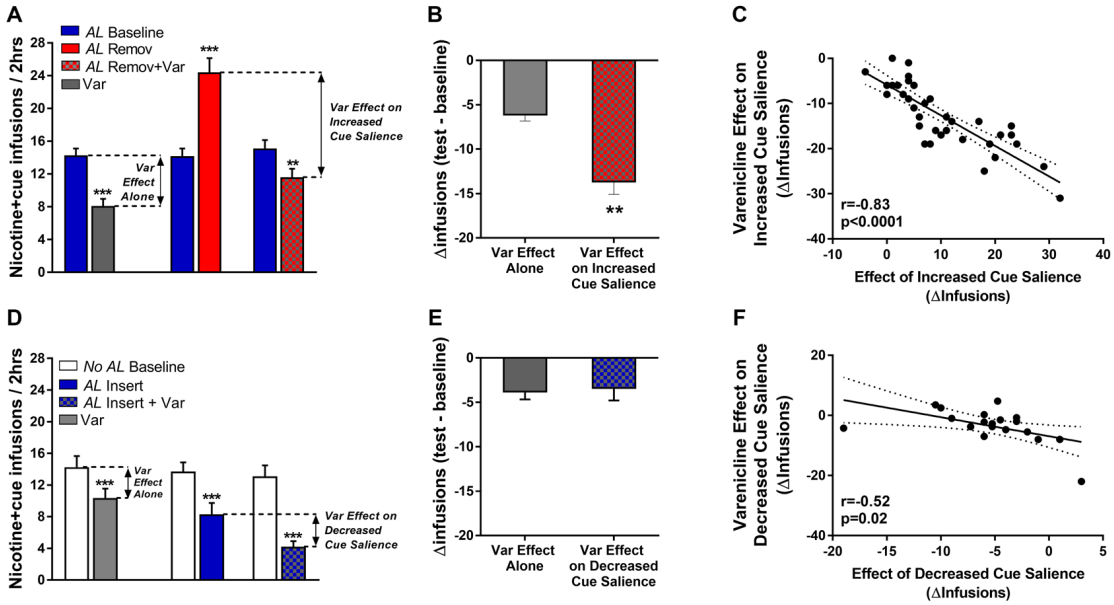

FIGURE 4 | Varenicline targets the reinforcement-enhancing effect of nicotine on its associated salient cue. **(A)** Infusions earned in rats trained for *nicotine + cue* self-administration in the presence of the interfering *AL* (*AL Baseline*), in response to Varenicline (*Var*), to *AL* Removal (*AL Remov*) or a combination of both (*AL Remov + Var*). **(B)** Comparison of Varenicline effect in *AL Baseline* condition (Infusions *Var AL Baseline* − Infusions *AL Baseline*) and in *Increased Cue Salience* condition [by *AL Removal*; calculated from the combined effect of *AL Removal* and *Varenicline* (Infusions *AL Remov + Var* − Infusions *AL Baseline*) minus the effect of *AL Removal* (Infusions *AL Remov* − Infusions *AL Baseline*)]. Varenicline absolute effect was amplified in the *Increased Cue Salience* condition (by *AL Removal)*. **(C)** Almost 1 to 1 negative correlation between the effect of *Increased Cue Salience* and the calculated effect of Varenicline on *Increased Cue Salience*. The individual increase in *nicotine + cue* infusions by *Increased Cue Salience* was antagonized by Varenicline. **(D)** Infusions earned in rats trained for *nicotine + cue* self-administration in the absence of the interfering *AL* (*No AL* Baseline), in response to Varenicline (*Var*), to *AL* Insertion (*AL Insert*) or a combination of both (*AL Insert + Var*). **(E)** Comparison of Varenicline effect in *No AL* baseline condition (Infusions *Var No AL* baseline—Infusions *No AL Baseline*) and in *Decreased Cue Salience* condition [by *AL Insertion*; calculated from the combined effect of *AL Insertion* and *Varenicline* (Infusions *AL Insert + Var* − Infusions *No AL Baseline*) minus the effect of *AL Insertion* (Infusions *AL Insert* − Infusions *No AL Baseline*)]. Varenicline absolute effect was similar in the two conditions. **(F)** Correlation between the effect of *Decreased Cue Salience* [by *AL Insertion*] and the calculated effect of Varenicline on Decreased Cue Salience (by *AL Insertion*). Bars denote group mean and error bars denote SEM. Data points reflect individual scores. $^{**}p < 0.01$, $^{***}p < 0.001$.

the molecular pharmacology of Varenicline is well-known (Coe et al., 2005; Rollema et al., 2007a), its psychopharmacological actions are still poorly understood. In this study, we evidenced that acute Varenicline reduced nicotine-induced enhancement of the reinforcing properties of a nicotine-paired cue during intravenous self-administration. This effect appeared to depend on how much nicotine-cue interactions were contributing to self-administration behavior at the individual level. Conversely, the decrease by acute Varenicline of self-administration of nicotine alone appeared not related to individual basal levels of self-administration.

Nicotine Alone Is a Poor Primary Reinforcer, but Is Strong Enough to Drive Self-administration in Certain Individuals, but Not in Others

Nicotine has weak primary reinforcement properties. Hence, classical nicotine self-administration has been developed to pair contingent nicotine IV delivery with the presentation of a salient visual cue light (Caggiula et al., 2002). A discrete cue light alone can act as a primary reinforcer in drug naïve rats (Deroche-Gamonet et al., 2002). In our study, we used the *saline + cue* condition as a control group evidencing the contribution of the cue in driving self-administration behavior. Comparison with the *nicotine + cue* group reveals the actual contribution of nicotine in *nicotine + cue* self-administration behavior.

In our study, by session 15, 100% of all rats trained in *nicotine + cue* condition showed criteria of significant self-administration behavior, but only 40% of all rats trained in the *nicotine* condition reached the same criteria. These results not only confirm the well-known observation described by Caggiula and colleagues, but it extends it with the observation that some rats appear much more sensitive to the reinforcing properties of nicotine, thus driving nicotine self-administration despite the lack of salient environmental cues, supporting that individuals may vary in the mechanisms that drive their nicotine-seeking (Garcia-Rivas and Deroche-Gamonet, 2019).

A Novel Procedure That Allows Targeting the Reinforcing-Enhancing Effects of Nicotine on Its Associated Salient Cue During Nicotine Self-administration

In a previous study, Palmatier et al. (2007) demonstrated that the reinforcement-enhancing effects of nicotine on visual cues

are dependent on the strength of the primary reinforcement of such cues in a nicotine-naïve state, with a stronger enhancing effect observed for visual cues with higher primary reinforcement properties. Further studies have assessed the effect of Varenicline on this nicotinic enhancement of cue reinforcement, but in conditions that are different from volitional nicotine intake (Levin et al., 2012; Barrett et al., 2018). Here, we developed a novel experimental approach that attempted a sudden increase in the visual salience of the nicotine-paired cue, through the removal of an interfering Ambient light (*AL*). This approach allowed us to explore the observations by Palmatier et al. (2007), but in the context of nicotine self-administration, and within the same individuals.

A possible explanation for the interfering effect of the *Ambient Light (AL)* in seeking behavior could be a non-specific aversive or stressful effect, rather than a reduction in the reinforcing effects of the cue. However, this explanation appears unlikely. The aversive effect of an ambient stressor would have impacted both active and inactive responding, while this is not the case. Critically, in the first *nicotine + cue* self-administration session, total responding was similar whether the *Ambient Light* was present or not. It is noteworthy that the presence of the *AL* delayed the acquisition of self-administration of *nicotine + cue*, which became equivalent to that of the *No AL* condition starting session 17. Overall, this data suggests that the effect of the *AL* is due to a reduction of the visual salience of the cue through visual interference, rather than a mere stress effect caused by the *AL*. Further studies, including progressive ratio schedules of reinforcement, could validate the interfering role of *AL* in cue reinforcement.

Importantly, the increase in self-administration due to removal of the visual interference was much more pronounced in *nicotine + cue* conditions compared to *saline + cue* conditions, supporting a nicotine-specific effect. This difference could be explained by the different value of the cue in these two conditions. In the *saline + cue* condition, the cue is acting as a primary reinforcer (Deroche-Gamonet et al., 2002). In the *nicotine + cue* condition, the cue is both a primary and a secondary reinforcer, and both reinforcing effects can be enhanced further by nicotine itself (Caggiula et al., 2009). However, it is more likely that the strong nicotine-specific increase in responding after *AL* removal is due to the magnifying effect by nicotine on a sudden increase in cue reinforcing effects, whether primary or secondary in nature. Supporting this view, previous studies show that nicotine can increase the reinforcement and incentive salience of cues that have already reinforcing value, whether primary or secondary (Donny et al., 2003; Chaudhri et al., 2006; Palmatier et al., 2007, 2013; Rupprecht et al., 2015). It thus follows that any increase in salience of nicotine-paired cues would be magnified even further by nicotine, as supported by our study. No other study to date has specifically addressed this possibility. By comparison, decreasing the cue salience by introduction of the *AL* has the same decreasing effect on both *nicotine + cue* and *saline + cue* self-administration, suggesting in this instance a non-specific decrease in visual perception, which is not altered by nicotine.

Varenicline Targets the Reinforcing Effects and Reinforcing-Enhancing Effects of Nicotine on Its Associated Cue

In accordance with the literature (Rollema et al., 2007b; O'Connor et al., 2010; Le Foll et al., 2012; Funk et al., 2016), we showed that Varenicline 1 mg/kg reduces *nicotine + cue* self-administration. We were interested in exploring whether such robust decrease in self-administration is due to Varenicline affecting nicotine reinforcement, nicotine-cue interactions, or a combination of both. Here we demonstrated that acute Varenicline also decreases behavior in rats self-administering nicotine alone, although to a lesser absolute extent. In the same conditions, acute Varenicline has no effect on the self-administration of the salient visual cue by itself.

A limitation in exploring Varenicline effects on the sole reinforcing effects of nicotine is that these are relatively weak, and even for those rats that acquired nicotine self-administration without the presence of a nicotine-paired cue, their baseline nicotine-seeking behavior is substantially lower than for *nicotine + cue* self-administration. This could compromise the detection of Varenicline effects, as decreases in responding are less evident when the baseline responding is already low. In trying to bypass this limitation, a recent article by Kazan and Charntikov (2019) studied the role of Varenicline in nicotine reinforcement through a behavioral economics approach. Briefly, they trained rats to self-administer *nicotine + cue* through daily escalated FR schedules of reinforcement, calculated the individual baseline demand for nicotine, and assessed the individual effect of Varenicline as a function of nicotine demand. They show that individual demand for nicotine predicted the individual reduction in self-administration after a Varenicline challenge. This could look contrary to our results (i.e. absence of correlation between basal self-infusions and Varenicline effect on basal self-administration in the *nicotine* group - experiment 1) because escalation of schedules of reinforcement is supposed to bring into evidence the role of nicotine reinforcement. However, the *nicotine + cue* protocol used by Kazan and Charntikov (2019) cannot disentangle the primary reinforcement of nicotine from the reinforcement-enhancing effect of nicotine on the associated visual stimulus. The same protocol with nicotine as the sole reinforcer would help clarify the case.

Our study also complements previous findings in clarifying the reinforcing-enhancing effects of Varenicline on a visual cue: namely, that these effects are only observed when individuals have been previously exposed to nAChR agonists. Contrary to our study, Clemens et al. (2017) and Barrett et al. (2018) showed that acute Varenicline increased the self-administration of a visual cue alone in the absence of nicotine. Furthermore, Levin et al. (2012) briefly reports in drug-naïve animals, the reinforcing-enhancing effects of Varenicline on visual cues. However, and differently to our case, in these studies, rats had been previously exposed to either nicotine or Varenicline. In Clemens et al. (2017), rats had been previously trained for *nicotine + cue* self-administration and Varenicline tested after seven self-administration of the cue alone, through a nicotine extinction-like procedure. In Barrett et al. (2018), Varenicline

was tested following a history of repeated passive exposure to nicotine administered after the cue self-administration sessions. In Levin et al. (2012), the authors make a brief comment that the reinforcing-enhancing effects of Varenicline were evident in the first seven sessions of repeated Varenicline exposure, although it remains unknown if the reported effects were already substantial during the first session. It is noteworthy that in these three cases, the reinforcing-enhancing effects of Varenicline appear similar, regardless of whether the nicotinic agonist was present at the moment of cue self-administration (Levin et al., 2012; Clemens et al., 2017) or disconnected from it (Barrett et al., 2018). In our study, the lack of previous history with nAChR agonists in *saline + cue* rats could thus explain the lack of previously described reinforcing-enhancing effects of Varenicline (Levin et al., 2012; Clemens et al., 2017; Barrett et al., 2018). This temporal requirement could most probably involve upregulation of α4β2-containing nAChRs, caused by chronic exposure to both nicotine (Marks et al., 1983; Buisson and Bertrand, 2001; Staley et al., 2006) and Varenicline (Marks et al., 2015). Nicotine, however, is known for its acutely reinforcing-enhancing effect of stimuli, even in drug-naïve individuals (Rupprecht et al., 2015; Perkins et al., 2017). This supports that Varenicline does not necessarily reproduce a nicotine-like increase in cue reinforcing effects, but requires a cholinergic system already sensitized to nicotinic agonists, which makes rats more sensitive to the reinforcing-enhancing effect of nicotinic agonists to cues. In addition, within the same study by Levin et al. (2012), Varenicline 1 mg/kg both failed and succeeded to increase the reinforcing effects of a visual stimulus in two distinct experiments with similar design, obscuring any consistent interpretation of the effect of Varenicline at this dose. Possibly, the effect of varenicline in enhancing the reinforcement of visual stimuli could be better seen at lower varenicline doses, as reported by Levin et al. (2012), which we failed to observe in this study. Further studies using different varenicline doses are needed to explore this possibility.

Varenicline Targets the Reinforcement-Enhancing Effect of Nicotine on Its Associated Cue During Self-administration

Using a novel visual interfering procedure, we evidenced that Varenicline appears to specifically reduce the reinforcement-enhancing effects of nicotine on surrounding cues during nicotine self-administration.

Varenicline effect on nicotine self-administration was bi-directional, depending on how individuals responded to the manipulation of the *AL*: the more *AL* removal increased self-administration, the stronger the effect of varenicline in opposing cue salience (**Figure 4C**), while the less AL insertion decreased self-administration, the stronger the effect of varenicline in decreasing cue salience (**Figure 4F**). This correlation was stronger for the *AL* removal condition. It is possible that the weaker correlation in the *AL* insertion condition is related to a lower number of rats tested. Nevertheless, these results add to the evidence shown by Kazan and Charntikov (2019), that Varenicline's effects appear dependent on individual differences in nicotine reinforcement. To our knowledge, we are the first to report an effect of Varenicline that is dependent on the strength of nicotine-cue interactions: a stronger nicotine-cue interaction is associated with a stronger Varenicline effect. This observation supports the rationale for individual variations in the mechanisms of nicotine-seeking (Garcia-Rivas and Deroche-Gamonet, 2019), with some individuals being more sensitive than others to the influence of the reinforcement-enhancing effect of nicotine on environmental cues, and who could differently benefit from Varenicline treatment.

It has been previously shown that the reinforcement-enhancing effect of nicotine on cues is not only dependent on α4β2-containing nAChRs (Liu et al., 2007), but also on the dopaminergic system (Palmatier et al., 2014). Given the precise molecular pharmacology of Varenicline, a possible mechanism for Varenicline could be antagonism at the α4β2-containing nAChRs located in the ventral tegmental area (VTA), thus reducing the nicotine-induced tonic firing of dopaminergic neurons, leading to decreased tonic release of dopamine in the nucleus accumbens (NAcc; Crunelle et al., 2010). Such a mechanism could also be involved in the effect of Varenicline on the primary reinforcing effects of nicotine, which are also thought to be dependent on VTA to NAcc signaling (Di Chiara, 2000; Picciotto and Corrigall, 2002). However, acute Varenicline appears to target the former, as a function of individual response, but not the latter. An alternative mechanism could involve α7 nAChRs, or other structures in the circuitry controlling nicotine-cue interactions, such as the basolateral amygdala, an area rich in α4β2- and α7 nAChRs (Feduccia et al., 2012) and also involved in drug-cue interactions (Janak and Tye, 2015).

In our study, we have investigated the psychopharmacological targets of Varenicline during early *nicotine + cue* self-administration. Future studies should address whether prolonged exposure to nicotine changes the way Varenicline affects *nicotine* and *nicotine + cue* self-administration. The differential effects of Varenicline in *nicotine + cue* self-administration in short vs. prolonged exposure to nicotine might depend on the experimental approach: George et al. (2011) reports that Varenicline does not differently affect rats with long access to nicotine (23-h sessions) compared to short access (1-h session). The study by Clemens et al. (2017) on the other hand, shows that after an extended training (40 sessions) with a short access protocol, Varenicline seems to also target the reinforcing properties of nicotine alone, compared to early training (20 sessions). However, the specificity of this Varenicline effect is problematic, as the decrease is seen both in active and inactive responding. These results warrant further exploration.

Furthermore, as a treatment for tobacco cessation, daily doses of Varenicline are recommended in the week leading up to a cessation attempt, with continuous daily administration over the following 11 weeks after cessation (Ebbert et al., 2010). While our study only assessed the effect of an acute exposure to 1 mg/kg Varenicline, further studies need to assess if prolonged exposure to Varenicline affects the psychopharmacological dimensions of nicotine-seeking during nicotine self-administration in a different way than those after

acute exposure. Studies with repeated Varenicline administration have been performed but focused on the reinforcing effects of a visual cue either in rats never exposed to nicotine (Levin et al., 2012) or previously administered with passive nicotine injections (Barrett et al., 2018).

Despite this, our results raise therapeutic implications. Increasing clinical and preclinical data suggests that smokers differ in the mechanisms that drive their nicotine-seeking (Garcia-Rivas and Deroche-Gamonet, 2019), with some smokers having stronger sensitivity to the primary reinforcing actions of nicotine (Hutchison et al., 2007; Esterlis et al., 2016), while others being more sensitive to the effects of nicotine on surrounding cues (Perkins, 2009; Perkins et al., 2017; Van Heel et al., 2017). Our results support individual variations in both nicotine reinforcing effects and nicotine-induced enhancement of cue reinforcing effects in the rat. Our data also suggest that individual variations in nicotine-induced enhancement of cue reinforcing effects, but not individual variations in nicotine reinforcing effects, would determine the amplitude of acute Varenicline-induced decrease in seeking during volitional administration of nicotine. Altogether, Varenicline might be more beneficial for smoking cessation in those who are especially sensitive to nicotine effects on surrounding cues, and not for those who are more sensitive to the primary reinforcing effects of nicotine. Further studies need to clarify more precisely the action of Varenicline, using a preclinical model that would allow for the fine exploration of individual differences in the mechanisms that drive nicotine-seeking (Garcia-Rivas et al., 2017).

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All procedures involving animal experimentation and experimental protocols were approved by the Animal Care Committee of Bordeaux (CEEA50, N° 50120168-A) and were conducted in accordance with the guidelines of the European Union Directive 2010/63/EU regulating animal research.

AUTHOR CONTRIBUTIONS

VG-R, NC and VD-G designed the experiments. VG-R, J-FF, NC, MC-G, PR and JT performed the research. VG-R, J-FF, NC and VD-G analyzed the data. VG-R and VD-G wrote the article.

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frontiers in Behavioral Neuroscience

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Individual Differences in Hatching Time Predict Alcohol Response in Zebrafish

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There are significant individual differences in response to alcohol: some people seem to exhibit higher alcohol sensitivity, while others are more resistant. These differences are related to alcohol metabolism, inherited traits, environmental/social pressure, personal habits and other indeterminate causes. In order to test how individual differences in hatching time are related to behavioral response to different alcohol concentrations, we separated zebrafish larvae into two categories according to egg emergence time: eggs hatched between 48 and 72 hours post-fertilization (hpf) were considered early emerging (EE), while those hatched from 72 to 96 hpf were considered late emerging (LE). On the 30th day post fertilization, EE and LE fish were exposed to four alcohol concentrations: 0.00% (control), 0.10%, 0.25% and 0.50%, and behavior was recorded for 60 min. We observed average and maximum swimming speed, distance traveled, and freezing time (immobility that indicates state of anxiety). For EE fish, 0.10% alcohol did not change behavior, while 0.25% and 0.50% increased freezing and decreased locomotion. By contrast, LE fish increased locomotion when exposed to both 0.10 and 0.25% alcohol, and increased freezing time at 0.50% alcohol. These results show that zebrafish behavioral profiles exhibit different sensitivities to alcohol, likely due to traits that can be tracked from early life stages and may indicate individuals' predisposition to alcohol tolerance and dependence.

Keywords: ethanol, egg emergence, anxiety, locomotion, personality, *Danio rerio*

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INTRODUCTION

Alcohol use is an age-old problem. It is the most commonly abused drug and has a massive impact on society (WHO, 2018). The neurological effects of alcohol manifest themselves in short and long-term use, ranging from increased aggressiveness, loss of motor control and single-event memory failure (Roseribloom et al., 2004; Quoilin et al., 2013; Amorim et al., 2017) to a highly debilitating state such as Wernicke-Korsakoff syndrome (Sullivan and Pfefferbaum, 2009). However, there are enormous individual differences in response to alcohol, with some people exhibiting higher alcohol sensitivity, while others are more resistant (van Beek et al., 2014). These differences are related to alcohol metabolism, inherited traits, environmental/social pressure, personal habits and other indeterminate causes (Bartholow et al., 2003; Buisman-Pijlman et al., 2014; Gullo and Potenza, 2014). Screening individual differences in order to detect traits associated with alcohol abuse are both difficult and expensive, but some profiles seem to indicate a greater likelihood of developing alcoholism and merit more thorough investigation (Cotton, 1979; Roman and Colombo, 2009; Araujo-Silva et al., 2018).

Individual variations in phenotype and behavior were long considered noise rather than the result of several different biological processes. However, in recent decades, the study of trait variability among individuals of the same species, so-called personality, temperament, or copying strategies (Gosling and John, 1999; Koolhaas et al., 1999; Réale et al., 2007) has been gaining ground since individuals exhibit different responses to similar environmental challenges (Gosling and John, 1999; Gosling et al., 2003), which reflect both genetic and environmental determinants. For instance, it is known that individuals differ in their metabolic rate (Braga Goncalves et al., 2015; Auer et al., 2018; Pettersen et al., 2018), vulnerability to disease (Cavigelli, 2005; MacKenzie et al., 2009), cortisol response to stress (Overli et al., 2002; Øverli et al., 2006, 2007; Frost et al., 2007; Kristiansen and Fernö, 2007; Silva et al., 2010) and more recently, a number of specific gene transcripts in the brain (MacKenzie et al., 2009) in addition to the response to drugs of abuse such as alcohol (Araujo-Silva et al., 2018).

In studies investigating individual differences in several species, including mammals, birds, and fish (Baugh et al., 2017; Araujo-Silva et al., 2018; Ferreira et al., 2018), a common idea is that the behavioral profile is usually accompanied by physiological responses. Several authors suggest that active vs. passive individuals can be characterized at either end of a continuum, with many intermediate profiles in between. These two extreme profiles, defined by Koolhaas et al. (1999) as "proactive" and "reactive," display opposite physiological and behavioral responses, such as sympathethic and parasympathetic reactivity (Verbeek et al., 2008), testosterone levels (Koolhaas et al., 1999), basal cortisol and hypothalamus-pituitary-interrenal (HPI) axis activity (Koolhaas et al., 1999; Øverli et al., 2005; Silva et al., 2010), reactivity to escape stressors (Silva et al., 2010), feeding motivation, exploration and risk-taking in novel environments (Øverli et al., 2006, 2007; Frost et al., 2007; MacKenzie et al., 2009), and aggressiveness (Øverli et al., 2004). As such, it seems that the concept of individual differences refers to interindividual variation in energy consumption to cope with situations throughout life; thus, the metabolic rate of the two most contrasting profiles should be markedly different.

Given that they appear very early in life, it has been reported that individual differences can be tracked from the spawning nest in salmonids, which may be linked to some behavioral and physiological characteristics in juvenile and adult fish (Vaz-Serrano et al., 2011; Andersson et al., 2013; Thörnqvist et al., 2015; Rosengren et al., 2017). While increased vulnerability to predation is an obvious disadvantage in early hatching time, benefits include increased access to territory and food (Brännäs, 1995). Screening behavior and physiological responses in early and late emerging fish suggest similarities with the proactive and reactive profiles: early emerging rainbow trout and Atlantic salmon were shown to be bolder, dominant and with lower brain serotonin levels during stress, resembling the proactive profile, while the opposite was observed for the late emerging fish (Metcalfe and Thorpe, 1992; Vaz-Serrano et al., 2011; Andersson et al., 2013; Thörnqvist et al., 2015). This evidence suggests that the timing of emergence is linked to boldness and proactive style, another trait that may indicate individual profile.

In recent years, the use of zebrafish (*Danio rerio*) as an animal research model to evaluate the effect of alcohol on behavior has increased (Gerlai et al., 2000; Irons et al., 2010; Tran and Gerlai, 2013; Chacon and Luchiari, 2014; Luchiari et al., 2015). Their high genetic and physiological similarity with humans enables translational research (Araujo-Silva et al., 2018). Furthermore, characteristics such as external fertilization and the transparency of the fish in the embryonic and larval stages make it possible to study the developing nervous system (Irons et al., 2010). Embryonic development is fast, whereby an ovule that has been fertilized develops into a larva with a heartbeat and eyes in 24–48 h (Kimmel et al., 1995), and a rich behavioral repertoire within a few days (Budick and O'Malley, 2000; Colwill and Creton, 2011). The zebrafish response to alcohol has been shown to resemble that of humans, making them a feasible model for the study of alcoholism, its variations and consequences (Gerlai et al., 2000). Thus, considering the differences between individuals in a population and that these differences may affect how they respond to a psychotropic drug as alcohol, we aimed at evaluating the effects of different alcohol concentrations (0.00%, 0.10%, 0.25% or 0.50%) on early and late emerging profiles in zebrafish (*Danio rerio*). Our hypothesis was that individual differences observed in early stages of development affect the way animals respond to alcohol later in life.

MATERIALS AND METHODS

Animal Housing, Maintenance and Breeding

Adult zebrafish (*Danio rerio*, wild-type, both sexes) were obtained from a local farm (Natal, Brazil) and held in 50 L tanks with a multistage filtration system at the fish vivarium of the Federal University of Rio Grande do Norte (UFRN). Temperature, pH, and oxygen were maintained at 28°C, 6.7 and 6 mg/L, respectively. A 12 h light-dark cycle was adopted. Fish were fed twice a day with commercial pelleted food (Alcon Basic®, 45% protein; 5% fat, Alcon, Brazil) and live brine shrimp (Premium grade, Brine Shrimp Direct, Ogden, UT, USA).

Every other day, two female and three male zebrafish from the stock were placed in breeding tanks (30 × 15 × 20 cm) filled with 3 L of system water. An acrylic plate full of small holes was placed on the bottom of the tank to prevent the fish from accessing the eggs. Plastic plants were used to enrich the tank and promote breeding. The breeding group were placed in the tanks at around 5 pm and kept in the same room conditions as the stock (28°C, 12L:12D). Fertilization was performed by natural spawning, which usually occurred during the first hour of daylight.

A total of 50 breeding groups (100 females and 150 males) were used to obtain the total number of eggs for this study. The eggs were collected, counted and transferred to Petri dishes, which were placed in an incubator at 28°C and checked daily for mortality and hatching time. Eggs were observed from 24 to 96 hours post-fertilization (hpf), every 2 h to remove hatched fry, which were placed in a separate Petri dish. Eggs hatched between 48 and 72 hpf were considered "early emerging" (EE),

while those hatched from 72 to 96 hpf were denominated "late emerging" (LE). EE and LE larvae were held in separated Petri dishes kept in the incubator until 120 hpf. Next, the larvae were transferred to plastic trays (BioPrátika 30.3 × 22.1 × 7.5 cm; filled with 1 L of system water) and exogenous feeding initiated. The larvae were fed powdered food dissolved in system water (Alcon Alevinos®, 44% protein; 5% fat, Alcon, Brazil) three times a day until 12 days post-fertilization (dpf). From 12 dpf on, the larvae were fed powdered food and brine shrimp until 30 dpf. A silicon tube connected to an air pump supplied oxygen to the water in the trays. At 15 dpf, fish were removed to tanks with water recirculating system where the volume was increased to 2 L and debris were washed away continuously. All the procedures were approved by the Animal Ethics Committee of UFRN (CEUA 122.055/2018).

Experimental Design and Alcohol Exposure

At 30 dpf, EE and LE larvae were divided into eight groups and exposed to four alcohol concentrations: 0.00% (control), 0.10%, 0.25% and 0.50%. Thus, different alcohol concentrations and hatching profiles could be tested from the groups formed (four EE groups and four LE groups): EE 0.00% alcohol (n = 13), LE 0.00% alcohol (n = 13), EE 0.10% alcohol (n = 13), LE 0.10% alcohol (n = 13), EE 0.25% alcohol (n = 13), LE 0.25% alcohol (n = 13), EE 0.50% alcohol (n = 13), and LE 0.50% alcohol (n = 13). For alcohol exposure, 99% absolute ethanol (Dinâmica, Química contemporânea Ltd, Brazil) was diluted into the system water to achieve the three concentrations used (0.10%, 0.25% and 0.50%).

Cell culture plates containing six wells were used for behavioral screening. The solution containing alcohol (0.00, 0.10, 0.25 or 0.50%) was used to fill the wells and fish was individually transferred to the well (one fish per well). Fish behavior was recorded from above for 60 min using a digital camera (Sony DCR-SX45 Digital Video Camera Recorder). The video files were transferred and analyzed using a video tracking program developed at MatLab (Pinheiro-da-Silva et al., 2017). Behavior was evaluated every min for 60 min, creating time course screening of the hatching profile of fish exposed to each alcohol concentration. The following parameters were quantified: average and maximum swimming speed, total distance traveled, and time spent immobile (freezing).

Statistical Analysis

Behavioral data were assessed to check for outliers, homogeneity, normality, zero trouble, collinearity and independent variables, as suggested by Zuur et al. (2010) Since our data were longitudinal (every minute for 60 min, obtaining repeated measures of the same animal), we used mixed effects modeling to develop a model for the response variable (each behavioral parameter evaluated) and explanatory variable (profile: EE or LE, alcohol concentration: 0.00, 0.10, 0.25, 0.50%, and time: 60 min). The mixed model used showed random effect factors due to the behavioral variation within the groups, fixed effect factors caused by the alcohol concentration effects observed, and standard error.

We used the glmmPQL command from the MASS package (Venables and Ripley, 2003) of the R program (Team, 2015) to develop the mixed model. The response variable freezing varied between 0 and 60 s, with a binomial distribution error and logit link function (according to Zuur et al., 2010). The response variables average speed, maximum speed and distance traveled were positive continuous quantitative data, not including zero ($Y > 0$); thus, a goodness-of-fit test was performed to determine the best distribution function. The gamma distribution function best fit these variable data (link function = inverse). In all cases, the *post hoc* comparisons between treatments of each model were made using Tukey's test in the "lsmeans" package (Lenth and Hervé, 2015). The significance level was set at $p < 0.05$.

Following the time-course analysis, we conducted a two-way analysis of variance (ANOVA) test to evaluate the main effect of alcohol concentration (four levels: 0.00, 0.10, 0.25, and 0.50%) and the emerging profile (two levels: early and late emerging) during the last 20 min of alcohol exposure, as well as the interaction between alcohol concentration and profile. Thus, the first response to the novel environment could be ignored and fish behavior under the influence of alcohol highlighted. When ANOVA exhibited statistical significance, we used Tukey's HSD *post hoc* test. The significance level was set at 0.05.

RESULTS

The locomotor parameters of the fish hatching profile exposed to each alcohol concentration for 60 min are presented in **Figure 1**. **Figure 2** depicts average locomotor response during the last 20 min of alcohol exposure.

Mixed model comparison showed that average zebrafish swimming speed during acute alcohol exposure was significantly changed due to fish profile (GLMM, $\chi^2 = 5.32$, $df = 1$, $p = 0.02$; **Figures 1A,B**) and alcohol concentration (GLMM, $\chi^2 = 37.38$, $df = 3$, $p < 0.001$; **Figures 1A,B**), but did not change over time (GLMM, $\chi^2 = 3.14$, $df = 1$, $p = 0.07$; **Figures 1A,B**). The interactions terms that showed statistical significance were profile vs. treatment (GLMM, $\chi^2 = 4.54$, $df = 3$, $p = 0.05$), profile vs. time (GLMM, $\chi^2 = 8.44$, $df = 1$, $p = 0.003$), and alcohol concentration vs. time (GLMM, $\chi^2 = 19.64$, $df = 3$, $p < 0.001$). The *post hoc* comparison test (lsmeans) between groups indicates that EE 0.01% showed the lowest average swimming speed, significantly different from EE 0.25%, EE 0.50%, LE 0.10%, LE 0.25% and LE 0.50%. Lsmeans also demonstrated that the highest average speed was obtained by LE 0.25%, significantly different from the other groups.

For maximum swimming speed, mixed model comparison indicated statistical significance due to alcohol concentration (GLMM, $\chi^2 = 12.17$, $df = 3$, $p = 0.007$; **Figures 1C,D**) and over time (GLMM, $\chi^2 = 14.37$, $df = 1$, $p < 0.001$; **Figures 1C,D**), but no change with respect to fish profile (GLMM, $\chi^2 = 0.41$, $df = 1$, $p = 0.52$; **Figures 1C,D**). The interaction terms that displayed statistical significance were alcohol concentration vs. time (GLMM, $\chi^2 = 27.19$, $df = 3$, $p < 0.001$), while the other interactions were not significant (profile vs. alcohol concentration: GLMM, $\chi^2 = 7.37$, $df = 3$, $p = 0.06$; profile vs. time: GLMM, $\chi^2 = 0.81$, $df = 1$, $p = 0.37$). The *post hoc* comparison test (lsmeans) showed that EE 0.10% obtained the highest maximum

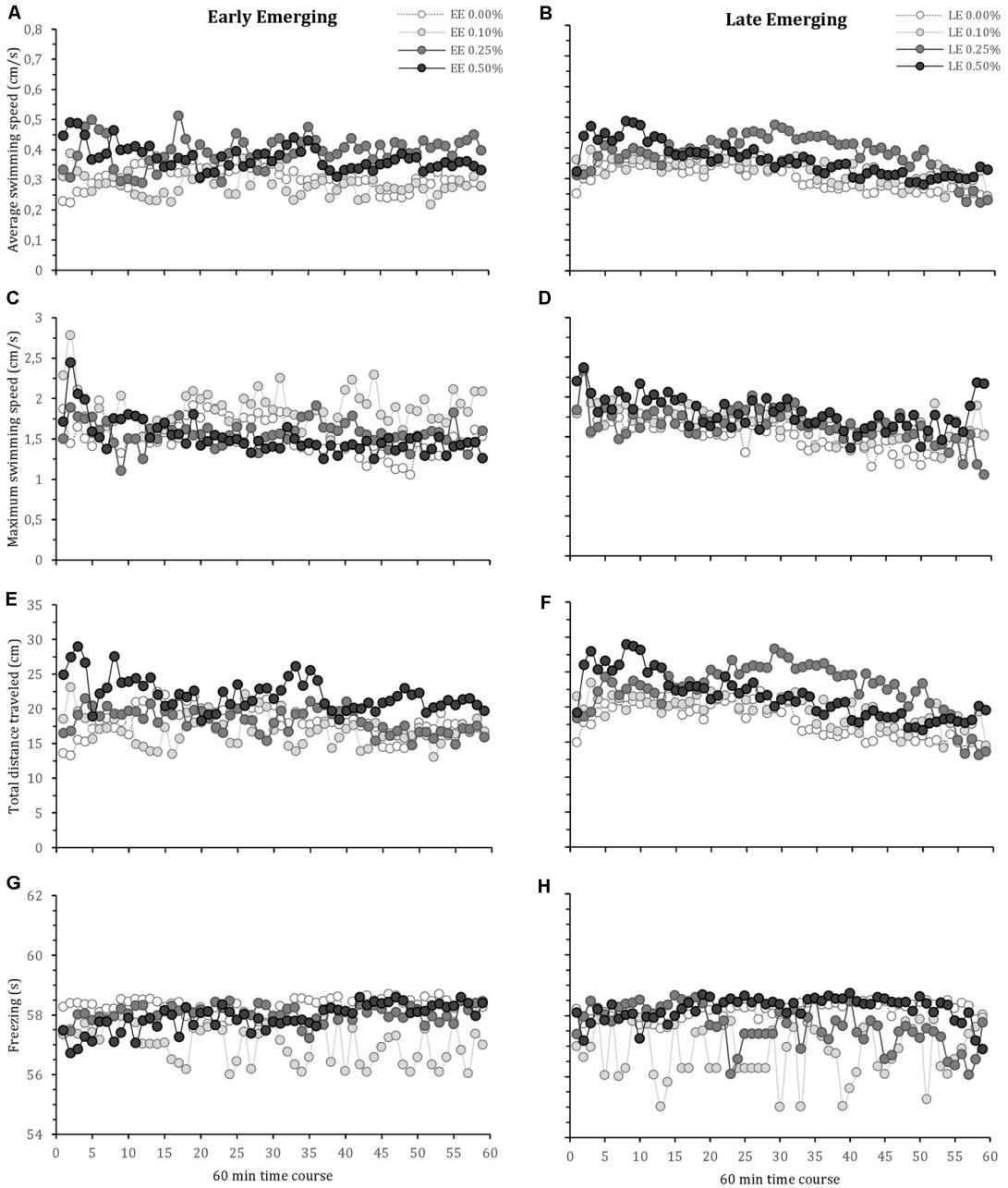

FIGURE 1 | Time-course behavioral changes during 60-min alcohol exposure in early and late emerging zebrafish. Early and Late emerging profiles were determined by the emergence time form the egg. Eggs hatched up to 72 hours post-fertilization (hpf) were considered early emerging (EE) and eggs hatched after 72 hpf were the late emerging (LE) fish. EE and LE larvae were kept up to 30 days old and then were exposed to alcohol concentrations of 0.00% (control), 0.10%, 0.25% or 0.50% for 60 min during which behavior was recoded. Graphs **(A)** and **(B)** show EE and LE fish average swimming speed, respectively. Graphs **(C)** and **(D)** present EE and LE fish maximum swimming speed, respectively. Graphs **(E)** and **(F)** are total distance traveled for EE and LE fish, respectively. Graphs **(G)** and **(H)** depict freezing time for EE and LE fish, respectively. Sample sizes (n) were 13 for each group. Mean are shown for every 1-min intervals of the total 60 min recording.

speed, followed by LE 0.50%, while the control groups (EE 0.00% and LE 0.00%) exhibited the lowest maximum speed (**Figure 2B**).

The mixed model comparison of total distance traveled showed statistical significance for fish profile (GLMM, $\chi^2 = 5.16$, $df = 1$, $p = 0.02$; **Figures 1E,F**) and alcohol concentration (GLMM, $\chi^2 = 39.61$, $df = 3$, $p < 0.001$; **Figures 1E,F**), but there was no change over time (GLMM, $\chi^2 = 1.21$, $df = 1$, $p = 0.27$; **Figures 1E,F**). The interaction terms showed statistical significance for profile vs. treatment (GLMM, $\chi^2 = 13.46$, $df = 3$, $p = 0.003$), profile vs. time (GLMM, $\chi^2 = 7.82$, $df = 1$, $p = 0.005$), and alcohol concentration vs. time (GLMM, $\chi^2 = 12.62$, $df = 3$, $p = 0.005$). Lsmeans *post hoc* test showed

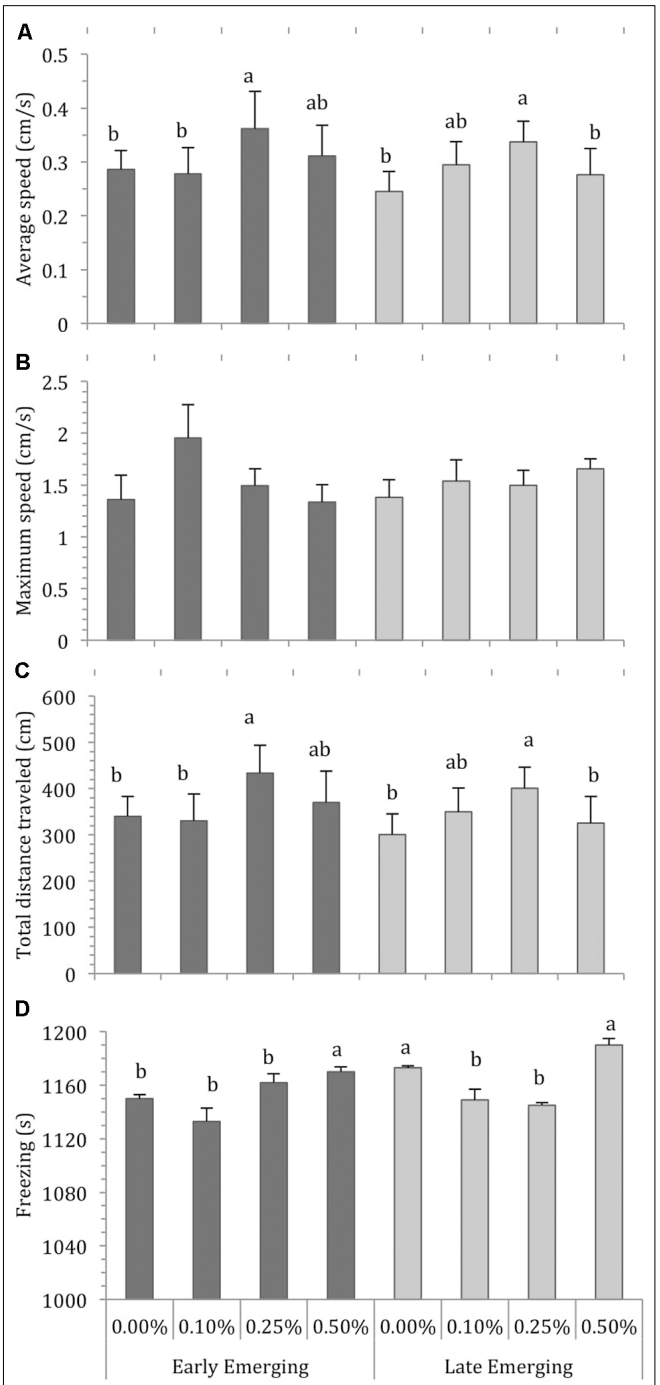

FIGURE 2 | Comparison of Early Emerging and Late Emerging zebrafish following alcohol exposure. Fish emerging profiles were determined according to eggs hatching time: individuals that hatched up to 72 hours post-fertilization (hpf) were the early emerging fish and those that hatched after 72 hpf were the late emerging fish. Early and late fish were exposed to alcohol concentration (0.00%, 0.10%, 0.25% or 0.50%) at 30 days post fertilization and behavior was recoded. Sample sizes (n) were 13 for each group. Bars indicates zebrafish locomotor behavior [mean + standard error of the mean (SEM)] during the last 20 min of 60-min alcohol exposure period: **(A)** average swimming speed, **(B)** maximum swimming speed, **(C)** total distance traveled, and **(D)** freezing. Different letters indicate statistical differences between groups analysis of variance (ANOVA, $p < 0.05$).

that EE 0.010% and LE 0.25% traveled the shortest and longest distance, respectively.

Freezing behavior, which reflects a state of immobility related to fear and anxiety usually observed when zebrafish are placed in a novel environment, is presented in **Figure 1D**. A mixed model comparison reveals statistical significance in fish profile (GLMM, $\chi^2 = 10.41$, $df = 1$, $p = 0.003$; **Figures 1G,H**), alcohol concentration (GLMM, $\chi^2 = 3.03$, $df = 3$, $p < 0.001$; **Figures 1G,H**) and over time (GLMM, $\chi^2 = 4.75$, $df = 1$, $p = 0.03$; **Figures 1G,H**). Alcohol concentration vs. time showed statistical significance (GLMM, $\chi^2 = 27.19$, $df = 3$, $p < 0.001$), the interactions between profile vs. alcohol concentration (GLMM, $\chi^2 = 3.37$, $df = 3$, $p = 0.006$) and profile vs. time (GLMM, $\chi^2 = 3.81$, $df = 1$, $p = 0.03$) were also was statistical significant. The *post hoc* comparison test (lsmeans) showed that EE 0.10% and LE 0.10% obtained the lowest freezing values, followed by LE 0.25%, while the other groups obtained the highest freezing values.

Depending on their profile, fish display the effects of alcohol concentration during the last 20 min of exposure because they are already habituated to the new environment and display no novelty-related anxiety. **Figure 2** presents the average values of the locomotor parameters measured during the last 20 min of alcohol exposure in fish from the early and late emerging profiles. Two-way ANOVA of the average swimming speed data revealed a significant effect of alcohol exposure ($F_{(3,103)} = 4.09$, $p = 0.008$). The main effect of emergence profile was non-significant ($F_{(1,103)} = 0.75$, $p = 0.39$). The profile vs. alcohol concentration interaction term was non-significant ($F_{(3,103)} = 0.29$, $p = 0.83$). Tukey's HSD test showed that EE 0.25% and LE 0.25% differed significantly ($p < 0.05$) from the other groups and LE 0.10% was significantly different from LE 0.00% (**Figure 2A**).

For maximum speed, two-way ANOVA found that the main effect of alcohol concentration was non-significant ($F_{(3,103)} = 0.86$, $p = 0.46$), as were the effects of profile ($F_{(1,103)} = 3.23$, $p = 0.07$) and interaction terms ($F_{(3,103)} = 0.78$, $p = 0.50$; **Figure 2B**). Two-way ANOVA analysis for total distance traveled showed a significant effect of alcohol concentration ($F_{(3,103)} = 4.58$, $p = 0.01$), but the effects of profile ($F_{(1,103)} = 0.74$, $p = 0.38$) and profile vs. alcohol were non-significant ($F_{(3,103)} = 0.29$, $p = 0.82$). Tukey's HSD test indicated that EE 0.25%, LE 0.10% and LE 0.25% differed significantly ($p < 0.05$) from the other groups (**Figure 2C**). Finally, analysis of freezing data showed that the effect of both alcohol exposure ($F_{(3,103)} = 27.94$, $p < 0.01$) and emergence profile ($F_{(1,103)} = 35.52$, $p < 0.01$) were significant. The interaction terms profile vs. alcohol concentation were significant ($F_{(3,103)} = 5.03$, $p < = 0.002$). Tukey's HSD test indicated that EE 0.50%, LE 0.00% and LE 0.50% differed significantly ($p < 0.05$) from the other groups (**Figure 2D**).

DISCUSSION

In this study, we showed the effects of alcohol exposure on different zebrafish profiles according to fry emerging time: early emerging (EE) and late emerging (LE). Early emerging (EE) zebrafish showed lower anxiety-like behavior compared to their

late emerging (LE) counterparts, suggesting that some behavioral reactions may be established very early in life. Moreover, these profiles responded differently to alcohol exposure, a psychoactive drug that alters brain biochemistry and ultimately reflects on animal behavior. EE zebrafish were affected by 0.25% and 0.50% alcohol, showing increased and decreased locomotion repectively but 0.10% alcohol did not change EE fish behavior. However, LE animals increased swimming and decreased freezing when exposed to 0.10% and 0.25% alcohol, while 0.50% alcohol caused increased freezing response. These results indicate that both profiles are affected by alcohol, but very low concentrations such as 0.10% are enough to cause behavioral changes in late emerging fish, while still tolerated by EE individuals, suggesting that different sensitivity to alcohol can be tracked from an early stage.

Individual differences in behavioral profile are molded by evolution (Sih et al., 2004; Colléter and Brown, 2011), thereby exerting strong genetic influence. In a natural environment, emergence from the spawning nest is a critical ontogentetic shift subjected to high selection pressure. From this moment on, individual experiences largely affect behavior and may have consequences for fitness (Dingemanse et al., 2004; Brown et al., 2007a,b). Several studies correlate emergence time from the egg with personality traits, including exploration, risk-taking, aggressiveness, and metabolic rate (Biro and Stamps, 2008). Early emerging fry usually require less time to overcome stress (Killen et al., 2011; Vaz-Serrano et al., 2011) higher aggression (Lahti et al., 2002; Killen et al., 2012), dominance behavior and boldness, resembling the proactive copying style (Martins et al., 2011; Vaz-Serrano et al., 2011; Andersson et al., 2013). For example, Metcalfe and Thorpe (1992) and Metcalfe et al. (1995) showed that early emerging Atlantic salmon are socially dominant, exhibit a higher metabolic rate and reach smoltification earlier than late emerging fry, while Rosengren et al. (2017) suggested higher risk behavior during stress in early emerging fish. Our results for EE and LE zebrafish exposed to 0.0% alcohol (control) show that the former are less sensible to environmental changes, displaying lower freezing behavior than LE. Zebrafish are naturally explorative, but usually increase fear/anxiety response (i.e., freezing) in novel places (Wong et al., 2010; Jesuthasan, 2012; Stewart et al., 2012), which is an adaptive behavior since unknown sites may pose unforeseen threats. However, the fear/anxiety response is expected to decrease over time, but EE and LE fish took different time periods to adjust to the new tank, which seems to be related to their profile. According to Vaz-Serrano et al. (2011) and Andersson et al. (2013), there is a correlation between emergence time and stress coping styles, the early and late emerging fish corresponding to the proactive and reactive profile, respectively. As such, LE zebrafish should take longer to reduce anxiety in a novel environment.

However, alcohol exposure showed potential to change this scenario. Alcohol is a biphasic drug that initially causes an anxiolytic effect, making one more explorative, less fearful and more likely to take risks (Addicott et al., 2007; Irons et al., 2010; Araujo-Silva et al., 2018). On the other hand, increasing alcohol concentration has the opposite effect: it heightens anxiety, decreases exploration and leads to a depressive state (Charness et al., 1989; Koike and Sobue, 2006; Campbeel et al., 2013; Amorim et al., 2017; Araujo-Silva et al., 2018). Very low alcohol concentrations are not expected to change behavior. A low dose is considered subclinical, that is, it causes slight neurochemical changes in brain function without affecting behavior (Careau et al., 2008). However, the low concentration used in the present study (0.10% alcohol) which did not alter EE response, led to decreased anxiety-like behavior and increased locomotion in LE fish. Not only did the very low concentration affect LE behavior, but the other alcohol concentrations also induced locomotor alterations.

Differences between the two profiles (EE vs. LE) may explain why they showed different responses to alcohol exposure. One possibility is the metabolic level exhibited by these profiles. Several studies have related early emergence to increased metabolic rate in fish (Vaz-Serrano et al., 2011; Andersson et al., 2013; Braga Goncalves et al., 2015; Rosengren et al., 2017; Auer et al., 2018; Pettersen et al., 2018), which suggests that EE fish may metabolize alcohol faster than LE, decreasing blood levels and accelerating drug excretion earlier than LE fish. This hypothesis seems plausible, since only the higher alcohol concentrations (0.25 and 0.50%) affected EE, while 0.10, 0.25 and 0.50% alcohol altered LE behavior, leading to the conclusion that slower alcohol processing allowed the lower concentration to enter the brain and affect behavior expression.

Although Careau et al. (2008) explicitly correlated metabolic traits to different personalities, more than only metabolism level is needed to explain why EE fish became less anxious when exposed to 0.25 and more anxious with 0.50% alcohol, while their LE counterparts reduced anxiety-like behavior under 0.10 and 0.25% and increased this response with 0.50% alcohol. Thus, we believe that the neurotransmission underlying individual differences may have affected the alcohol outcomes.

Acute alcohol exposure undoubtedly acts on several brain neurotransmitter systems, promoting behavioral modifications that depend on the amount of alcohol available and neural activity level. For instance, a small amount of alcohol is considered anxiolytic (Tran et al., 2016), since it inhibits the glutamatergic system and stimulates the GABAergic system (Rico et al., 2011) causing an initial relaxation effect. Additionally, alcohol activates serotonin and dopamine release, two important neurotransmitters related to arousal states (i.e., locomotor activity and responsiveness; Chiu and Prober, 2013) and anxiety behavior (Kalueff et al., 2007; Banerjee, 2014), respectively. This response was observed in LE zebrafish exposed to both 0.10 and 0.25% alcohol, given that they reduced freezing behavior and increased exploration, corroborating other authors who reported the anxiolytic effects of lower alcohol doses and anxiogenic effects of higher doses (Gerlai et al., 2000; Mathur and Guo, 2011; Amorim et al., 2017).

In fact, the neurotransmitter systems have been suggested to differ between individual personalities. Lower serotonin and higher dopamine levels characterize proactive and bolder individuals, while reactive and shyer animals exhibit higher serotonin and lower dopamine levels (Koolhaas et al., 1999, 2010; Silva et al., 2010; Backström and Winberg, 2017). Thus, when alcohol interacts with serotonergic and dopaminergic brain

activity, the outcomes of bold and shy animals are expected to be complete opposites. For instance, alcohol increases dopamine transmission, which is suggested to be related to the user's initial feeling of pleasure, and likely much more intense in shy than bold animals, due to the lower dopamine levels in the former. In this respect, we believe that alcohol is a psychoactive drug, perceived differently by each profile, making shyer individuals more prone to developing dependence than their bold counterparts.

In the present study, EE and LE zebrafish showed different responses to alcohol. Our results suggest that 0.10 and 0.25% alcohol decreased anxiety-like behavior in LE zebrafish, while 0.50% alcohol increased anxiety. By contrast, 0.10% alcohol did not change EE behavior, but 0.25% alcohol exerted anxiolytic effects and 0.50% induced an anxiogenic response. Thus, alcohol responsiveness may be related to the intrinsic characteristics of individuals, including genetic predisposition, metabolic rate and neurotransmitter level in the brain. Alcohol, one of the most abused drugs in the world, is responsible for more than 3 million deaths a year (WHO, 2018), making prevention and effective treatments a daunting challenge. In this regard, using zebrafish to show that individual features observed since very early in life may be an influencing factor on alcohol responsiveness and dependence could lead to new studies on the mechanisms of susceptibility to alcoholism.

CONCLUSION

This study is the first to show that early life traits may be related to alcohol responsiveness, and it is important to underscore that the environment has a significant impact on behavior and decision making. Thus, life experiences should also be considered an indicator of alcohol dependence. Furthermore, future research focusing on individual differences in levels of neurotransmitters such as dopamine and serotonin before and after alcohol exposure may help understand why some profiles show greater predisposition to alcoholism than others, in addition to suggesting prospective treatments. Knowledge on how different individuals cope with the environment in order to survive and succeed is essential to understanding vulnerability to diseases such as alcoholism, a condition that the zebrafish has been contributing to elucidate, reinforcing its value in translational research.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All the procedures were approved by the Animal Ethics Committee of the Federal University of Rio Grande do Norte (CEUA 122.055/2018).

AUTHOR CONTRIBUTIONS

ML-F and AL conceived and designed the experiments and wrote the article. ML-F and HA-S perfomed the experiments. HA-S analyzed the data.

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REVIEW
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Understanding Addiction Using Animal Models

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Drug addiction is a neuropsychiatric disorder with grave personal consequences that has an extraordinary global economic impact. Despite decades of research, the options available to treat addiction are often ineffective because our rudimentary understanding of drug-induced pathology in brain circuits and synaptic physiology inhibits the rational design of successful therapies. This understanding will arise first from animal models of addiction where experimentation at the level of circuits and molecular biology is possible. We will review the most common preclinical models of addictive behavior and discuss the advantages and disadvantages of each. This includes non-contingent models in which animals are passively exposed to rewarding substances, as well as widely used contingent models such as drug self-administration and relapse. For the latter, we elaborate on the different ways of mimicking craving and relapse, which include using acute stress, drug administration or exposure to cues and contexts previously paired with drug self-administration. We further describe paradigms where drug-taking is challenged by alternative rewards, such as appetitive foods or social interaction. In an attempt to better model the individual vulnerability to drug abuse that characterizes human addiction, the field has also established preclinical paradigms in which drug-induced behaviors are ranked by various criteria of drug use in the presence of negative consequences. Separation of more vulnerable animals according to these criteria, along with other innate predispositions including goal- or sign-tracking, sensation-seeking behavior or impulsivity, has established individual genetic susceptibilities to developing drug addiction and relapse vulnerability. We further examine current models of behavioral addictions such as gambling, a disorder included in the DSM-5, and exercise, mentioned in the DSM-5 but not included yet due to insufficient peer-reviewed evidence. Finally, after reviewing the face validity of the aforementioned models, we consider the most common standardized tests used by pharmaceutical companies to assess the addictive potential of a drug during clinical trials.

Keywords: addiction models, preclinical studies, DSM-V, drug seeking behavior, relapse activity

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Abbreviations: 5-CSRTT, 5-Choice Serial Reaction Time Task; AMPA, Receptor: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BLA, Basolateral amygdala; bHR, Bred high-responder; bLR, Bred low-responder; BP, Break-point; CIE, Chronic intermittent ethanol; CNS, Central nervous system; CPA, Conditioned Place Aversion; CPP, Conditioned place preference; CS, Conditioned stimulus; CSA, Controlled Substances Act; DID, Drinking In the Dark; DS, Discriminative stimulus; DSM, Diagnostic and Statistical Manual; EA, Exercise addiction; GD, Gambling disorder; GT, Goal-tracker; FDA, Food and Drug Administration agency; HR, High-responder; IGT, Iowa gambling task; IntA, Intermittent access training; LgA, Long-access training; LR, Low-responder; NAc, Nucleus accumbens; OFC, Orbitofrontal cortex; PavCA, Pavlovian conditioned approach training; PFC, Prefrontal cortex; rGT, Rodent gambling task; SA, Self-administration; ShA, Short-access training; ST, Sign-tracker; THC, Δ^9-tetrahydrocannabinol; VTA, Ventral tegmental area.

INTRODUCTION

"*All models are wrong, some models are useful.*"
George Box

The main difficulty with modeling drug addiction using nonhuman animals is capturing an inherently complex behavioral pathology using relatively simple behavioral protocols (Spanagel, 2017; Müller, 2018). Environmental circumstances, behavioral traits and genetic factors all interplay with one another and affect an individual's susceptibility to acquiring and maintaining the use of an addictive substance, as well as relapse propensity (Everitt and Robbins, 2016). Regardless, creating better preclinical models of drug addiction is essential for elucidating the neurobiological mechanisms that contribute to addiction-related behaviors, and creating better treatment options for those afflicted with addiction. In this review article, we build on the existing literature (García Pardo et al., 2017; Lynch, 2018) and discuss the various models that exist for studying addiction-related behaviors in animals, including individual variation in addiction-related behaviors, and commonalities between drug addiction and certain behavioral addictions. We then focus on how these models are used to assess abuse potential by pharmaceutical companies.

In models such as behavioral sensitization and conditioned place preference (CPP), an animal is non-contingently administered a drug, allowing drug delivery independent from the motivation to take the drug to be assessed. More commonly used are drug self-administration (SA) models, whereby drug delivery is contingent upon the animal's motivation to take the drug. SA models are constantly evolving, with the emphasis being placed on the importance of the duration of drug experience (i.e., session length) and the temporal pattern of drug delivery as a means to better model the transition to addiction. The motivation for drug-taking can also be assessed using multisymptomatic training paradigms and behavioral economics. Factors that contribute to reinstated drug-seeking behavior, including environmental contexts, cues, stressors and the drug itself are briefly discussed, along with procedures where drugs are challenged by alternative rewards.

Independent of modeling addiction-related behaviors, we emphasize the importance and advantages of modeling individual variation within these behaviors, as is seen in humans. For example, measures of impulsivity have been shown to predict addiction liability. We also discuss two well-established models that capture individual variation in addiction-related behaviors: the high-responder/low-responder model, and the sign-tracker/goal-tracker model. While the high-responder/low-responder model captures individual variation in the acquisition of drug-taking behavior, the sign-tracker/goal-tracker model captures individual variation in relapse propensity. Thus, the two models independently capture two different phases of addiction.

Next, we discuss behavioral addictions that share several similarities with drug addiction, with an emphasis on gambling disorder. There is a high prevalence rate between those diagnosed with gambling disorder and those with a substance abuse disorder (17% for illicit drugs, 28% for alcohol dependence according to Lorains et al., 2011) and not surprisingly the criteria to diagnose the two are very similar (for review see Rash et al., 2016). As with drug addiction, accurately modeling behaviors associated with gambling disorder using rodents are complex. The growing prevalence and efforts to assess and diagnose exercise addiction are also briefly discussed.

We end this review with a discussion on the models used by pharmaceutical companies to assess the abuse potential of possible medications for the treatment of addiction. It is for this reason that critical evaluation of models used to assess addiction-related behaviors is particularly necessary, as these models are being used to evaluate treatment efficacy.

NON-CONTINGENT MODELS: BEHAVIORAL SENSITIZATION, CONDITIONED PLACE PREFERENCE AND RUNWAY MODEL

Models based on non-contingent (i.e., experimenter-administered) drug exposure are simple and quick to set up. Because of these advantages, many studies have used them to identify key reward-related neurobiological substrates and how drug exposure alters them. See **Table 1** for a succinct summary of advantages and limitations of these models.

Behavioral Sensitization

The behavioral sensitization model is at the heart of the incentive-sensitization theory of addiction developed by Robinson and Berridge (1993). The model is based on the potentiation of drug-induced locomotion after repeated non-contingent exposure to a constant drug dose. Behavioral sensitization is usually divided into two phases: the induction (or initiation) and the expression phase. During the induction phase, it is possible to measure the molecular and cellular modifications directly induced by drug exposure. The expression, tested with a drug challenge and after a variable withdrawal, is generally attributed to the long-term effects of the aforementioned drug-induced changes. Behavioral sensitization requires D1-dopaminergic receptor activation in the ventral tegmental area (VTA; Vezina, 1996) and AMPA-mediated glutamatergic transmission in the nucleus accumbens (NAc), the latter being shared with most models of drug seeking (Bell and Kalivas, 1996; Pierce et al., 1996; Famous et al., 2008). Sensitization to all drugs of abuse has also been shown to correlate with a sustained hyper-reactivity of noradrenergic and serotonergic systems in the locus coeruleus and dorsal raphe, respectively (Tassin, 2008; Doucet et al., 2013).

Behavioral sensitization has many advantages. First, drug delivery is simple, as it relies on experimenter-administered intraperitoneal injections. Depending on the experimental timeline and withdrawal periods, sensitization can be rapidly induced since potentiation can be measured after only a few drug injections, or even a single injection (Magos, 1969; Robinson et al., 1982; Vanderschuren et al., 1999a; Valjent et al., 2010). However, it is possible to use sensitization to study the long-term effects of chronic drug-exposure, one rat study showing amphetamine sensitization lasting up to a

year (Paulson et al., 1991). But beyond these technical strengths, an important characteristic of behavioral sensitization is that most drugs of abuse, including cocaine (Post and Rose, 1976), amphetamine (Segal and Mandell, 1974), morphine (Babbini and Davis, 1972), ethanol [in certain mouse strains only, never shown in rats (Didone et al., 2008; Bahi and Dreyer, 2012a,b)], and nicotine (Clarke and Kumar, 1983), induce sensitization in rodents. Notably, no behavioral sensitization has been shown with Δ^9-tetrahydrocannabinol (THC; Varvel et al., 2007). Throughout the drug spectrum, sensitization remains sensitive to individual vulnerabilities to stress and genetics factors, as demonstrated by the wide range of behaviors observed with different rodent strains (Phillips et al., 1997).

Most importantly, drugs often cross-sensitize, which means that potentiated response to one drug is observed after induction with another drug. Cross-sensitization has indeed been observed between psychostimulants such as amphetamine and cocaine (Bonate et al., 1997; Vanderschuren et al., 1999a), but also across different drug classes like amphetamine and morphine (Vezina and Stewart, 1990; Vanderschuren et al., 1997), with the exception of one study in rats where cross-sensitization was lacking (Vanderschuren et al., 1999b). Surprisingly, although THC does not induce behavioral sensitization as mentioned earlier (Varvel et al., 2007), it cross-sensitizes with opioids (Lamarque et al., 2001; Cadoni et al., 2008) and amphetamine (Lamarque et al., 2001) in rats. However, cannabinoid agonist HU 210 failed to cross-sensitize with morphine or alcohol (Hagues et al., 2008). Interestingly, pre-exposing animals to drugs, thus inducing sensitization, potentiates CPP (Lett, 1989; Gaiardi et al., 1991; Shippenberg and Heidbreder, 1995; Shippenberg et al., 1996; Meririnne et al., 2001; Harris and Aston-Jones, 2003a,b) and SA behaviors (Horger et al., 1990, 1992; Piazza et al., 1990; Valadez and Schenk, 1994; Pierre and Vezina, 1998; Covington and Miczek, 2001) compared to non-sensitized controls. These results support the idea that chronic non-contingent drug exposure used in the sensitization model allows studying the neurobiological pathways shared by all drugs.

Despite all the advantages of this model, behavioral sensitization faces major downsides that contributed to the field increasingly shifting towards other models. Before all else, the face validity of this model is limited as sensitization in humans is challenging to demonstrate, although some studies measured a potentiation of certain symptoms such as activity and energy levels, mood or speech quantity after repeated amphetamine challenges that could be compared to behavioral sensitization (Strakowski et al., 1996; Strakowski and Sax, 1998; Boileau et al., 2006). Chronic amphetamine use has also been linked to progressive augmentation in paranoid behaviors (Kalivas and Stewart, 1991). Another characteristic of this model contrasting with the clinic resides in the fact that only a few drug injections can induce sensitization in rodents, whereas in humans a large number of exposures over time often precede abuse. Because of this, it has been argued that this model can be useful to better understand the initial phases of drug intake that influence, but does not provide a complete picture, of the transition to substance use disorder (Vanderschuren and Pierce, 2010). Finally, cross-sensitization is not limited to drugs, but also extends to stressors such as foot shock (Herman et al., 1984; Sorg, 1992), restrain stress (Robinson et al., 1985) or tail pinch (Antelman et al., 1980). In addition to stress, amphetamine-sensitized animals show facilitation of sexual behavior (Fiorino and Phillips, 1999a,b), whereas rats sensitized to morphine display increased interest for food, as well as sexual and social behaviors (Nocjar and Panksepp, 2007). These results suggest that drug-sensitization induces an unspecific activation of the reward pathways, which contradicts Diagnostic and Statistical Manual (DSM) criteria of addicted individual's disinterest for natural, social or professional rewards (APA, 2013).

Conditioned Place Preference (CPP)

CPP (also known as place preference conditioning, or PPC) allows testing the rewarding or aversive properties of an experience or a stimulus. A multitude of variations of the CPP model exist, yet the standard use remains to associate an experience, such as non-contingent drug delivery, to a recognizable context, often a chamber with definitive cues identifiable by the animal. In parallel, animals are also exposed to a different neutral context. After a first phase of pre-conditioning to assert no innate preference of the animal for one of the contexts, the acquisition phase consists of pairing the drug to one context. Pairing can be achieved after single or repeated exposures, depending on the drug doses or timing exposures. On test day, or post-conditioning phase, the animal is allowed to explore the contexts freely. When the time spent in the paired context is longer to the time spent in the unpaired one, the stimulus is concluded to be rewarding. The opposite result grants aversive effects to the stimulus, defined as Conditioned Place Aversion (CPA). CPP was first shown with morphine (Beach, 1957), and a multitude of studies later showed that all drugs of abuse, including cocaine, amphetamine, methamphetamine, nicotine, alcohol and cannabis also induced CPP (Bardo and Bevins, 2000; García Pardo et al., 2017). Along with SA, CPP is the most commonly used model to test abuse potential of a new drug during clinical trials (see later section). The popularity and abundant use of this model relies on its simplicity and promptness. Moreover, the drug of interest is typically not administered on testing day, thus allowing the determination of its rewarding properties and measurement of enduring neurobiological changes induced by the drug without causing massive neurotransmitter release. It also provides a tool to definitively establish aversive effects through avoidance, which lacks in the self-administering paradigm (i.e., lack of responding to a drug can be interpreted as the drug having aversive or neutral properties). Importantly, individual differences and susceptibilities to the rewarding effects of drugs can be measured using this paradigm. While reviewing how the novelty-seeking endophenotype impacts psychostimulant responses, Arenas et al. (2016) summarized the potentiated responses of high responding rats to sub-threshold doses of amphetamine and cocaine compared to low responding animals (see the "Individual Variation in Addiction-Related Behaviors" section below for more detailed definition of the High-responder/Low-responder model). This potentiated response

could be linked to the corticotropin-releasing factor (CRF) since a study found that mice continually overexpressing CRF show potentiated cocaine CPP only in mice displaying low behavioral reactivity to novelty in contrast to mice with high locomotor response to novelty (Kasahara et al., 2015). More recently, a study established a positive correlation between rats exhibiting risk-taking behaviors and methamphetamine-induced CPP (Takahashi et al., 2019), corroborating the ability to detect individual differences using this paradigm. However, CPP is not specific to abused substances, since natural rewards such as food (Cason et al., 2010), novelty (Klebaur and Bardo, 1999), physical exercise (wheel running; Antoniadis et al., 2000) and sexual behavior (Paredes, 2009) induce robust CPP. Similar to the behavioral sensitization and SA models, CPP heavily relies on motor capacities, thus requiring appropriate controls to assess possible sedative or anxiolytic effects of the drug that are not present when testing the animal in a drug-free state.

Using receptor-specific agonists and antagonists, many studies (for an extensive review see Tzschentke, 2007) have established a necessary role of the usual suspects within reward circuitry (dopaminergic, glutamatergic, GABergic, cholinergic, noradrenergic and serotonergic systems) in the induction and maintenance of CPP. Consistent with this idea of shared reward-related mechanisms, inducing CPP often potentiates the behavior observed in other models of addiction as seen in a study where expression of cocaine behavioral sensitization is only observed in the compartment paired with the drug during CPP, and not in a novel compartment (Duvauchelle et al., 2000). Using CPP and CPA can also help elucidate heterogeneous behaviors in other models. Supporting this, the authors of a study comparing animals self-administering cocaine at a high and stable level to animals failing to do so elegantly show that self-administering animals exhibit cocaine CPP, while non-self-administering ones show CPA (Rademacher et al., 2000). These results argue that cocaine effects are appetitive for some animals but aversive for others. However, inducing CPP with one reward does not always predict a potentiated reward-induced behavior in other models, as reported in a study that found no correlation between the magnitude of novelty-induced CPP and the degree of amphetamine SA (Klebaur et al., 2001). Another study reported that ethanol consumption during the SA pre-exposure phase negatively correlates with ethanol-induced CPP in mice (Nocjar et al., 1999).

In some studies, CPP is also used to model relapse and dissect the neurobiology of drug-seeking (for extensive review, see Aguilar et al., 2009). Prior to CPP reinstatement, animals undergo extinction training, consisting in either exposing the animal to the previously reward-paired context without administering the reward or administering sham injections in the paired and unpaired contexts (Epstein et al., 2006; Aguilar et al., 2009). CPP reinstatement, shown for cocaine, amphetamine, methamphetamine, morphine, heroin, nicotine, ethanol and MDMA, can be induced by a priming dose of drug or different types of stress, including footshock, immobilization and forced swim (Liu et al., 2008; Aguilar et al., 2009). A study further demonstrated robust cocaine CPP reinstatement induced by conditioned fear stimuli, in this case an odor or a tone previously paired with footshock (Sanchez and Sorg, 2001). In a study testing the establishment, maintenance, extinction and reinstatement of cocaine CPP, authors showed that once developed, place preference endures for several weeks and is rapidly reinstated after extinction training following cocaine priming injections (Mueller and Stewart, 2000). Similar to the induction and maintenance part of CPP, several neurotransmitters have been shown to drive reinstatement, including glutamatergic, dopaminergic and noradrenergic transmissions. The role of these systems seems to be highly dependent on reinstatement modality, i.e., drug priming or stress (Aguilar et al., 2009).

The Runway Model

The runway model has been used to evaluate opponent aversive and rewarding components of drugs. It was developed to study goal-directed behaviors with natural rewards (Hull, 1934; Crespi, 1942; Miller, 1944). From the start compartment, animals learn to cross a 6-foot long straight corridor (the runway) to reach the goal compartment, where the reward is delivered (Ettenberg, 2004, 2009). Prior to drug exposure, food-deprived animals are trained to enter the goal compartment for food pellets. Subsequently, entering the goal compartment is associated with drug delivery. The run time, i.e., the time the trained animal takes to reach the goal compartment, is the resultant of approach and avoidance behaviors and is interpreted as an index of motivation/aversion for the reward. While in some cases the drug-seeking is delivered in a noncontingent fashion, the model has been adapted to more recent techniques such as i.v. drug self-administration (Geist and Ettenberg, 1990) or optogenetics (Jhou et al., 2013). The runway model allows characterization of the ambivalent properties of drugs, which have been shown with most drugs including cocaine, amphetamine, heroin, morphine, MDMA and ethanol (for review see Ettenberg, 2009).

CONTINGENT MODELS OF ADDICTION-RELATED BEHAVIORS

The models presented here rely on operant learning during repeated exposure to the drug of interest. During SA sessions an animal performs an action (e.g., lever deflection or nose port entry) in order to receive an infusion of the drug. There is consensus in the addiction field that initial exposures to the drug mainly impact the prefrontal cortex (PFC), driving goal-directed behavior, and the mesolimbic regions, including the NAc, are key regions in the integration of reinforcing stimuli (Hopf and Lesscher, 2014). As the training develops, the dorsal striatum has been shown to take a major role in maintaining drug intake (Belin and Everitt, 2008). Below we discuss the main SA paradigms currently used in drug addiction research, followed by a discussion on the various ways to assess the motivation for taking a drug and relapse propensity. See **Table 1** for a brief summary of advantages and limitations of drug self-administration models and **Table 2** for models of motivation and drug-seeking and relapse models mentioned below.

TABLE 1 | Brief summary of preclinical behavioral models most currently used to study the neurobiology of addiction: non-contingent drug administration and contingent drug self-administration (SA).

Model	Advantages	Limits
Non-contingent drug administration		
Behavioral sensitization	• Long-lasting • Shared by all drugs • Cross-sensitization amongst drugs	• Lack of animal-driven behavior • Stereotypies at high doses • Not exclusive to drugs of abuse • Poor face validity
Conditioned Place Preference (CPP)	• Drug-free testing • Establishes rewarding or aversive properties	• Lack of animal-driven behavior • Not exclusive to drugs of abuse
Runway Model	• Can be adapted to contingent drug delivery • Drug-free testing • Establishes rewarding or aversive properties	• Requires initiation training with other rewards • Not exclusive to drugs of abuse
Contingent—drug self-administration (SA)		
Short Access (ShA)	• Short training sessions • Reliably shows escalation of intake and relapse behavior	• Does not capture compulsive drug-taking behavior
Long Access (LgA)	• Greater escalation of intake, higher break-points and greater drug-induced reinstatement compared to ShA	• Long training sessions
Intermittent Access (IntA)	• Captures temporal pattern of drug intake observed in humans • Greater motivation to work for the drug and cue-induced reinstatement compared to ShA and LgA	• More complex behavioral training compared to ShA and LgA
2-Bottle choice-Ethanol	• Simple set up, no invasive surgery • Good correlation between intake and blood concentration • Good face validity	• Low consumption • Initiation training with other rewards
Drink in the Dark (DID)-Ethanol	• No confounding with other reward • Good face validity for binge-drinking	• Strain-specific
Chronic Intermittent Ethanol (CIE)	• Good face validity (increased voluntary consumption)	• Long protocol

Drug Self-administration Models

Animal drug SA paradigms have significantly evolved since the inception of the technique in 1962 (Weeks, 1962), as more attempts are being put forth to more accurately model drug-taking behavior in humans. A commonly used paradigm, short-access (ShA) training, involves SA sessions that generally last between 1 and 3 h. ShA paradigms reliably show an increase in drug-taking behavior and reinstatement of drug-seeking behavior, two features of human addiction. However, despite its proliferous use, arguments have been made that ShA sessions capture drug-taking behavior, but not behavior that is representative of the transition to addiction (Ahmed and Koob, 1998). That is to say, ShA may only address recreational drug use, and not the escalation to compulsive drug taking that is seen in human addicts (for review see Roberts et al., 2007). To address this limitation, a long-access (LgA; Spanagel et al., 1996) training procedure was developed that consists of SA training sessions lasting a minimum of 6 h (Ahmed and Koob, 1998), with some lasting as long as 12 h (Lucantonio et al., 2015; Cocker et al., 2019). Compared to ShA sessions, rats undergoing LgA training show greater escalation in drug-taking behavior (Ahmed and Koob, 1998; Mantsch et al., 2004; Mandt et al., 2015), are more motivated to work for an infusion of cocaine (Paterson and Markou, 2003; Hao et al., 2010), and show greater cocaine-primed drug-seeking behavior (Mantsch et al., 2004; Knackstedt and Kalivas, 2007). The neurobiological mechanisms mediating behavior as a result of these two training paradigms also appear to differ, specifically in respect to neuroplasticity within the striatum (Purgianto et al., 2013; Ducret et al., 2016).

Rats have continuous access to a drug during LgA and ShA training, however, the temporal pattern of drug delivery has been argued to also play a critical role in the transition to addiction. Human addicts have been reported to take cocaine intermittently whereby a large quantity of cocaine is consumed within a short time span, followed by a period of no drug use before consuming cocaine again (Allain et al., 2015). This intermittent pattern of drug-taking is believed to cause a constant spiking of brain-cocaine concentration levels and contribute to addiction-related behaviors (Zimmer et al., 2011). The intermittent access (IntA) training procedure emulates this behavior in a rodent model. During this task, rats are allowed to self-administer during 5 min bins that are separated by 25 min periods where the drug is not available (Zimmer et al., 2011). This procedure results in a high level of consumption during drug-available periods (Allain et al., 2018) and fluctuations in brain-cocaine concentration levels (Zimmer et al., 2012). While rats trained

TABLE 2 | Brief summary of preclinical behavioral models most currently used to study the neurobiology of addiction: motivation and seeking in contingent models, models including alternative rewards.

Model	Advantages	Limits
Contingent—motivation for drug taking		
Progressive Ratio (PR)	• Simple task • Can be done several times throughout training	• Direct comparisons between different reinforcers not possible • Reward on-board during test
Behavioral Economics	• Can measure several parameters of motivation within one session • Can be done several times throughout training • Direct comparisons between different reinforcers possible	• Complex data analysis
DSM Related	• Modeled directly from DSM criteria • Good face validity	• Requires the use of several different behavioral paradigms • Long protocol
Contingent—drug seeking and relapse		
Cued-Reinstatement	• Two types: discrete and discriminative cues • Simple set-up • Good face validity	• Action precedes the reward-cue in discrete cue-induced reinstatement (usually reverse in human population)
Context-Reinstatement	• Can assess the effects of a compilation of cues on different sensory modalities on drug-seeking behavior	• Complex SA procedures (i.e. multiple contexts needed)
Prime-Reinstatement	• Simple set-up • Good face validity	• Route of administration normally different than during SA • Reward on-board during test
Stress-Reinstatement	• Environmental and interoceptive stressors can be used • Shown to increase relapse across several classes of drugs	• Complex training with certain stressors • Difficult to model complex human psychological stressors
Contingent drug seeking in presence of alternative rewards		
Any model presenting competing choice between drugs and other rewards	• Good face validity: models closer to human experience (not-limited to drug exposure exclusively) • Myriad of alternative rewards available (appetitive foods, social interactions, enriched environment, etc.)	• Complex study of the neurobiology of each reward • Might require longer training and protocols • Might require larger n and additional controls

to self-administer cocaine using LgA procedures consume more drug, rats trained using IntA show greater motivation to work for cocaine compared to rats in LgA or ShA training (Zimmer et al., 2012). Rats also show greater cue-induced drug-seeking behavior following IntA training compared to LgA or ShA (Kawa et al., 2016, 2019). IntA results in greater dopamine concentrations within the NAc core following a single infusion of cocaine compared to LgA training, and dopamine levels correlated with several measures of motivation for cocaine (Kawa et al., 2019). IntA training, but not LgA or ShA, using psychostimulants also results in sensitization of NAc dopamine transports (Calipari et al., 2013, 2014). Taken together, it is apparent that while ShA, LgA and IntA all produce drug-taking and drug-seeking behavior, the behavioral paradigms differ in several other measures of motivation for a drug and resultant neurobiological effects, both of which are factors that should be taken into consideration during experimental design.

MOTIVATION FOR DRUG-TAKING BEHAVIOR

Motivation for a drug can be measured independent of the quantity of drug consumed or pattern of intake. Progressive ratio tests and behavioral economics can be used to assess the reinforcing properties of a reward as the price and/or demand for the drug is manipulated. Adapting DSM criteria on substance use disorders to behavioral tests are also used to more directly translate data from rodent models to human addiction.

Progressive Ratio

Progressive ratio schedules are within-session procedures where the cost of a reward exponentially increases with each subsequent trial (Hodos, 1961; Roberts and Richardson, 1992; Richardson and Roberts, 1996). Using this paradigm, the motivation of the animal to work for a reward can be measured, with the maximum number of responses an animal makes in order to receive the reward referred to as the "break-point" (BP). BPs can be taken at several time points in an experiment, yielding insight into how the reinforcing properties of a drug change over the course of drug SA training. Behavior during this test is particularly sensitive to drug dose (Roberts et al., 1989), injection speed (Woolverton and Wang, 2004; Liu et al., 2005), and availability of drug during SA as well as length of forced abstinence (Morgan et al., 2002, 2005). Due to these factors, comparing BPs for the same reward between studies is often difficult. Furthermore, BPs are not comparable between different reinforcers, as they are not

standardized to a baseline threshold, as is common using demand curves (discussed below). Nevertheless, a progressive ratio test is a useful tool to assess the motivation of an animal to work for a drug, and thus track the transition to addiction in animal models (for review see Roberts et al., 2007).

Behavioral Economics: Demand Curve Analysis

Behavioral economics approaches, specifically demand curve analyses, have become more widely used due to their unique ability to measure several parameters of motivation for a drug during SA (Bickel et al., 1993, 2011; Hursh and Winger, 1995). A demand curve is the effort an individual is willing to expend for a reward at various prices (Hursh, 1980), and so the cost or price of a reward is a function of that effort (Hursh et al., 1988). Demand curves can be generated within a single SA session using a threshold procedure (Oleson and Roberts, 2009; Oleson et al., 2011; Bentzley et al., 2013). These sessions typically last 110 min, and every 10 min the dose of drug available decreases according to a quarter logarithmic scale. At the conclusion of the session, a demand curve is fit to the data, and several variables are generated that yield insight into the reinforcing properties of the reward (Hursh and Silberberg, 2008). Recent models of demand curve analysis have used a focused fitting approach, whereby data points that are generated when brain-cocaine concentrations greatly fluctuate, generally at the start of the session when the animal is "loading" on the drug or toward the end of the session when the price is beyond what the animal is willing to work for the drug, are removed from analysis (Bentzley et al., 2013). This has been shown to result in a demand curve that more accurately represents the behavior of the subject (Bentzley et al., 2013).

The following variables are calculated from the demand curve: Q_o, P_{max}, O_{max} and α. Q_0 is a measure of the "hedonic set point" (Ahmed and Koob, 1998, 1999), or the drug intake when the effort to acquire the drug is low. It thus acts as a general measure of consummatory behavior (Oleson et al., 2011). Because the price of the reward is low, Q_o is a function of demand only. In contrast, P_{max} is a function of elasticity. Elasticity refers to the rate at which the slope of the demand curve changes as the price for the reward increases (Hursh, 1980). A demand curve showing more elasticity is indicative of an individual showing less effort to consume the reward as the price increases. P_{max} is the maximum price (responses/mg reward) an individual will pay to consume the reward, or rather the maximum effort the animal will expel to maintain its hedonic set point (i.e., Q_o; Hursh, 1991). It is not too surprising then that P_{max} values have been shown to correlate with the BP in a progressive ratio test (Rodefer and Carroll, 1997; Bickel and Madden, 1999; Lenoir and Ahmed, 2008; Oleson and Roberts, 2009). The O_{max} value, or the maximum number of responses made at P_{max}, is a function of both demand and elasticity (Hursh and Winger, 1995). This value is unique in that it is the only variable generated from a demand curve that reliably predicts the success of drug addiction treatment (MacKillop and Murphy, 2007). The last variable, α, is known as the "essential value" of a reward and is the slope of the demand curve (Hursh and Silberberg, 2008). The motivation to continue to work for the drug as price increases is inversely related to α, such that rewards with a higher essential value have smaller α values, and show less elasticity (Bentzley et al., 2013). Alpha can also be tracked across several time periods throughout SA training to see how the essential value of the drug changes with increased drug experience (Christensen et al., 2008b). An advantage of α in comparison to the other variables is that it is inherently normalized to Q_o, allowing for α values to be directly compared between reinforcers. In fact, food as a reward has a greater essential value compared to both cocaine (Christensen et al., 2008a) and methamphetamine (Galuska et al., 2011). Though P_{max} and O_{max} are not normalized to Q_o, they can manually be such that these values are also able to be compared across different rewards (Ko et al., 2002; Winger et al., 2006; Wade-Galuska et al., 2007). Overall, though data analysis is complex, demand curve analysis affords researchers the ability to parse the multiple components of motivation for drug-taking behavior within a single session and allows for the direct comparison of these components between different rewards.

Modeling DSM-Related Drug Addiction Behaviors

Another consideration when modeling addiction-related behaviors in animal models is incorporating the diagnostic criteria within the DSM for substance use disorders in humans. To that end, efforts have been put forth to model some of the criteria in animal models in order to create better preclinical models of drug addiction (Deroche-Gamonet et al., 2004; for review see Belin-Rauscent et al., 2016). Criterion often modeled includes: compulsive drug-seeking behavior when the drug is not available, high levels of motivation for the drug, and continuing to take drug despite the co-occurrence of adverse consequences (Deroche-Gamonet et al., 2004). In a rodent model, these criteria are applied by measuring drug-seeking behavior during periods of signaled no drug availability, using progressive ratio tests, and pairing a foot shock with reward consumption (for review see Belin-Rauscent et al., 2016). By using this multi-symptomatic model, rats can be separated based on the number of criteria met for a substance use disorder diagnosis, and interestingly the percent of rats that meet all criteria is very similar to the percent of human drug addicts that meet DSM criteria, further strengthening the validity of using this model (Deroche-Gamonet et al., 2004). Another advantage of this model is the ability to assess individual differences in behavioral traits and neurobiological factors that may contribute to an addicted phenotype (Belin et al., 2008, 2009, 2011; Kasanetz et al., 2010; Kawa et al., 2016).

MODELS OF REINSTATEMENT OF DRUG-SEEKING BEHAVIOR

The biggest obstacle in the treatment of drug addiction is the high rates of relapse following exposure to environmental stimuli (e.g., cues, contexts, stressors) associated with prior drug-taking behavior (Shaham et al., 2003; Bossert et al., 2013). The most common methods for examining relapse behavior in animal models is *via* tests for cue-induced [both discrete (Meil and See, 1996) and discriminative (Weiss et al., 2000)],

context-induced (Crombag and Shaham, 2002), drug-primed (de Wit and Stewart, 1981) and stress-induced (Shaham and Stewart, 1995) reinstatement. Tests for reinstatement generally occur following a period of abstinence, such as forced abstinence, voluntary abstinence, extinction training, or a combination of these procedures. While each reinstatement test isolates a specific factor that contributes to drug-seeking behavior, in humans, several of these factors likely co-occur and result in relapse. However, by studying each of these models separately, we are able to assess similarities and differences in the neurobiological mechanisms that mediate each mode of relapse (for review see Crombag et al., 2008; Bossert et al., 2013).

Cues associated with the drug-taking experience can result in craving (Childress et al., 1988, 1993) and ultimately drug relapse (see Bossert et al., 2013). There are two common types of cues used during SA training that differ based on their contingency of presentation: a discrete cue and a discriminative stimulus (DS). A discrete cue is one that is localizable and directly tied to operant responding for drug delivery (e.g., light above the lever), thus presentation of a discrete cue is contingent upon drug-taking behavior. During discrete cue-induced reinstatement, the action during SA that resulted in drug delivery and presentation of the discrete drug-associated cue now only results in the presentation of the cue. Therefore, during these tests, the conditioned reinforcing property, or the ability of the cue to invigorate ongoing behavior, of the reward-paired cue is being assessed. In humans, however, though presentation of the reward-cue invigorates drug-seeking behavior, oftentimes drug-seeking behavior precedes presentation of a reward-cue. While using a single discrete cue can evoke drug-seeking behavior, using a compound discrete stimulus, such as a cue-light and tone pairing, results in a more robust reinstatement (Kruzich et al., 2001). In contrast to a discrete cue, a DS signals when the reward is, or is not, available during SA training sessions. That is, a DS operates as an "occasion setter" (Crombag et al., 2008), signaling when operant responding for the drug will (positive DS), or will not be (negative DS), reinforced (e.g., house light turning on and off). Thus, presentation of the DS is not contingent upon drug-taking behavior. During discriminative cue-induced reinstatement tests, the positive or negative DS is noncontingently presented, and the ability of the DS to affect subsequent drug-seeking behavior is analyzed. While the use of a DS is oftentimes classified as a type of cue-induced reinstatement, some have argued that it shares more properties with context-induced reinstatement (Weiss, 2005; Trask et al., 2017).

Like cues, exposure to contexts associated with the drug-taking experience can result in relapse in humans (Wikler, 1973; O'Brien et al., 1992). A context is a compilation of several cues where no single cue predicts drug availability more than another. To test the ability of a context to invigorate drug-seeking behavior, animals are trained to self-administer drugs in one context (Context A), extinguished in a separate context (Context B), and then reintroduced to Context A for the reinstatement test (Bouton and Bolles, 1979; Crombag and Shaham, 2002; Fuchs et al., 2005). In addition to environmental cues and contexts evoking drug-seeking behavior, interoceptive cues associated with the drug experience following abstinence can also result in craving (Jaffe et al., 1989) and elevated intake (de Wit and Chutuape, 1993). Tests for drug-primed reinstatement involve the non-contingent delivery of the drug prior to being tested in extinction conditions, thus the reinstating properties of a drug are being tested while the drug is on board. In contrast to intravenous delivery as during SA, the drug prime is usually administered subcutaneously or intraperitoneally, leading to possible pharmacokinetic differences in the drug's effect. Regardless, prime reinstatement remains the only test that isolates the ability of the drug itself to invigorate drug-seeking behavior.

The ability of different environmental and interoceptive stressors to invigorate drug-seeking behavior has also been explored. During these tests, a stressor is generally administered to the animal *prior* to the session starting. The main stressors used are food deprivation (Shalev et al., 2000), intermittent foot-shock (Shaham and Stewart, 1995), and administration of yohimbine (Shepard et al., 2004), a drug that causes anxiety- and stress-like effects. While it is difficult to model the complex psychological and physical stressors relevant to the human condition using animal models, the aforementioned stressors have been shown to potentiate drug-seeking behavior across several classes of drugs (for review see Mantsch et al., 2016).

There are several similarities and differences between the neurobiological mechanisms that mediate drug-seeking behavior using the different models of reinstatement. However, such detail is not within the scope of the current review, but several reviews exist that proficiently address the neurobiology associated with different animal relapse models (see Crombag et al., 2008; Bossert et al., 2013; Mantsch et al., 2016).

ALTERNATIVE REWARDS

Most operant models currently used to dissect the neurobiology underlying abuse disorders present very restricted options to animals; they can choose between self-administering the reward or not. However, a growing number of studies prove that when given broader choices, the vast majority of animals recoil from drug rewards.

Early in the development of the addiction field, a few rare studies measured the desire of dogs or chimpanzees pre-treated with drugs to choose cocaine or morphine over food (Tatum and Seevers, 1929; Spragg, 1940), in an attempt to model addiction-like phenotypes in animals. More recently, Lenoir et al. (2007) published an elegant study that surprised many: when given the mutually exclusive option between saccharin-sweetened water or cocaine, 94% of the animals preferred the sweet water over intravenous cocaine. Rats established this preference after multiple cocaines and sweet water samplings, consistently over 15 days of training. Importantly, when cocaine was present, maximal lever sampling and locomotor sensitization confirmed its rewarding and locomotor effects. Based on these results and subsequent work, Ahmed et al. (2013) argued that the field might be limited by using models lacking competing choices to study addiction, a disorder altering value-based decision-making. To study craving after experiencing several rewards, one study tested reinstating

animals after they underwent food and cocaine SA, followed by choice tests and extinction training (Tunstall and Kearns, 2014). While the majority of rats chose to self-administer food, cocaine-primed reinstatement induced a significant increase in lever pressing of the cocaine-associated lever. Footshock and food-primed induced reinstatements however only induced a mild, non-specific increase of responding in both levers. The authors conclude that cocaine seeking can prevail over food seeking when cocaine is on board during primed reinstatement. A follow-up study established that when choosing between cocaine or grain pellets, rats still preferred pellets. However, cue-induced reinstatement following extinction training showed cocaine craving, as measured by a significant increase in lever pressing for the previously cocaine-associated lever (Tunstall and Kearns, 2016). When choosing between grain and sucrose pellets, the majority of rats self-administered sucrose over grain pellets and also responded more to the sucrose-associated lever during cued-reinstatement. The cocaine/sucrose paradigm was not tested. These results argue for a strengthening of the cocaine-associated cue despite cocaine not being the preferred option during SA.

Recently, Venniro et al. (2018) elegantly developed an operant model of choice between drugs and social interaction and showed that operant social reward prevented methamphetamine and heroin SA, even in rats exhibiting a high addiction score (Deroche-Gamonet et al., 2004). It also prevented methamphetamine incubation of craving and relapse, through protein kinase C-δ -expressing neurons in the central amygdala and inhibition of activity in the anterior ventral insular cortex (Venniro et al., 2018). These results are consistent with what is observed in humans, where greater social support and integration predicted lower risk of relapse for alcohol, opiates and cigarette smoking (Havassy et al., 1991). Another innovative study showed how social interactions profoundly affect decision-making and firing of dopaminergic cells in the VTA by analyzing the behavior of mice living in Souris City, a large environment shared by a large community of peers (Torquet et al., 2018). Based on measurements obtained after experimenter-induced social re-organizations, the authors highlight the importance of social environments on animals' individual profiles and goal-directed decision-making.

The effects of alternative rewards are not only observed in contingent models. Solinas et al. (2009) showed that upgrading mice home cages to an enriched environment not only reduced the reinforcing effect of psychostimulants, as it had been previously shown (Bardo et al., 2001; Bezard et al., 2003; El Rawas et al., 2009; Solinas et al., 2009), but completely eliminated cocaine-induced behavioral sensitization and CPP (Solinas et al., 2008). These results were later extended to additional drugs including methamphetamine, heroin and nicotine (Sikora et al., 2018).

Despite the fact that exposing animals to multiple rewards makes dissecting the neurobiological effects of each reward more complex, it brings preclinical models closer to the intricate human experience. Akin to Portugal, a few countries combat addiction and the social marginalization associated with it by offering treatment, support services and enforcing harm reduction policies (Cabral, 2017). These could be considered the clinical equivalent of enriched environment or social interaction, and the success of such drug policy supports the idea that, by implementing similar strategies, drug abuse can be greatly decreased.

MODELS OF ALCOHOL INTAKE

Similar to other drugs, alcohol abuse is a complex disorder impacted by social, economic and neurobiological factors (Goltseker et al., 2019). Aside from a few examples (Augier et al., 2014; de Guglielmo et al., 2017), voluntary alcohol consumption is typically weak, and often requires water-depriving the animals to incentivize drinking or initially pairing ethanol with a more salient reward, such as sucrose (Koob and Weiss, 1990; Becker, 2013; Goltseker et al., 2019). Since this "initiation training" introduces animals to multiple rewards and this can be problematic (see "Alternative Rewards" section above), a few rodent strains showing high preference for alcohol have been selectively bred (Li et al., 1979; Stewart and Li, 1997; Bell et al., 2006). Despite low levels of behavior, many studies use two-bottle choice and drinking in the dark (DID) models, both based on voluntary consumption. In the two-bottle choice model, animals are usually first presented with two bottles of water, later replacing one water solution bottle by another containing increasing percentages of alcohol (García Pardo et al., 2017). Access to alcohol can be continuous or intermittent, presenting the alcohol bottle only every other day (Brancato et al., 2016). Two-hours alcohol exposure is sufficient to measure significant correlations between alcohol intake and blood ethanol concentrations (Griffin, 2014). Several studies show that long-term exposure to intermittent alcohol access induces binge-drinking, potentiated alcohol preference and high blood alcohol concentrations (Wise, 1973; Carnicella et al., 2014). Because of these behaviors, this model mimics closely what is observed in humans, for whom the drinking pattern is a key factor in the development of alcohol use disorder (Kranzler and Soyka, 2018).

The DID model (Rhodes et al., 2005) takes advantage of rodent's nocturnal activity to replace the home cage water bottle by a bottle containing a high concentration (20%) ethanol solution for a short period of time (2–4 h). This ethanol exposure promotes binge drinking and pharmacologically relevant blood ethanol concentrations, high enough to cause behavioral evidence of intoxication (Thiele and Navarro, 2014). This model aims to mimic the rapid and massive consumption most often observed in adolescent alcohol drinking. It does not require any modification of the alcohol solutions with other rewards or progressive increase of alcohol percentages, and binge drinking can be observed in 4 days, making it a simple and prompt model to use. However, the model seems to be somewhat restricted by mouse strain specificity. In the original study presenting the model (Rhodes et al., 2005), the DID paradigm induced binge drinking in the high ethanol drinking strain (C57BL/6J), yet the behavior was not observed in any of the other 11 inbred mice strains tested.

Chronic intermittent ethanol (CIE) is another recent model of alcohol use disorder gaining popularity (Griffin, 2014). This paradigm combines voluntary drinking and repeated exposure to alcohol vapor. After a 4-week training period of daily 2 h voluntary alcohol drinking, mice enter a cycle of 16 h vapor exposure, followed by 8 h of control air exposure (Lopez and Becker, 2005). After repeating the cycle 4–5 times (one cycle is enough to measure significant effects, but repeated cycles potentiate the behavioral outcome), animals are then tested in limited access sessions, similar to the ones performed during the training phase. CIE-animals exhibit a significant increase in voluntary ethanol drinking compared to controls, thus modeling the increase experienced by humans developing alcohol use disorder, that have been shown to be driven by neuroadaptations in glutamatergic and CRF signaling (Griffin, 2014). Since low voluntary alcohol consumption in most rodent strains is a notable limitation to study binge drinking (see discussion above), a non-contingent version of the CIE model, the Chronic-Intermittent Ethanol Administration (CIEA) paradigm, has been developed in rats (Nogales et al., 2014; Contreras et al., 2019) and mice (Sanchez-Roige et al., 2014; Lacaille et al., 2015; Monleón et al., 2019). The protocol follows the CIE timeline or a variation of it, i.e., repeated cycles of exposure to i.p. ethanol injections (3–4 g/kg) for several consecutive days intertwined with repeated days of non-exposure. CIEA is easy and inexpensive, and in combination with simple behavioral paradigms such as locomotion or elevated plus-maze, allows studying the neurobiology of binge drinking.

Alternative models focus on ethanol seeking behavior as a way to replicate relapse. Similar to other drugs, seeking behavior can be induced by priming injections of ethanol, stress, ethanol-paired cues or a combination of these factors (Le et al., 1998; Liu and Weiss, 2002; LeCocq et al., 2018). Using an alcohol-preferring rat strain, Giuliano et al. (2015) developed a different model of cue-induced alcohol-seeking. The procedure begins with a long exposure (18 sessions) to a 2-bottle choice procedure, followed by training to instrumental response to access alcohol paired with an alcohol-associated conditioned stimuli (CS). Alcohol seeking is measured during 20 min cycles where the drug is no longer present and contingent presentations of the CS act as a reinforcer. At the end of the seeking period, ethanol is re-introduced to avoid the CS losing its reinforcing properties. This experimental design aims to model alcohol craving by creating unusually high levels of alcohol-seeking behavior induced by the CS, followed by high alcohol consumption following the craving, thus closely mimicking craving leading to ethanol consumption in humans. Other models focus on compulsive-like alcohol intake, by incorporating aversive consequences to consumption, such as pairing alcohol intake with bitter quinine or footshocks (Hopf and Lesscher, 2014). These paradigms aim to model drug use despite negative consequences, one of the key symptoms listed in the DSM-5 to characterize substance abuse (APA, 2013). Quinine-resistant alcohol consumption seems to require long periods of ethanol exposure to develop, since it is measured after long cycles (8 months) of free access to alcohol (Spanagel et al., 1996; Fachin-Scheit et al., 2006; for review see Hopf and Lesscher, 2014) or after at least 3 months of intermittent access (Hopf et al., 2010). The models pairing footshocks to ethanol intake in alcohol-preferring rats show that the punishment context-dependently decreases subsequent alcohol SA (Marchant et al., 2013), however, some rats show footshock-resistant alcohol intake (Seif et al., 2013).

INDIVIDUAL VARIATION IN ADDICTION-RELATED BEHAVIORS

It is widely acknowledged that regardless of the class of addictive drug, a minority of people who use the drug develop compulsive drug-seeking behaviors indicative of substance use disorder. Appropriately modeling this variation in animals can yield powerful insight into the neurobiological mechanisms that mediate addiction propensity. Fortunately, animal models exist that capture the individual variation inherently present in the human population. Impulsivity, a trait associated with addiction liability, can be assessed using an array of behavioral paradigms. Individual variation in the acquisition of drug-taking behavior can be captured using the high-responder (HR)/low-responder (LR) model, while individual variation in relapse propensity can be assessed using the sign-tracker (ST)/goal-tracker (GT) model. The HR/LR and ST/GT models are particularly useful as they capture individual variation in two distinct phases of drug addiction: acquisition and relapse, respectively.

Models of Impulsivity

Although not specific to addiction, impulsivity, or the tendency to act prematurely without foresight, is often impaired in individuals with substance abuse disorders (Dalley et al., 2011; Ersche et al., 2012; Kaiser et al., 2016) and constitutes one of the risk factors for addictive behaviors (Dalley et al., 2007; Voon and Dalley, 2016; Kozak et al., 2019). This is particularly the case in adolescence and young adulthood, a critical period of substance experimentation, brain development and elevated impulsive behavior (Rømer Thomsen et al., 2018). Similarly, in preclinical models, adolescent animals (largely defined as postnatal day 21–60), tend to display more impulsive patterns of responding compared to adults (Burton and Fletcher, 2012; Hunt et al., 2016).

Adapted from a human task, the 5-choice serial reaction time task (5-CSRTT) rodent model was originally presented by Carli et al. (1983) in rats and has since become one of the most commonly used models to study attentional performance and motor impulsivity in rodents (Higgins and Silenieks, 2017). Rodents are first trained (20–30 sessions, around 100 trials/session) to respond to a visual stimulus on top of one of the five nose-pokes arranged on one wall of the testing chamber. Responding to the stimulus by poking the associated nose-poke is rewarded by food or liquid reward. This task requires the animal to maintain attention to the five nose-pokes and their corresponding visual cues. Poking during inter-trial intervals, i.e., between the last nose-poke and the presentation of a new stimulus, is recorded as a premature and impulsive response that is not rewarded and followed by a time-out. One of the advantages of this test resides in the possibility to control nearly every parameter, from the randomization of the visual stimuli

to the length of the limited hold of the nose-poke and time out periods (Higgins and Silenieks, 2017). However, interpretation of the data requires researchers to take into account the extensive influence of attention when drawing conclusions, as any disorder disrupting attention might heavily alter the results. In this regard, using mice in this task could appear more delicate than using rats, since mice have shorter attention spans (Kentros et al., 2004; Hok et al., 2016). However, mice performances in this task are equivalent to rats, a few studies showing indeed superior motor control of task performance (Humby et al., 1999; Sanchez-Roige et al., 2012; Cope et al., 2016; Higgins and Silenieks, 2017). Exposure to drugs of abuse is typically correlated to robust increases in impulsive behavior, often specifically in adolescent rodents but not in adults, measured with the 5-CSRTT or the two-choice reaction time task, a simplified version of the former (Burton and Fletcher, 2012; Siemian et al., 2017; Moazen et al., 2018; Xue et al., 2018). Interestingly, a study compared cocaine and a cocaine-associated cue to compete for attention, and concluded that while cocaine severely disrupted the well-learned sustained attention task in rats, the cocaine-associated cue induced cocaine seeking but failed to impair the task (Pitchers et al., 2017c).

Another model focuses on response inhibition, i.e., the ability to inhibit a pre-potent (planned or already initiated) action by measuring action restraint and/or cancellation (Bari and Robbins, 2013). The animal is required to withhold responding for a set duration in order to receive a reward. Any premature response during the waiting interval resets the waiting time and increases the delay to reward.

Using these models, the field has established that impairments of the PFC, that undergoes profound pruning during adolescence (Drzewiecki et al., 2016), are both risk factors and consequences of impulse-control disorders, akin to what is observed in substance abuse (Goldstein and Volkow, 2011). Other studies show a role of VTA dopamine, locus coeruleus norepinephrine neurons and cholinergic neurotransmission on attention, impulsive and motivational control (Balachandran et al., 2018; Fitzpatrick et al., 2019; Sarter and Lustig, 2019).

High-Responder/Low-Responder Model

Rats are characterized as HRs or LRs based on their cumulative locomotor movements during a locomotor test in a novel, inescapable environment, with HRs showing greater locomotor activity compared to LRs. This separates rats based on novelty-induced "sensation-seeking" behavior, a trait associated with drug addiction (Piazza et al., 1989; Dellu et al., 1996). This model captures individual variation in the acquisition of drug-taking behavior, specifically psychostimulants. Relative to LRs, HRs acquire cocaine (Piazza et al., 2000; Mantsch et al., 2001; Ferris et al., 2013), amphetamine (Piazza et al., 1989, 1990, 1991, 1998; Klebaur et al., 2001; Cain et al., 2008) and nicotine SA (Suto et al., 2001) at a faster rate. HRs also show greater behavioral sensitization to repeated amphetamine injections compared to LRs (Hooks et al., 1992). Despite acquiring drug-taking at different rates, outbred HRs and LRs do not differ in other addiction-related behaviors following prolonged cocaine SA, including the motivation to work for the drug, or drug-seeking behavior during tests of cocaine-primed and cue-induced reinstatement (Deroche-Gamonet et al., 2004). However, work using a model of rats selectively bred based on locomotor response to a novel environment [bred high-responder (bHR)/bred low-responder (bLR) model] has challenged this view, as these two phenotypes do differ in several addiction-related traits. Compared to bLR, bHR show higher levels of impulsivity (Flagel et al., 2010), attribute incentive motivational value to food and cocaine cues (Flagel et al., 2010), and acquire cocaine SA at a faster rate (Davis et al., 2008; Flagel et al., 2016). Recent work has shown that after prolonged SA training bHR initially acquires cocaine SA at a faster rate and show greater compulsive drug-seeking behavior when drug is not available compared to bLRs (Flagel et al., 2016). bHRs also show greater drug-seeking behavior during tests of cocaine-primed and cue-induced reinstatement compared to bLRs (Flagel et al., 2016). While the data from the selectively bred rat line (Flagel et al., 2016) contrasts those of an outbred population of rats (Deroche-Gamonet et al., 2004), it appears that this model may still be relevant for assessing individual variation in addiction-related behavior beyond the acquisition of drug-taking.

Work focusing on the neurobiological mechanisms underlying phenotypic differences between HRs and LRs has focused mainly on the mesolimbic dopamine system. Following drug experience, both outbred and selectively bred HRs and LRs differ in several dopamine parameters within the NAc (Rougé-Pont et al., 1993; Chefer et al., 2003; Flagel et al., 2010; Ferris et al., 2013; Waselus et al., 2013; Mabrouk et al., 2018), as well as dopamine firing rates in the VTA (McCutcheon et al., 2009, HRs and LRs only). Differences also exist in basal levels of epigenetic modification within the NAc in bHRs and bLRs (Chaudhury et al., 2014), and these differences persist following cocaine experience (Flagel et al., 2016). In addition to the mesolimbic dopamine system, HRs and LR differentially engage the hypothalamic-pituitary-adrenal axis (Piazza et al., 1991; Kabbaj et al., 2007), which is also believed to contribute to differences in addiction-related behaviors between the two phenotypes.

Sign-Tracker/Goal-Tracker Model

The sign-tracker (ST)/goal-tracker (GT) model is used to assess individual variation in the motivational value of a reward-paired cue during a Pavlovian conditioned approach (PavCA) task (Flagel et al., 2007; Robinson and Flagel, 2009; Meyer et al., 2012). During Pavlovian learning, a once neutral stimulus that reliably precedes the delivery of a reward becomes attributed with a predictive value and is transformed into a CS (Pavlov, 1927). However, in addition to a predictive value, the CS can also be attributed with an incentive motivational value and invigorate behavior on its own (Robinson and Berridge, 1993; Berridge, 2001). During PavCA training, rats that attribute a predictive value to the CS are called GTs, whereas those that attribute both a predictive and incentive motivational value to the CS are STs. Using this model, the neurobiological mechanisms underlying the predictive vs. the incentive motivational value of a reward cue have been explored. STs engage regions within the "motive circuit" (Kalivas and Volkow, 2005) to a greater

extent than GTs in response to both food (Flagel et al., 2011a; Haight et al., 2017) and drug-paired cues (Yager et al., 2015). Dopamine transmission within the NAc core (Flagel et al., 2007, 2011b; Saunders and Robinson, 2012) and PFC (Pitchers et al., 2017b) also mediates sign-tracking behavior, whereas cholinergic transmission within the PFC mediates goal-tracking behavior (Pitchers et al., 2017b). Collectively, work has shown that goal-tracking behavior is reliant on "top-down" cortical processing (for review see Kuhn et al., 2018a; Sarter and Phillips, 2018; Campus et al., 2019), whereas sign-tracking behavior engages "bottom-up" subcortical processing (for review see Flagel and Robinson, 2017; Kuhn et al., 2018a). It is proposed that the imbalance between "top-down" and "bottom-up" processing results in the behavioral differences between the phenotypes.

It has been postulated that attributing an excessive incentive motivational value to a reward-cue can lead to maladaptive behaviors such as drug addiction. In fact, STs and GTs differ in several addiction-related behaviors. For example, in addition to sign-tracking to cues associated with a food reward, STs also sign-track to cues associated with cocaine (Uslaner et al., 2006; Yager and Robinson, 2013) and opioid (Yager et al., 2015) reward delivery. Relative to GTs, STs are also more impulsive (Flagel et al., 2010; Lovic et al., 2011) and will work harder for an infusion of cocaine (Saunders and Robinson, 2011). However, the two phenotypes do not differ in the rate of cocaine SA (Saunders and Robinson, 2010; but see Beckmann et al., 2011; Saunders et al., 2013; Kawa et al., 2016; Kuhn et al., 2018b) or operant extinction (Saunders and Robinson, 2011; Kawa et al., 2016; Kuhn et al., 2018b), but do differ in reinstatement of drug-seeking behavior. STs show greater rates of both cocaine-primed (Saunders and Robinson, 2011) and cue-induced (Saunders et al., 2013; but see Kawa et al., 2016) reinstatement of drug-seeking behavior compared to GTs following ShA training. Work has shown that enhanced dopamine transmission within the NAc core contributes to higher cue-induced drug-seeking behavior observed in STs (Saunders et al., 2013). Additionally, the paraventricular nucleus of the thalamus, a region within the motive circuitry (Kelley et al., 2005) that has recently gained attention for mediating motivated behaviors including addiction-related behaviors (for review see Millan et al., 2017), is also a key node regulating this individual variation in cue-induced drug-seeking behavior (Kuhn et al., 2018b).

Though STs are more susceptible to both cocaine-primed and cue-induced reinstatement, GTs show greater drug-seeking behavior during a test for context-induced reinstatement (Saunders et al., 2014), and in response to discriminative stimuli associated with reward delivery, an effect mediated by cholinergic transmission within the PFC (Pitchers et al., 2017a). Compared to STs, GTs more readily utilize cortical processing (for review see Sarter and Phillips, 2018), and it is postulated that this cortical engagement allows them to disentangle the complex nature of contexts and discriminative stimuli better than STs, resulting in more drug-seeking behavior. This difference in relapse propensity between STs and GTs relative to the type of reinstatement implies that both phenotypes are sensitive to addiction-related behaviors; however, the environmental contingencies and neurobiological mechanisms mediating these effects differ. These findings suggest that in contrast to the HR/LR model, the main strength of the ST/GT model is elucidating the neurobiological mechanisms associated with individual variation in relapse propensity. This model also has translational validity, as work in humans has shown sign- and goal-tracking behavior (Garofalo and di Pellegrino, 2015; Joyner et al., 2018; Schad et al., 2019), though linking a specific conditioned response in humans with addiction-related behaviors has yet to be explored.

ADDITIONAL MODELS OF BEHAVIORAL ADDICTIONS

Gambling Disorder

Due to its diagnostic similarities with substance-use disorders, gambling disorder (GD; Langdon et al., 2019) was moved to a new category entitled "Substance-related and Addictive Disorders" in the most recent DSM (APA, 2013). In fact, GD is currently the only behavioral addiction that is diagnosable in the DSM, and interestingly there is a high level of comorbidity between individuals diagnosed with GD and drug addiction (Lorains et al., 2011). GD is characterized as compulsive gambling behavior that results in distress and causes disruptions to an individual's personal and professional life (APA, 2013). The Iowa Gambling Task (IGT), a neuropsychological battery used to assess decision-making strategies, is clinically used to study GD. During this task, an individual selects a card from one of four decks, each of which has a different probability of a reward to punishment ratio assigned to it resulting in "safe" and "risky" options (Bechara et al., 1994). Individuals with GD choose cards from riskier decks more often than healthy control, resulting in fewer winnings (Cavedini et al., 2002). In fact, performance on this task has been associated with treatment success (Alvarez-Moya et al., 2011).

The rodent gambling task (rGT; Zeeb et al., 2009) is the most commonly used adaptation of the IGT. During this task, rats have the option to poke into four different ports, and each port has a specific reward (e.g., sugar pellets) and punishment (e.g., time-out period between trials) ratio associated with it. Rats are given a fixed amount of time to complete the task and choosing from the riskier ports (i.e., more sugar pellets but a longer time-out period) results in a lower net gain compared to selecting the safer ports. Studies have found that the orbitofrontal cortex (OFC) and the basolateral amygdala (BLA) mediate the acquisition of adaptive decision making during this task (Zeeb and Winstanley, 2011, 2013). However, only the BLA is needed for the continued expression of the adaptive strategy suggesting that the OFC is initially recruited to establish the behavior, but the BLA maintains it (Zeeb and Winstanley, 2011; Zeeb et al., 2015). The insular cortex has also been shown to mediate decision making during the rGT (Ishii et al., 2015; Pushparaj et al., 2015; Daniel et al., 2017). These data complement studies in humans demonstrating that various regions of the PFC are

recruited during the IGT (Fellows and Farah, 2005; Tanabe et al., 2007; Lawrence et al., 2009; Power et al., 2012), and the amygdala mediates GD in humans (Bechara et al., 1999; Takeuchi et al., 2019).

While the rGT emulates aspects of GD and appears to have translational significance, it has been criticized for only modeling poor decision-making strategies, and not behaviors exclusively associated with GD (for review see Winstanley and Clark, 2016). To better address this, additional rodent models have been developed to model specific aspects of GD. For example, the rodent betting task models "escalation of commitment", or rather the phenomenon that people become more cautious as the stakes get higher (Staw, 1981). Like humans, as stakes get higher rats will more often select a certain reward as opposed to the chance of receiving a higher reward with the risk of receiving nothing at all (Cocker et al., 2012). The BLA (Tremblay et al., 2014), regions within the PFC (Barrus et al., 2017), and dorsal striatal dopamine levels (Cocker et al., 2012) appear to mediate behavior during this task. Gambling tasks have also been created to mimic the behavior of "loss-chasing," or continuing to gamble in an effort to earn back previous losses, which is commonly observed in individuals with GD (Toce-Gerstein et al., 2003; Strong and Kahler, 2007). During this paradigm, rats intermittently must choose between withholding responding during a time-out period, or to gamble with the chance of avoiding the time-out period with the risk of doubling it (Rogers et al., 2013). The BLA (Tremblay et al., 2014), as well as serotonin and dopamine transmission (Rogers et al., 2013), have been shown to mediate behavior during this task. Lastly, a task known as the rat slot machine task has been used to model the "near-miss effect" (Peters et al., 2010) seen in humans whereby barely losing a gamble motivates continual gambling (Kassinove and Schare, 2001; Clark et al., 2009). During this task, rats must first poke into all available ports, after which the lights within each port either turn on or stay off. If all lights come on, that is considered a "win" trial and the rat must press on a lever in order to receive the reward. Compared to other "loss" trials, rats show greater lever pressing when a "near miss" occurs (e.g., lights in all ports come on except one vs. only one light coming on; Winstanley et al., 2011). Dopamine signaling has been implicated in mediating behavior during this task (Winstanley et al., 2011; Cocker et al., 2014, 2017), particularly within the insular cortex (Cocker et al., 2016).

Other factors that affect gambling behavior in humans are also being taken into consideration when modeling GD, such as the motivational salience of audiovisual cues that are commonly present in casinos. Similar to behavior in drug addiction, cues and contexts associated with gambling have been shown to affect gambling behavior (for review see Barrus et al., 2016), and behavioral paradigms in rodents have been created to emulate these effects (Barrus and Winstanley, 2016; Adams et al., 2017; Langdon et al., 2019). Taken together, it is evident that no single behavioral paradigm can capture the complexity of GD in rodents. Thus, similar to studying drug addiction-related behaviors in animal models, a battery of assessments are used in order to better model and assess the neurobiological underpinnings of GD.

Exercise Addiction

Though not included in the DSM, exercise addiction (EA) has been reported to affect 0.3–0.5% of the general population and 1.9–3.2% of individuals who regularly exercise (Berczik et al., 2012; Griffiths et al., 2015). The main issue in diagnosing individuals with EA is that diagnostic criteria have not yet been agreed upon. Several screening tools have been developed to assess EA in humans, including the Exercise Addiction Inventory and the more commonly used Exercise Dependence Scale (for review see Hausenblas et al., 2017). Several studies have proposed that criteria for EA be adapted from those used to diagnose substance use disorders per the DSM, including measures of tolerance, withdrawal, and decreased involvement in other activities (for review see Freimuth et al., 2011). Independent of diagnostic criteria, studies have identified behavioral traits associated with EA, such as obsessive-compulsive behavior, loneliness and anxiety (Macfarlane et al., 2016; Lukács et al., 2019). Several other disorders, including substance use disorders, eating disorder, and other behavior addictions such as shopping and sex addiction have been found to co-occur with EA (for review see Freimuth et al., 2011). Despite not having a standardized diagnostic procedure, rodent models of EA exist, primarily composed of assessing wheel-running behavior. In fact, several studies using rodent wheel-running models have yielded evidence of behaviors akin to symptoms of EA aligning with those observed in substance use disorders (for review see Richter et al., 2014). Though still being fully conceptualized, it is evident that EA is garnering more attention and is being more rigorously assessed in both clinical and preclinical models.

MODELS USED BY PHARMACEUTICAL COMPANIES TO ASSESS ABUSE POTENTIAL AND THEIR FACE VALIDITY

Regardless of the scientific question, all animal models strive to represent part of the neurobiological mechanisms that guide human behavior. Models are limited in this regard, but their use is needed to assess the abuse potential of new drugs. We describe in this section a general overview of the procedures commonly used by pharmaceutical companies during a new drug safety evaluation to assess that risk. In 2017, the Food and Drug Administration agency (FDA) released an updated guide for industry gathering nonbinding recommendations on how to evaluate whether a new drug product acting on the central nervous system (CNS) has abuse potential and required to be subject to control under the Controlled Substances Act (CSA; U.S. Department of Health and Human Services, 2017). FDA does not recommend that every drug under development undergo an evaluation of abuse potential, but proposes focusing on the new CNS-active molecular entities that have not previously been assessed by FDA for abuse potential. After assessing if a new drug, or any of its major metabolites, is CNS-active through chemistry, pharmacokinetics and receptor-binding studies, the next step includes abuse-related animal behavioral studies, most commonly performed in rats. A first

set of safety studies measure the effects of the drug on general behavior, such as a motor performance test. As an example, drug-induced hyperactivity is recorded as an abuse-related signal. Specific abuse-related studies evaluate: (1) the rewarding and reinforcing properties of the drug; and (2) the similarity of the effects of the new drug to established drugs of abuse, defined as drug discrimination. The rewarding and reinforcing properties are measured using SA starting on fixed ratio 1, which increases with continual training. Drug discrimination generally involves training animals to self-administer a training drug, usually a drug of abuse of similar classification than the newly tested drug, with a known mechanism of action. When the new drug induces the same SA behavior than the training drug, it is then hypothesized that drugs share a pharmacological mechanism of action (Carter and Griffiths, 2009). However, since certain classes of drugs, such as hallucinogenic $5HT_{2A}$ agonists or cannabinoids, are poorly self-administered if at all (Yanagita, 1986; Fantegrossi et al., 2004; Heal et al., 2018), conditioned place-preference studies are alternatively recommended to establish potential rewarding properties of a new drug (Heal et al., 2018). These studies are usually performed at the end of phase 2 of the clinical trial, using the final therapeutic doses previously selected. When the studies establishing drug reward properties are completed, the FDA additionally recommends performing a physical dependence study, to identify potential withdrawal syndromes and behavioral disruptions upon abrupt drug discontinuation.

The decision to test the abuse potential of a new drug in humans depends on the results of the above-mentioned animal studies, a thorough comparison of the preclinical and clinical studies related to the drug and profiles of abuse- and euphoria-related adverse events established in healthy individuals and individuals with the disease of study. If all of these markers point to abuse-related signals, the drug is then tested in humans reporting numerous recent recreational experiences with drugs in the same drug class as the tested drug, through human abuse potential additional studies.

The two major models used to classify and regulate drug use, SA and CPP, present different levels of face validity. SA, including multiple-choice SA procedures (Griffiths et al., 1993), presents a high level of face validity and predictive validity (Haney and Spealman, 2008; Carter and Griffiths, 2009) and drugs self-administered in rodents correspond with those having reinforcing properties in humans (Schuster and Thompson, 1969; Griffiths and Ator, 1980; Haney and Spealman, 2008; Carter and Griffiths, 2009). In several studies, preclinical identification of drugs reducing craving and/or intake successfully translated to humans (Heilig et al., 2016). This was the case for the α2-adrenergic agonist clonidine that reduced cocaine and heroin craving in a human laboratory setting and clinical trial (Jobes et al., 2011; Kowalczyk et al., 2015). Similarly, glucocorticoid receptor antagonist mifepristone was found to decrease alcohol intake in rats, and also lessened cue-induced alcohol craving and intake in individuals with alcohol use disorder (Vendruscolo et al., 2012, 2015).

It is critical to remember that although useful, simple rodent models fail to encapsulate the complexity of human life. Factors such as drug availability, economic and socio-cultural levels influence the opportunities and experiences available to individuals and thus impact the risk of abuse. Preclinical models offering alternative rewards or including behavioral economics tackle some of these factors (see above), but these models are mostly limited to laboratory use currently and are not yet mainstreamed for pharmacological testing. Some authors additionally argue that, albeit the current models used to assess abuse potential are valid and reliable, they often lack accuracy assessing the propensity to develop addiction and the severity in which it will manifest, two parameters deeply influenced by individual vulnerability (Conway et al., 2010). They argue the need for better indices accounting for the complexity and multifactorial traits that impact the development of addiction in humans, in order to develop improved prevention approaches and treatment strategies.

One way to address these limitations is to introduce genetic variability in rodent models. While genetic factors contribute to susceptibility to drug addiction (for review see Nestler, 2000; Bevilacqua and Goldman, 2009), the heritability of drug use varies with hallucinogens showing the lowest level of heritability and cocaine showing the highest (Goldman et al., 2005). Rat lines exist that inherently capture genetic diversity, such as the heterogeneous stock rat, allowing for better representation of genetic and behavioral variation present in the human population (Solberg Woods, 2014; Woods and Mott, 2017). For a more targeted assessment of the role a gene may have in a behavioral trait, transgenic models, specifically genetic knockout and overexpressing models, can be used. In contrast to manipulating the genetic background of an animal to assess changes in behaviors, models can also be created by selectively breeding animals based on a specific behavioral trait, such as in the bHR/bLR model, and then assessing genetic differences between phenotypes. Lastly, to gain a broader understanding of the genetic differences that may contribute to certain phenotypic traits in animal models, quantitative trait loci analysis can be used. Using this technique, genetic variants can be identified in animals that differ in behaviors associated with addiction. While genetics plays a role in the predisposition for addiction-related behaviors, previous work has also focused on the epigenetic effects drugs of abuse have on the long-term transcriptional regulation of genes and how this contributes to addiction (Robison and Nestler, 2011). By focusing on the genetic basis for addiction-related behaviors, in conjunction with implementing better preclinical models of addiction, pharmaceutical companies can identify more successful therapeutics interventions in the treatment of drug addiction.

CONCLUSION

Despite the complexity of substance-related and addictive disorders, we have highlighted in this review currently used preclinical models aiming to mimic as closely as possible the behaviors observed in humans. As models become more complex, the tools used to study the underlying neurobiological substrates also improve, moving the field forward towards future therapeutic opportunities.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Behavioral Traits Associated With Resilience to the Effects of Repeated Social Defeat on Cocaine-Induced Conditioned Place Preference in Mice

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The relationship between stress and drug use is well demonstrated. Stress-induced by repeated social defeat (RSD) enhances the conditioned place preference (CPP) induced by cocaine in mice. The phenomenon of resilience understood as the ability of subjects to overcome the negative effects of stress is the focus of increasing interest. Our aim is to characterize the behavior of resilient animals with respect to the effects of RSD on the CPP induced by cocaine. To this end, 25 male C57BL/6 mice were exposed to stress by RSD during late adolescence, while other 15 male mice did not undergo stress (controls). On the 2 days following the last defeat, all the animals carried out the elevated plus maze (EPM) and Hole Board, Social Interaction, Tail Suspension and Splash tests. Three weeks later, all the animals performed the CPP paradigm with a low dose of cocaine (1 mg/kg). Exposure to RSD decreased all measurements related to the open arms of the EPM. It also reduced social interaction, immobility in the tail suspension test (TST) and grooming in the splash test. RSD exposure also increased the sensitivity of the mice to the rewarding effects of cocaine, since only defeated animals acquired CPP. Several behavioral traits were related to resilience to the potentiating effect of RSD on cocaine CPP. Mice that showed less submission during defeat episodes, a lower percentage of time in the open arms of the EPM, low novelty-seeking, high social interaction, greater immobility in the TST and a higher frequency of grooming were those that were resilient to the long-term effects of social defeat on cocaine reward since they behaved like controls and did not develop CPP. These results suggest that the behavioral profile of resilient defeated mice is characterized by an active coping response during episodes of defeat, a greater concern for potential dangers, less reactivity in a situation of inevitable moderate stress and fewer depressive-like symptoms after stress. Determining the neurobehavioral substrates of resilience is the first step towards developing behavioral or pharmacological interventions that increase resilience in individuals at a high risk of suffering from stress.

Keywords: resilience, social defeat stress, cocaine, mice, conditioned place preference, reward, vulnerability

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Abbreviations: CPP, conditioned place preference; EPM, elevated plus maze; FG, frequency of grooming; ISI, index of social interaction; NS, novelty-seeking; PND, post-natal day; RSD, repeated social defeat; TI, time of immobility; TST, tail suspension test; %TOA, percentage of time in open arms.

INTRODUCTION

According to the World Health Organization, the global prevalence of cocaine use was estimated at roughly 0.4% of the global population aged 15–64 in 2016 (about 18.2 million users), with higher incidence rates in developed societies (World Drug Report, 2018). Individual and environmental variables act as risk factors, facilitating the initiation and maintenance of drug use, the transition to addiction, and relapse after detoxification (Dellu et al., 1996; Enoch, 2006). Among the environmental factors affecting vulnerability to drug addiction, exposure to stress plays a primary role. Traumatic life events during critical periods of development have a profound influence on the development of personality (Kim et al., 2009; Congdon et al., 2012; Oshri et al., 2013) and increase the risk of suffering from mental and drug-use disorders (Kessler et al., 2010; Sayed et al., 2015).

Chronic social stress, including problems with social interaction (family or friend relationships, work-place stress, bullying, etc.) is the most frequent type of stress faced by human beings. In preclinical studies with rodents, chronic social stress is modeled by the repeated social defeat (RSD) paradigm. Brief episodes of aggression from a more aggressive conspecific, together with social subordination, induce anxiety- and depression-like symptoms (Bartolomucci et al., 2009; Nestler and Hyman, 2010; Hollis and Kabbaj, 2014; Czéh et al., 2016; Vannan et al., 2018). Exposure to RSD has also been shown to increase the rewarding effects of drugs of abuse (Ellenbroek et al., 2005; Burke et al., 2011; Aguilar et al., 2013; García-Pardo et al., 2015, 2017; Newman et al., 2018). Moreover, several studies performed in our laboratory using the conditioned place preference (CPP) paradigm have demonstrated that mice exposed to RSD during late adolescence exhibit an enhanced sensitivity to the rewarding effects of low doses of cocaine in adulthood (Montagud-Romero et al., 2016a,b; Rodríguez-Arias et al., 2017; García-Pardo et al., 2019).

In spite of the close relationship between life adversity and psychopathology, not all individuals exposed to stress develop a mental disorder. In fact, most are resilient and display an adaptive response to stress that ensures a relatively normal physical and psychological function (Southwick and Charney, 2012). Thus, resilience can be defined as "the process of adapting well in the face of adversity" (Charney, 2004), or as the capacity to overcome the deleterious consequences of stress, which result in the development of psychiatric disorders in more vulnerable individuals. It is unclear why some individuals are more resilient to the impairing effects of stress than others, but neurochemical, genetic, and epigenetic processes seem to be associated with resilience to stress-related disorders (Cadet, 2016; Osório et al., 2016).

The RSD paradigm has proven to be a useful model for studying the mechanisms involved in susceptibility or resilience to the negative consequences of social stress (Nestler and Hyman, 2010). As in humans, individual differences exist in the development of psychopathology after RSD exposure. Only the subgroup of mice characterized as susceptible to the effects of RSD on social interaction with a conspecific (social avoidance) exhibit a wide variety of deleterious consequences, including anhedonia- and anxiety-like symptoms, elevated reactivity of the hypothalamic-pituitary-adrenal (HPA) axis and other behavioral and physiological alterations (Berton et al., 2006; Krishnan et al., 2007; Nestler and Hyman, 2010; Russo et al., 2012; Russo and Nestler, 2013).

Resilience could also explain why not all individuals who undergo stressful experiences become addicted to drugs of abuse. Using the RSD model, Krishnan et al. (2007) demonstrated that only mice characterized as susceptible (mice that displayed social avoidance after RSD exposure) developed cocaine-induced CPP. Similarly, animals vulnerable to the effects of RSD on social interaction were shown to increase alcohol self-administration in comparison to non-stressed controls or resilient animals that did not develop social avoidance after RSD (Nelson et al., 2018). Both studies suggest that resilient mice that do not display a deficit of social interaction after stress are also resilient to the rewarding effects of drugs of abuse. These are the only studies to have identified animals that were susceptible or resilient to the influence of RSD on the rewarding effects of drugs of abuse. As Cadet (2016) noted, most neuroscience research has focused on identifying negative or pathological elements underlying a subject's vulnerability to drug addiction; however, the characterization of the traits that confer resilience against the consequences of social stress on the effects of drugs of abuse could be a more effective approach to preventing and treating addictive disorders. Identifying predictive behavioral patterns of resilience is the first step towards developing early, individualized preventive strategies that enhance resilience and promote a resilient personality in individuals at risk who are exposed to significative levels of stress.

Thus, the aim of this work was to determine the existence of individual differences in response to RSD and to characterize the behavioral profile of animals that are resilient to the long-term effects of social defeat on cocaine-induced CPP. For this purpose, a group of late adolescent mice were exposed to RSD (four episodes separated by intervals of 72 h), while another group did not undergo stress. The behavior of the defeated mice was evaluated during the first and fourth episodes of defeat and they were segregated in two subgroups according to the time they spent engaged in defense/submission. The short-term effects of RSD were evaluated to compare the behavior of defeated mice to that of control mice in the elevated plus-maze and the hole board and in social interaction, tail suspension and splash tests, 24–48 h after the last episode of defeat. According to the behavior of the defeated mice in these behavioral tests, they were segregated into two subgroups: one affected by RSD (vulnerable mice), and the other behaving like the control group (resilient mice). Three weeks after the last episode of defeat, acquisition of CPP after conditioning with a low dose of cocaine was evaluated in all the mice in order to identify the behavioral traits that confer resilience to the long-term effects of RSD on the CPP induced by cocaine. A lack of CPP was used to define the animals that were resilient to the effects of RSD on cocaine reward since non-stressed mice did not develop CPP with the dose of cocaine employed.

MATERIALS AND METHODS

Subjects

Forty male mice of the C57BL/6 strain and 15 male mice of the OF1 strain (Charles River, France) were used in the study. They arrived in the laboratory on a postnatal day (PND) 21 and were housed for 26 days before initiation of the experimental procedures. Experimental mice (C57BL/6) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents (OF1) were individually housed in plastic cages (23 × 32 × 20 cm) in order to induce heightened aggression (Rodríguez-Arias et al., 1998). To reduce their stress levels in response to experimental manipulations, grouped mice were handled for 5 min per day on each of the 3 days prior to initiation of the experimental procedures. All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30); and food and water available *ad libitum*, except during behavioral tests. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. The protocol was approved by the Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

Drugs

Animals were injected intraperitoneally with 1 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) or (NaCl 0.9%) in a volume of 0.01 ml/g of weight. The physiological saline was also used to dissolve the cocaine. The dose of cocaine was selected on the basis of previous studies (Rodríguez-Arias et al., 2017; García-Pardo et al., 2019).

Experimental Design

After an adaptation period, the experimental mice (C57BL/6) were assigned to two groups: one non-stressed control group (n = 15) and another subsequently exposed to four episodes of RSD (n = 25) on PND 47, 50, 53 and 56. On PND 57–58, all mice underwent different behavioral tests: elevated plus maze (EPM), hole board, social interaction, tail suspension, and splash tests. Afterward, all mice were housed in the vivarium for 3 weeks, after which they underwent the CPP procedure (see **Figure 1**). All experiments took place during the dark period (8.30–16.30) and in a different environment to that of the confrontation sessions. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h prior to testing.

Experimental Protocols

Repeated Social Defeat (RSD)

The RSD procedure consisted of four encounters (separated by intervals of 72 h, PND 47, 50, 53 and 56) with a conspecific isolated mouse (OF1), which resulted in the defeat of the experimental animal. Each encounter lasted for 25 min and consisted of three phases, which began by introducing the experimental animal (intruder) into the home cage of the aggressive opponent (resident) for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall, which allowed social interaction and threats from the aggressive male resident. The wire mesh was then removed from the cage and the confrontation between the two mice began and lasted for 5 min. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. Intruder mice were exposed to a different aggressor mouse during each episode of social defeat. The criterion

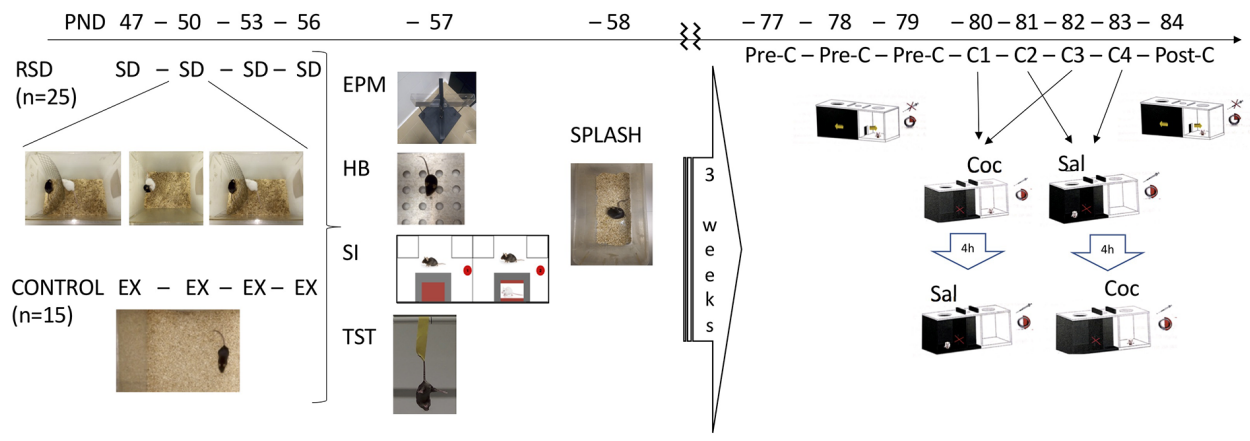

FIGURE 1 | Experimental design. Two groups of mice were used. One group was exposed to repeated social defeat (RSD, n = 25). On postnatal day (PND) 47, 50, 53 and 56, experimental mice were introduced into the cage of an aggressive opponent. The physical contact between them was allowed for only 5 min when the experimental mouse experienced social defeat (SD). On the same PND, the other group (CONTROL, n = 15) explored (EX) an empty cage. On PND 57, all mice performed the elevated plus maze (EPM) and the hole board (HB), social interaction (SI) and tail suspension test (TST). On PND 58, all mice performed the splash test. After an interval of 3 weeks, all mice underwent the conditioned place preference (CPP) paradigm. On PND 77, 78 and 79, they underwent the pre-conditioning (Pre-C) phase. On PND 80, 81, 82, 83 they performed four conditioning sessions (C1-C4) receiving 1 mg/kg of cocaine (Coc) or saline (Sal) before being placed to the drug- or saline-paired compartment, respectively. On PND 84, mice underwent the post-conditioning (Post-C) phase.

used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Ribeiro Do Couto et al., 2006). All experimental mice displayed defeat, given that they all faced resident mice with high levels of aggression. The first and fourth agonistic encounters were videotaped and evaluated by an observer who was blind to the treatment (Brain et al., 1989) using a computerized system (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time spent in avoidance/flee and defense/submission by the experimental mice and the time spent in threat and attack by the resident aggressive mice were measured, as were the latencies of these behaviors. The control (non-stressed) group underwent the same protocol, without the presence of a "resident" mouse in the cage (exploration).

Elevated Plus Maze (EPM)

The effects of RSD on anxiety were evaluated using the EPM paradigm on PND 57. This test is based on the natural aversion of mice to open elevated areas, as well as on the natural spontaneous exploratory behavior they exhibit in novel environments; therefore, it measures the extent to which rodents avoid high open spaces. The apparatus consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), and the junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. The total time spent in the open and closed arms, the number of entries into the open and closed arms, and the percentage of time and entries into the open arms are commonly considered indicators of open space-induced anxiety in mice. Thus, anxiety levels are considered to be lower when the measurements in the open arms are higher and the measurements in the closed arms are lower, and vice versa (Rodgers and Johnson, 1995; Rodgers and Dalvi, 1997). Moreover, the total entries into the closed arms are regarded as locomotor activity scores (Campos et al., 2013; Valzachi et al., 2013).

At the beginning of each trial, subjects were placed on the central platform facing an open arm and were allowed to explore for 5 min. The maze was cleaned with a 7% alcohol swab after each test, and the device remained untouched until completely dry. The behavior of the mice was video recorded and later analyzed by an investigator blind to the experimental conditions, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The measures recorded during the test period were frequency of entries and time spent in each section of the apparatus (open arms, closed arms and central platform). An arm was considered to have been visited when the animal placed all four paws on it. The following measures were taken into account for the statistical analyses: the latency to first enter the open arms, the time and percentage of time [(open/open + closed) × 100] spent in the open arms, the number and the percentage of open arm entries and total entries into the arms.

Hole Board Test

The novelty-seeking of mice was evaluated in the hole board test 24 h after the last defeat or exploration (PND 57). This test was carried out in a square box (28 × 28 × 20.5 cm) with transparent Plexiglas walls and 16 equidistant holes of 3 cm in diameter on the floor (CIBERTEC SA, Madrid, Spain). Photocells below the surface of the holes detected the number of times that mice performed a head-dip. At the beginning of the test, mice were placed in the same corner of the box and were allowed to freely explore the apparatus for 10 min. The latency to the first dip and the frequency of dips were automatically recorded by the apparatus.

Social Interaction Test

Twenty-four hours after the last defeat or exploration (PND 57), the social behavior of the mice was evaluated in an open field (37 × 37 × 30 cm). A perforated plexiglass cage (10 × 6.5 × 30 cm) was placed in the middle of one wall of the open field. After habituation to the room, each animal was placed in the center of the open field and was allowed to explore it twice, under two different experimental conditions. The first time (object phase), the perforated plexiglass cage was empty. After 10 min of exploration, the experimental mouse was returned to its home cage for 2 min. Next, a mouse of the OF1 strain was confined to the perforated cage (to safeguard the experimental mouse from attack) and the experimental mouse was reintroduced in the open field for 10 min (social phase). The OF1 mouse was unfamiliar to the experimental mouse (i.e., it was different from the one used in the RSD episodes). In both phases, the time spent in the 8 cm area surrounding the perforated cage—the interaction zone—was registered and automatically sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). An index of social interaction (ISI) was obtained [time spent in the interaction zone during the social phase/(time spent in the interaction zone during the social phase + time spent in the interaction zone during the object phase); Henriques-Alves and Queiroz, 2016]. The ISI is commonly used as the social preference-avoidance index (Krishnan et al., 2007).

Tail Suspension Test (TST)

The tail suspension test (TST) measures the behavioral variable of immobility, which is considered to represent despair (Pollak et al., 2010). It is based on the observation that rodents, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable, stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung in an uncontrollable fashion by their tail (Cryan et al., 2005). This has been used as a measure of behavioral depression because, when antidepressant treatments are given prior to the test, the subjects engage in escape-directed behaviors for longer periods of time than after treatment with a vehicle (Pollak et al., 2010).

Twenty-four hours after the last defeat or exploration (PND 57), we investigated whether our procedure of social defeat modified the length of time spent in immobile positions in the

TST. Following the protocol described by Vaugeois et al. (1997), mice were suspended by the tail, using adhesive tape, from a hook connected to a strain gauge that recorded their movements during a 6-min test period. The behavior displayed by the mice was video recorded and later analyzed by an observer blind to the treatment received by the animal, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The parameters considered for the statistical analyses were the total time spent immobile and the latency to show immobility.

Splash Test

The splash test consisted of spraying a 10% sucrose solution on the dorsal coat of a mouse placed in a transparent cage (15 × 30 × 20 cm) with regular bedding to stimulate grooming behavior. The behavior of the mice was videotaped for 5 min and later analyzed by an observer blind to the treatment received by the animal using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The latency to the first grooming, the time spent engaged in this behavior and its frequency were recorded. An increase in the latency of grooming and a decrease in the time and/or frequency of grooming is interpreted as depressive-like behavior (Smolinsky et al., 2009).

Conditioned Place Preference (CPP)

Three weeks after the last episode of social defeat, the animals carried out the CPP procedure. For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 34.5 cm high). The compartments had different colored walls (black vs. white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (Cibertec SA, Madrid, Spain).

The CPP consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference (for detailed explanations of the procedure, see Maldonado et al., 2007). In brief, during pre-conditioning (Pre-C), the time spent by the animal in each compartment during a 15-min period was recorded. Animals showing a strong unconditioned aversion or a preference for a given compartment were excluded from the study. In the second phase (conditioning), which lasted for 4 days, experimental animals received saline before being confined to the vehicle-paired compartment for 30 min and, after an interval of 4 h, were injected with 1 mg/kg of cocaine immediately before being confined to the drug-paired compartment for 30 min. During the third phase, or post-conditioning (Post-C), the time spent by the untreated mice in each compartment was recorded during a 15-min period.

Statistical Analysis

The effects of RSD on the different behavioral measures (with the exception of CPP) were evaluated by means of unpaired Student *t*-tests, comparing the non-stressed control group to the defeated group (control vs. RSD). In the case of CPP, a mixed two-way ANOVA with a within-subjects variable Days with two levels (Pre-C and Post-C) and a between-subjects variable Stress with two levels (Control and RSD) was used. *Post hoc* comparisons were performed with Bonferroni tests, which allow multiple hypotheses to be tested simultaneously, limiting the type I error rate without increasing the probability of a type II error occurring.

With the data obtained in the defeat episodes and in the behavioral tests performed 24 or 48 h afterward (EPM, hole board, social interaction, tail suspension and splash tests), the group of defeated mice was separated into two subgroups according to the median of the whole group. Mice with scores higher than the median were assigned to the High Score group and those with lower scores to the Low Score group. For example, defeated mice were defined as high or low novelty-seeking (NS) according to their head-dip scores (below or above the defeated group median) in the hole board test. We have previously used this median-split analysis to study the effects of NS on the behavioral effects of different drugs of abuse (Arenas et al., 2014; Montagud-Romero et al., 2014; Mateos-García et al., 2015; Rodríguez-Arias et al., 2015, 2016). A one-way ANOVA with a between-subjects variable—Group, with three levels (Control, Defeated High Score and Defeated Low Score)—was performed for the following measures: time in defense/submission in the first episode of defeat, percentage of time in the open arms of the EPM, number of dips in the hole board, time of immobility in the TST, and grooming (frequency and time) in the splash test. The *post hoc* comparison was performed with the Tukey test. To determine the possible behavioral markers of resilience to the effects of social defeat on cocaine CPP, a mixed two-way ANOVA with a within-subjects variable—Days, with two levels (Pre-C and Post-C)—and a between-subjects variable—Group, with three levels (Control, Defeated High Score and Defeated Low Score)—was used. *Post hoc* comparisons were performed with Bonferroni tests.

In order to determine whether there was a relationship among the performances of mice in the different procedures, Pearson correlation tests were used. In the case of CPP, the conditioning score (time spent in Post-C minus time spent in Pre-C) was calculated. All statistical analyses were performed with the SPSS program.

RESULTS

Effects of RSD on the CPP Induced by Cocaine

The ANOVA of the CPP data showed a significant effect of the variable Days ($F_{(1,38)} = 5.634$; $p < 0.05$) and the Interaction Days × Stress ($F_{(1,38)} = 4.186$; $p < 0.05$). RSD increased the rewarding effects of cocaine since only defeated mice spent more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$). Conversely, mice not exposed to defeat (Control group) did not show CPP (**Supplementary Figure S1**).

Behavioral Profile of Mice During Social Defeats and Resilience to Cocaine CPP

After the behavioral analysis of defeat episodes, defeated mice were divided into two subgroups according to their Defense/Submission scores during the first episode of defeat (below or above the median of the defeated group, 20.11 s, Low or High Defense/S. Student *t*-test showed a significant difference between these two subgroups of defeated mice (Low and High Defense/S) with respect to the Time spent in Defense/Submission in the first episode of defeat ($t_{(26)} = -5.878$; $p < 0.001$).

The behavioral profile of mice during the defeat episodes is related to their subsequent resilience or vulnerability to developing cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of the Time spent in submissive behavior during the first episode of defeat showed that the variable Days ($F_{(1,37)} = 11.179$; $p < 0.01$) and the interaction Days × Group ($F_{(2,37)} = 3.297$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons revealed that only the High Defense/S group, which spent more time in defensive/submissive behavior, showed CPP ($p < 0.05$, significantly longer time in the drug-paired compartment in Post-C than in Pre-C). The control group (non-defeated mice) and the Low Defense/S group (defeated mice that showed less time in defensive/submissive behaviors) did not develop CPP (see **Figure 2**).

Besides the Time spent in Defense/Submission, the behavioral analysis of defeat episodes revealed other differences among the mice that were resilient or vulnerable to the effects of RSD on the CPP induced by cocaine. Student's *t*-tests showed significant differences between the two subgroups of defeated mice in the first episode of defeat with respect to Latency of Submission ($t_{(26)} = 2.322$; $p < 0.05$), Time spent in Flight ($t_{(26)} = 4.519$; $p < 0.001$) and Time receiving Threat from the opponent ($t_{(26)} = -4.01$; $p < 0.001$). Moreover, subgroups of defeated mice showed differences in the fourth episode of defeat in the Time spent in Defense/Submission ($t_{(26)} = -2.075$; $p < 0.05$) and in the Latency of Attack from the opponent ($t_{(26)} = -2.334$; $p < 0.05$). As can be seen in **Figure 3**, the behavioral profile of resilient mice was characterized by lower submission and more avoidance/flee. In addition, they received lower levels of threat but were attacked faster.

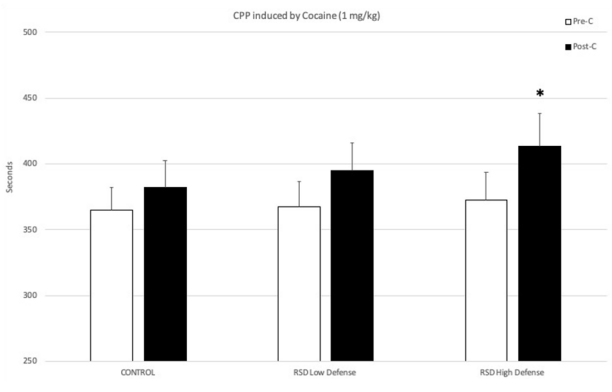

FIGURE 2 | Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice during the first episode of defeat. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The group of defeated mice was divided into two subgroups according to the time spent in defense/submission in the first episode of defeat: RSD Low Defense and RSD High Defense. After defeat, mice were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). *$P < 0.05$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

Elevated Plus Maze and Resilience to Cocaine CPP

RSD induced anxiogenic-like effects in the EPM. Student's *t*-tests showed significant differences between defeated and control mice in several measures related to the open arms. In comparison to controls, mice exposed to RSD showed a decrease in the Time ($t_{(42)} = 3.407$; $p < 0.001$) and Percentage of time ($t_{(42)} = 3.143$; $p < 0.01$) spent in the open arms, an increase in the latency to enter the open arms ($t_{(40)} = -3.174$; $p < 0.01$), and a reduced number of Entries ($t_{(42)} = 5.780$; $p < 0.001$) and Percentage of entries ($t_{(42)} = 3.493$; $p < 0.001$) into the open arms. Furthermore, RSD decreased the total number of Total entries into the arms ($t_{(42)} = 5.410$; $p < 0.001$; **Figure 4**).

In order to evaluate resilience to the effects of RSD in the EPM, defeated mice were divided into two subgroups according to their scores of Percentage of time in the open arms (below or above the median of the defeated group, 25.92%), and Low or High %TOA. A one-way ANOVA showed a significant effect of the variable Group ($F_{(2,41)} = 41.326$; $p < 0.001$). Tukey *post hoc* comparisons indicated that the Low %TOA group was significantly different from the control and High %TOA groups (ps < 0.001; **Figure 5A**). Thus, there was a group of mice that were resilient to the effects of RSD on the EPM and did not show a decrease in the percentage of time spent in the open arms.

However, resilience to the anxiogenic-like effects of RSD in the EPM is inversely related to resilience to the long-term effects of RSD on cocaine-induced CPP. The ANOVA of the CPP data of controls and the two groups of defeated mice (Low and High %TOA) showed that the variable Days ($F_{(1,38)} = 8.046$; $p < 0.01$) and the Interaction Days × Group ($F_{(2,38)} = 3.806$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons showed that only the mice that spent the higher percentage of time in the open arms (High %TOA) developed CPP ($p < 0.001$, more time in the drug-paired compartment in Post-C than in Pre-C). The control group (non-defeated mice) and the group of defeated mice that spent the lower percentage of time in the open arms (Low %TOA) did not develop CPP (see **Figure 5B**).

Besides the percentage of time in the open arms, there were other differences in the open arm measures between mice that were resilient and vulnerable to the long-term effects of RSD on cocaine-induced CPP. Student's *t*-tests indicated significant differences between both groups of defeated mice with respect to the time spent ($t_{(26)} = -5.937$; $p < 0.001$), number of entries

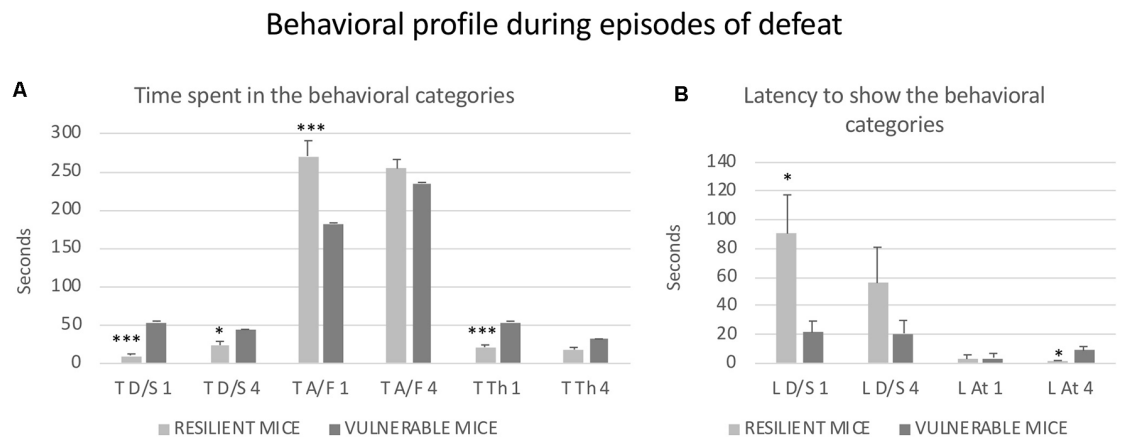

FIGURE 3 | Behavioral profile during episodes of the defeat of mice that were resilient to the long-term effects of RSD on a cocaine-induced CPP. All mice were exposed to RSD (n = 25) and later conditioned with cocaine. **(A)** Bars represent the mean (±SEM) time (in seconds) spent in the behavioral categories of defense/submission (T D/S), avoidance/flee (T A/F) and threat (T Th) analyzed during the first and fourth episodes of defeat. **(B)** Bars represent the mean (±SEM) latency (in seconds) to show defense/submission (L D/S) and attack (L At). As can be seen in **Figure 2**, defeated mice that did not acquire cocaine-induced CPP were defined as resilient mice (light gray bars), while defeated mice that developed cocaine-induced CPP were defined as vulnerable mice (dark gray bars). $^{*}P < 0.05$, $^{***}P < 0.001$, significant difference vs. vulnerable mice.

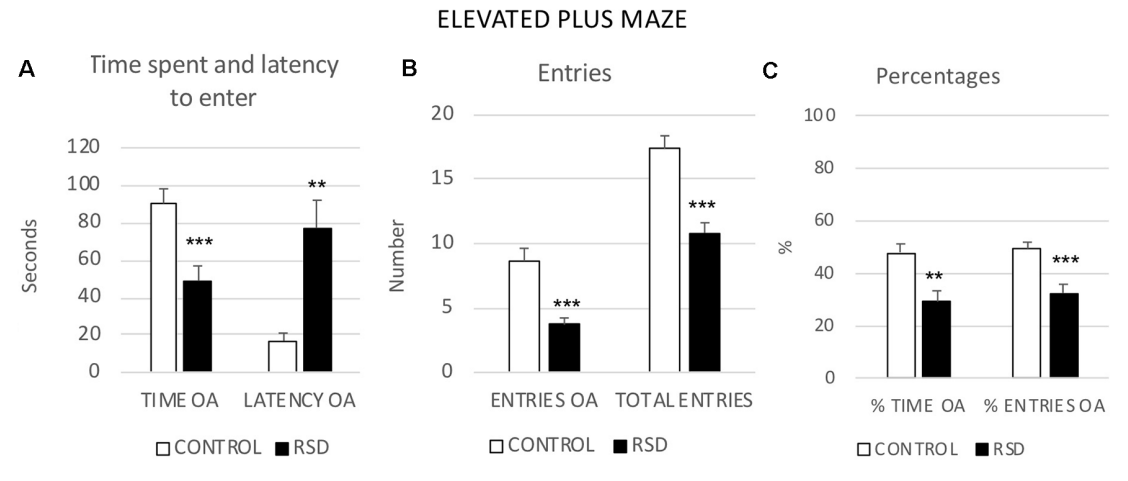

FIGURE 4 | Effects of RSD on the EPM. One group of mice was not exposed to stress (CONTROL, n = 15) and the other group was exposed to RSD (n = 25). The behavior of mice in the EPM was evaluated. **(A)** Bars represent the mean (±SEM) time (in seconds) spent in the open arms (OA) of the maze and the mean (±SEM) latency to enter to the OA. **(B)** Bars represent the mean (±SEM) number of entries into the arms of the maze. **(C)** Bars represent the mean (±SEM) percentages of time spent in and entries into the OA. $^{**}P < 0.01$, $^{***}P < 0.001$, significant difference with respect to CONTROL group.

($t_{(26)} = -3.341$; $p < 0.01$) and percentage of entries into the open arms ($t_{(26)} = -4.619$; $p < 0.001$). It appeared that mice that were resilient to the long-term effects of RSD on cocaine-induced CPP engaged less in the exploration of the open arms (see **Supplementary Figure S2**).

Hole Board Test and Resilience to Cocaine CPP

No significant effects of RSD were observed in the latency to the first dip, but defeated mice showed an almost significant reduction in the number of dips ($t_{(40)} = 1.930$, $p < 0.06$; **Figure 6A**, second bar).

In order to evaluate resilience to the effects of RSD in the hole board test, defeated mice were divided into two subgroups according to their dip scores (below or above the median of the defeated group, 26 dips), Low novelty-seeking (Low NS) or High NS. A one-way ANOVA revealed a significant effect of the variable Group ($F_{(2,39)} = 12.91$, $p < 0.001$). Tukey *post hoc* comcrent from the control group and from the High NS group ($ps < 0.001$; **Figure 6A**). Thus, this group of mice was resilient to the effects of RSD on the hole board test and did not show a decrease in the number of dips.

Resilience to the effects of RSD in the hole board test is inversely related to resilience to the long-term effects of RSD

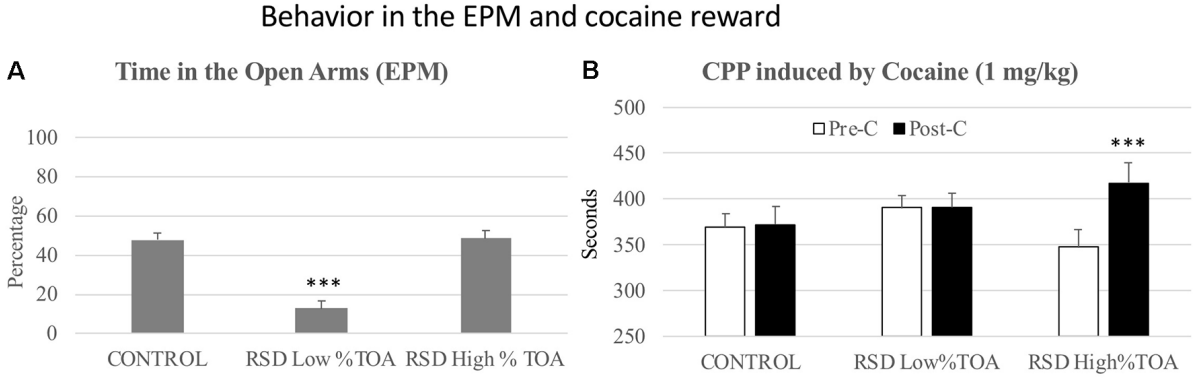

FIGURE 5 | Behavior in the elevated plus-maze and cocaine reward. **(A)** Resilience to the short-term effects of RSD on the EPM. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The group of defeated mice was divided into two subgroups according to the percentage of time they spent in the open arms (%TOA): RSD Low %TOA and RSD High %TOA. Bars represent the mean (±SEM) percentage of TOA. ***$P < 0.001$, significant difference vs. the CONTROL and RSD High %TOA groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the EPM. Mice (CONTROL, RSD Low % TOA and RSD High % TOA groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). ***$P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

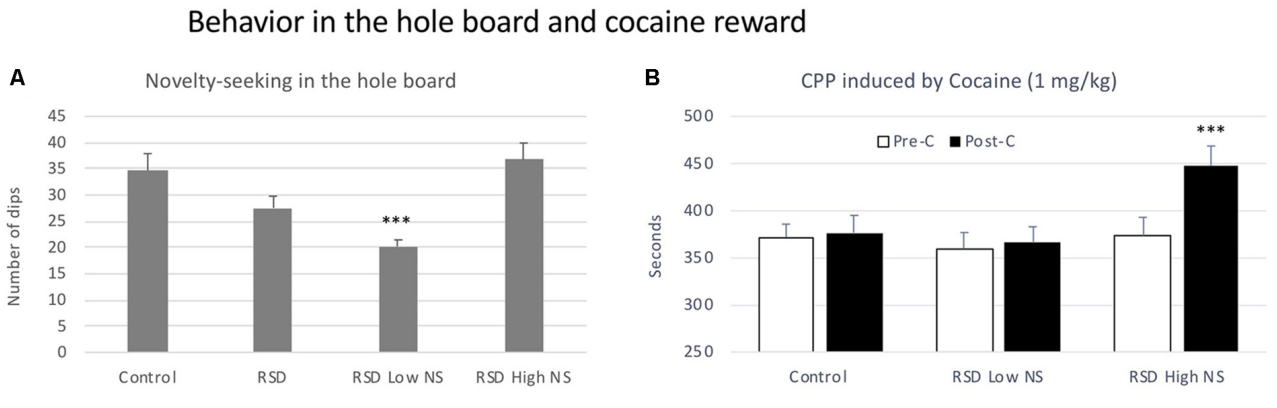

FIGURE 6 | Behavior in the hole board test and cocaine reward. **(A)** Short-term effects of RSD on novelty-seeking behavior in the hole board. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The behavior of mice in the hole board test was evaluated. The group of defeated mice (RSD, second bar) was divided into two subgroups according to the number of dips they performed: RSD Low NS and RSD High NS. Bars represent the mean (±SEM) number of dips. ***$P < 0.001$, significant difference vs. the CONTROL and RSD High NS groups. **(B)** Effects of RSD on cocaine-induced CPP according to the novelty-seeking (NS) profile of mice. Mice (CONTROL, RSD Low NS and RSD High NS groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). ***$P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of the number of dips (Low and High NS) showed that the variable Days ($F_{(1,38)} = 9.41$, $p < 0.004$) and the Interaction Days × Group ($F_{(2,38)} = 3.65$, $p < 0.04$) were significant. Bonferroni *post hoc* comparisons revealed that only mice in the RSD High NS group developed CPP ($p < 0.001$, more time in the drug-paired compartment in Post-C than in Pre-C). The control group (non-defeated mice) and the group of defeated mice with fewer dips (RSD Low NS) did not develop CPP (see **Figure 6B**).

Social Interaction and Resilience to Cocaine CPP

Mice exposed to RSD showed a reduced ISI when they were exposed to an aggressive OF1 mice ($t_{(39)} = 2.924$; $p < 0.01$; **Figure 7A**, second bar). However, this reduction was not observed in all the defeated mice. According to their ISI score (below or above the median of the defeated group, 0.43), defeated mice were separated into two groups: Low ISI or High ISI. A one-way ANOVA revealed a significant effect of the variable Group ($F_{(2,39)} = 42.231$, $p < 0.001$). Tukey *post hoc* comparisons indicated that the Low ISI group was significantly different from

the control and High ISI groups (*p*s < 0.001; **Figure 7A**). Thus, there was a group of defeated mice that was resilient to the impairing effects of RSD on social interaction and that did not engage in less social interaction.

The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in the function of their ISI showed that the variable Days ($F_{(1,37)} = 12.032$; $p < 0.001$) and the interaction Days × Group ($F_{(2,37)} = 3.508$; $p < 0.05$) were significant. *Post hoc* comparisons revealed that only the RSD Low ISI group displayed CPP ($p < 0.05$, significantly higher time spent in the drug-paired compartment in Post-C than in Pre-C). The control group of mice not exposed to defeat and the group of defeated mice that showed a higher social interaction index (RSD High SI group) did not develop CPP (see **Figure 7B**).

Tail Suspension Test and Resilience to Cocaine CPP

With respect to the control group, RSD reduced the Time spent immobile by the mice ($t_{(42)} = 4.452$; $p < 0.0$; **Figure 8A**, second bar), but did not affect the Latency to show this behavior (data not shown).

In order to evaluate resilience to the effects of RSD in the TST, defeated mice were divided into two subgroups according to their scores of Time spent immobile (below or above the median of the defeated group, 141 s, Low TI or High TI. One-way ANOVA showed a significant effect of the variable Group ($F_{(2,41)} = 27.728$; $p < 0.001$). Tukey *post hoc* comparisons indicated that the group that spent less time in immobility (Low TI) was significantly different from the control and High TI groups (*p*s < 0.001;

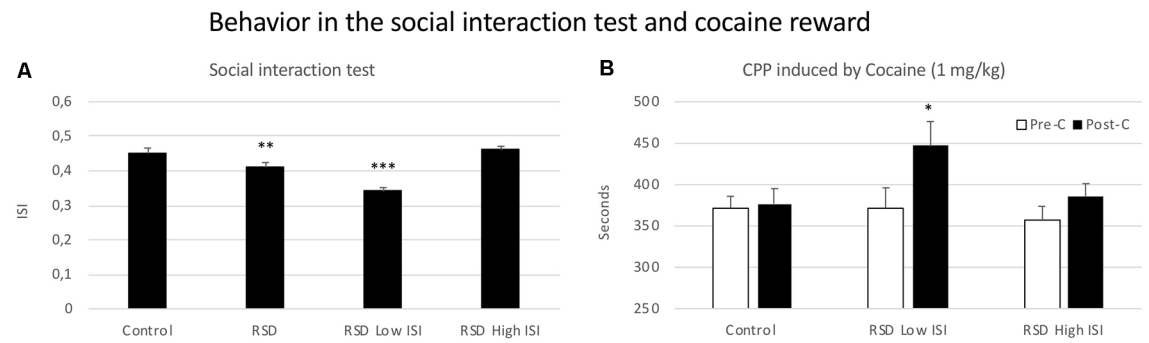

FIGURE 7 | Behavior in the social interaction test and cocaine reward. **(A)** Short-term effects of RSD on the social interaction test. One group of mice was not exposed to stress (CONTROL, *n* = 15) and the other group was exposed to RSD (*n* = 25). The behavior of mice in the social interaction test was evaluated. The group of defeated mice (RSD, second bar) was divided into two subgroups according to their index of social interaction (ISI): RSD Low ISI and RSD High ISI. Bars represent the mean (±SEM) ISI. $^{**}P < 0.01$, significant difference with respect to the CONTROL group; $^{***}P < 0.001$, significant difference with respect to the CONTROL and RSD Low ISI groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the social interaction test. Mice (CONTROL, RSD Low ISI and RSD High ISI groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). $^{*}P < 0.005$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

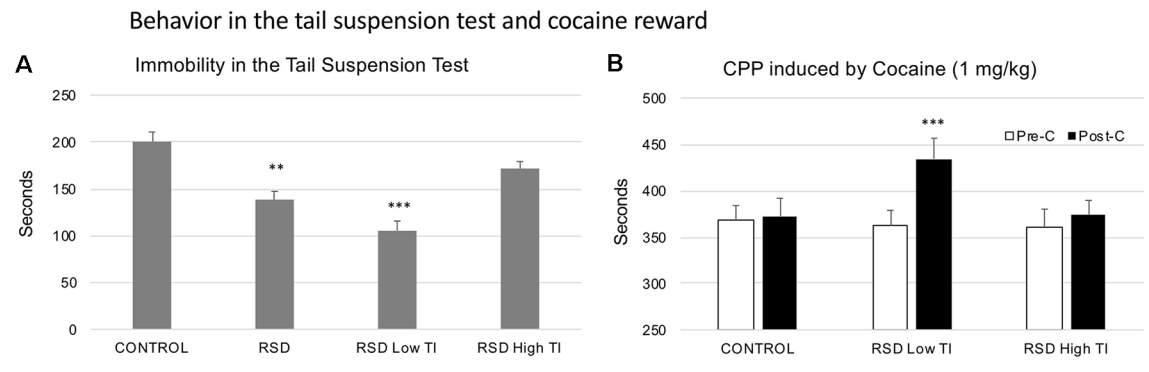

FIGURE 8 | Behavior in the TST and cocaine reward. **(A)** Short-term effects of RSD on the TST. One group of mice was not exposed to stress (CONTROL, *n* = 15) and the other group was exposed to RSD (*n* = 25). The behavior of mice in the TST was evaluated. The group of defeated mice (RSD, second bar) was divided into two subgroups according to the time spent immobile (TI): RSD Low TI and RSD High TI. Bars represent the mean (±SEM) time (in seconds) spent immobile. $^{**}P < 0.01$, significant difference with respect to CONTROL group; $^{***}P < 0.001$, significant difference with respect to the CONTROL and RSD High NS groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the TST. Mice (CONTROL, RSD Low TI and RSD High TI groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). $^{***}P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

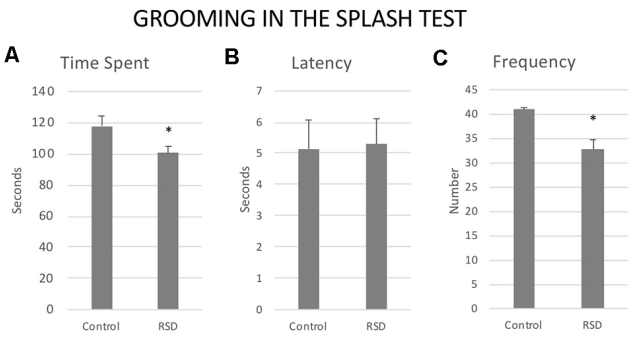

FIGURE 9 | Effects of RSD on grooming behavior in the splash test. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$) on PND 47, 50, 53 and 56. The grooming behavior of mice in the splash test was evaluated. **(A)** Bars represent the mean (±SEM) time (in seconds) spent grooming. **(B)** Bars represent the mean (±SEM) latency (in seconds) to groom. **(C)** Bars represent the mean (±SEM) number of times that the mice perform grooming. $^*P < 0.05$, significant difference with respect to Control group.

Figure 8A). Thus, there was a group of mice that was resilient to the effects of RSD on the TST and that did not show a decrease in immobility.

Resilience to the effects of RSD in the tail suspension is associated with resilience to the long-term effects of RSD on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of the Time spent immobile showed that the variable Days ($F_{(1,38)} = 11.029$; $p < 0.01$) and the Interaction Days × Group ($F_{(2,38)} = 3.320$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons showed that only the Low TI group developed CPP (more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$). The control (non-defeated mice) and the RSD High TI groups did not develop CPP (see **Figure 8B**).

Splash Test and Resilience to Cocaine CPP

Exposure to RSD reduced the Frequency ($t_{(40)} = 2.37$; $p < 0.05$) and the Time spent in Grooming ($t_{(40)} = 2.407$; $p < 0.05$). No significant effects were observed with respect to the Latency to the first grooming ($t_{(40)} = -0.115$; $p < 0.9$; **Figure 9**).

In order to evaluate resilience to the effects of RSD in the splash test, defeated mice were divided into two subgroups according to their scores of Frequency of grooming (below or above the median of the defeated group, 33.8 times), Low FG or High FG. One-way ANOVA showed a significant effect of the variable Group ($F_{(2,39)} = 15,758$; $p < 0.001$). Tukey *post hoc* comparisons indicated that the group RSD Low FG differed significantly from the control and the RSD High FG groups ($ps < 0.001$; **Figure 10A**). Thus, this group of mice was resilient to the effects of RSD on the splash test and did not show a decrease in grooming.

Resilience to the effects of RSD in the splash test is associated with resilience to the long-term effects of RSD on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of their frequency of grooming showed that the variable Days ($F_{(1,36)} = 7.82$, $p < 0.01$) and the Interaction Days × Group ($F_{(2,36)} = 3.230$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons showed that only the RSD Low FG group developed CPP (more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$). The control (non-defeated mice) and the RSD High FG groups did not develop CPP (see **Figure 10B**).

When defeated mice were divided into two subgroups according to their scores in Time spent grooming (below or

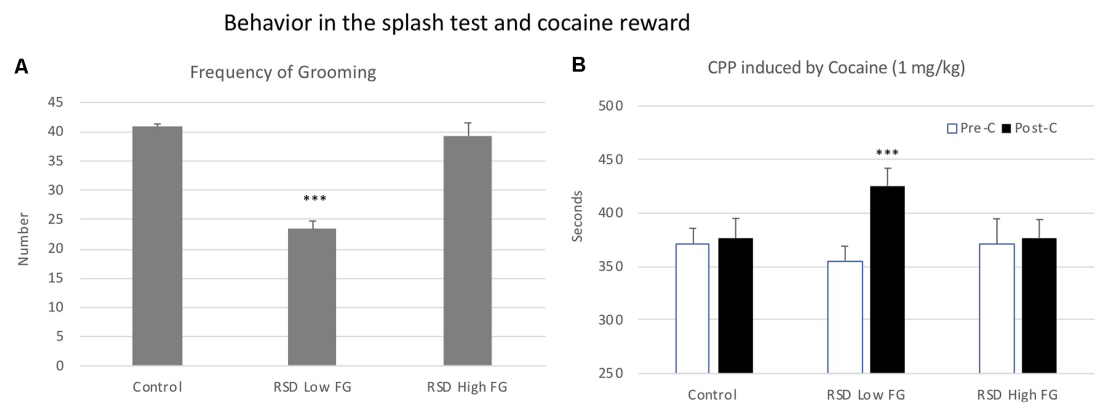

FIGURE 10 | Behavior in the splash test and cocaine reward. **(A)** Resilience to the short-term effects of RSD on grooming behavior in the splash test. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The grooming behavior of mice in the splash test was evaluated. The group of defeated mice was divided into two subgroups according to their frequency of grooming (FG): RSD Low FG and RSD High FG. Bars represent the mean (±SEM) number of times that the mice performed grooming behavior. $^{***}P < 0.001$, significant difference vs. the CONTROL and RSD High FG groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the splash test. Mice (CONTROL, RSD Low FG and RSD High FG groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and in the post-conditioning test (Post-C, black bars). $^{***}P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

above the median of the defeated group, 103.22 s), Low TG or High TG, the one-way ANOVA showed a significant effect of the variable Group ($F_{(2,39)}$ = 16, 32; $p < 0.001$) and Tukey *post hoc* comparisons indicated that the group RSD Low TG (mean 81.38, SD 3.35) was significantly different from the control (mean 118, SD 6.3) and the RSD High TG (mean 116, SD 4.38) groups ($ps < 0.001$). However, no influence of this behavioral trait on the CPP induced by cocaine was observed. The ANOVA of the CPP data of control, RSD Low TG and RSD High TG showed that only the variable Days was significant ($F_{(1,36)} = 5.53$, $p < 0.05$; data not shown).

Correlations Between Measurements in the Different Behavioral Tests

A limited number of significant correlations were observed among the performances of mice in the different behavioral procedures (see **Supplementary Table S1**). There was a correlation between the percentage of time in the open arms in the EPM and the time spent immobile in the TST ($r = 0.504$; $p < 0.01$), as well as a negative correlation between the time spent in submission and the ISI ($r = -0.403$; $p < 0.05$). The CPP score correlated with the number of dips ($r = 0.421$; $p < 0.05$), with mice with a higher novelty-seeking proving to be more vulnerable to developing cocaine-induced CPP. Furthermore, there was a significant inverse correlation between the ISI and the CPP score ($r = -0.393$, $p < 0.05$), since mice with reduced social interaction were more likely to show CPP.

DISCUSSION

The results of the present work reveal individual differences in the response of mice to RSD exposure during late adolescence. During defeat experiences, some mice displayed less defense/submission and more avoidance/flee behaviors, while others were characterized by the opposite pattern. In the short term, RSD induced anxiety-like symptoms in the EPM, social avoidance in the social interaction test, hyperreactivity in the TST and depressive-like symptoms in the splash test. In the long term, RSD increased the sensitivity of mice to the rewarding effects of a low dose of cocaine in the CPP paradigm. However, only one subgroup of mice showed anxiety- or depression-like symptomatology, a reduction of novelty-seeking, deficits of social interaction, increased reactivity to stress, and greater vulnerability to cocaine-induced CPP (vulnerable mice), while another subgroup remained resilient to the effects of RSD. More importantly, the behavioral profile of the mice in the short-term response to RSD was predictive of subsequent resilience to the long-term influence of RSD on cocaine reward. The defeated mice characterized by lower levels of defensive/submissive behavior, less interest in the open arms in the EPM, less novelty-seeking behavior, a greater level of social interaction, greater immobility in the TST and a higher frequency of grooming in the splash test were resilient to the RSD-induced potentiation of cocaine CPP.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With the Behavioral Profile of Mice During Social Defeat Episodes

After the behavioral analysis of the defeat episodes, defeated mice could be segregated into two subpopulations. In the first episode of defeat, one group (resilient mice) displayed a more active coping response characterized by a longer latency to show submission, less time engaged in defense/submission and more time in avoidance/flee, while the other group (vulnerable mice) showed the opposite behavioral profile. In the fourth episode of defeat, resilient mice showed less defense/submission and were attacked faster by the opponent, which suggests that they managed the stressful situation better than vulnerable mice. The coping response of experimental animals exposed to stress has been used in other studies to distinguish between resilient and vulnerable individuals. For example, male rats were classified as having an active or passive coping strategy according to their latency of submission (Wood et al., 2010, 2013; Pearson-Leary et al., 2017; Grafe et al., 2018; Corbett et al., 2019) and the index of flee behavior in a social interaction test performed after RSD has also been applied to mice (Henriques-Alves and Queiroz, 2016).

Since an active coping strategy has been related with resilience to the negative consequences of stress (Feder et al., 2009; Wu et al., 2013; Wood and Bhatnagar, 2015), we hypothesized that the behavioral profile of mice during defeat episodes is predictive of the long-term effects of RSD on the CPP induced by cocaine. As expected, RSD exposure during late adolescence increased the sensitivity of mice to the rewarding effects of cocaine in adulthood, since only defeated mice developed CPP after conditioning with a dose that was ineffective in non-stressed control mice. These results are in line with and extend our previous findings in OF1 strain mice (Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017; Ferrer-Pérez et al., 2018; García-Pardo et al., 2019). However, this is the first study to demonstrate that certain mice are resilient to the long-term RSD-induced potentiation of cocaine reward. In a previous study, Krishnan et al. (2007) exposed mice to 10 episodes of social defeat and segregated them (on day 11) into susceptible and unsusceptible subjects according to the presence or absence of social avoidance; in other words, only susceptible mice (showing social avoidance) exhibited cocaine-induced CPP. Whether the effect of defeat on cocaine reward continued to be present long after RSD was not evaluated since both resilient and vulnerable mice performed the CPP procedure 24 h after the last session of defeat (Krishnan et al., 2007). Our results indicate that the behavioral profile of late adolescent mice during episodes of defeat is an early predictor of their subsequent susceptibility or resilience to the effects of RSD on cocaine-induced CPP in adulthood. Vulnerable defeated mice with higher levels of submission developed CPP. Conversely, defeated mice that developed a more active coping strategy during defeat episodes were resilient, as they behaved like control mice and did not acquire CPP. These results are in accordance with those observed by Yanovich et al. (2018),

TABLE 1 | Summary table of results.

Behavioral test		Resilient mice	Vulnerable mice
Conditioned place preference	Cocaine CPP	=	↑
Agonistic encounters	Defense/Submission	↓	↑
Elevated plus-maze	Open Arms Measures	↓	=
Hole board test	Novelty-seeking	↓	=
Social interaction test	Social investigation	=	↓
Tail suspension test	Immobility	=	↓
Splash test	Grooming	=	↓

After RSD exposure, vulnerable mice developed CPP with a low dose of cocaine, that did not induce CPP in controls and defeated resilient mice. Reduced defensive/submissive behavior during episodes of defeat, avoidance of the open arms of the elevated plus-maze and lower novelty-seeking were behavioral traits predictive of resilience to the effects of RSD on cocaine CPP. Defeated resilient mice behaved as controls in the social interaction, tail suspension and splash test. Conversely, increased defensive/submissive behavior during episodes of defeat, hyperreactivity in a stressful situation (tail suspension test) and depressive-like behaviors (social avoidance and anhedonia after RSD) were behavioral traits predictive of vulnerability to the effects of RSD on cocaine CPP.

who reported that only selectively bred submissive (but not dominant) mice displayed a marked increase in cocaine CPP after exposure to chronic mild stress. A specific coping strategy is considered to be adaptive (i.e., it reduces the impact of stress on the subject) depending on the environment and the type of stressor (Wood and Bhatnagar, 2015); our results suggest that, in conditions of repeated exposure to brief episodes of social stress, passive coping (such as submissive behaviors and immobility) is less adaptive.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Inversely Related With Resilience to the Anxiety-Like Behavior Induced by RSD in the EPM

Our results show that RSD induces a behavioral profile in the EPM argued to be indicative of anxiety (Campos et al., 2013). In comparison to non-stressed controls, defeated mice spent less time and a lower percentage of time in the open arms of the EPM, performed fewer entries and percentage of entries into these arms, and displayed longer latency to visit an open arm for the first time. These results are in agreement with previous studies reporting that different procedures of social stress induce anxiety-like symptomatology in the EPM (Rodgers and Cole, 1993; Lehmann and Herkenham, 2011; Iñiguez et al., 2014; Duque et al., 2017).

Nevertheless, not all defeated mice showed an aversion for the open arms. Subpopulations could be segregated into those that are susceptible and resilient to the short-term effects of RSD on the EPM. Resilient mice spent a similar percentage of time in the open arms to the control group, which was not exposed to RSD. In contrast, vulnerable mice spent a clearly lower percentage of time in the open arms in comparison to controls and to the other group of defeated mice. Kaufmann and Brennan (2018) also identified a subgroup of defeated mice that spent less time in the open arms (which were also vulnerable to the social avoidance induced by RSD) and another subgroup that was resilient to both deficits. Other studies have also affirmed the existence of animals that are resilient to the effects of several types of social stress on the EPM. For example, using the predator odor stress model, rats were segregated as susceptible or resilient based on EPM behavior and context avoidance (Brodnik et al., 2017). Similarly, in another study, rats were classified as vulnerable or resilient to the effects of RSD on anxiety according to the behavior they displayed in the EPM, dark/lightbox and acoustic startle response test (0 or 1 symptom = resilient rat, 2 or 3 symptoms = vulnerable rat; Le Dorze and Gisquet-Verrier, 2016). In this way, it would seem that some animals are resilient to the anxiety-like behavior induced by social stress.

Due to the close association between anxiety and cocaine use disorders (Vorspan et al., 2015), it can be hypothesized that subjects that are resilient to the effects of RSD on anxiety in the EPM are also resilient to the long-term effects on cocaine reward. However, our results do not support this theory. Unexpectedly, the defeated mice that did not develop cocaine CPP were those that spent a lower percentage of time in the open arms. In contrast, the defeated mice spending a higher percentage of time in the open arms (which were, thus, resilient to the short-term effects of social defeat) showed an enhanced vulnerability to cocaine and developed CPP. No previous studies have evaluated whether the behavioral profile in the EPM after exposure to RSD is related to subsequent vulnerability or resilience to developing cocaine-induced CPP. Krishnan et al. (2007) did not observe a relationship between the expression of anxiety-like symptoms in the EPM and the acquisition of cocaine-induced CPP in mice exposed to RSD. In the study in question, vulnerable mice (which displayed social avoidance and cocaine-induced CPP) and resilient mice (that did not show these effects) exhibited an increase in the time spent in the closed arms in the EPM (Krishnan et al., 2007). Conversely, in a more recent study, rats that were vulnerable to the stress induced by exposure to the odor of a predator were more sensitive to the effects of cocaine (Brodnik et al., 2017). Seven days after stress exposure, male rats were segregated into resilient or susceptible groups according to the time they spent in the open arms of the EPM and in the compartment associated with the predator's odor. In comparison to resilient rats, the hyperactivity induced by cocaine and the reinforcing effect of this drug in the self-administration paradigm were enhanced in susceptible rats (Brodnik et al., 2017). These divergent results may be due to differences in the methodology (species, type of stress, the time elapsed between stress exposure and behavioral testing, etc.). However, from our point of view, the most

important factor is the criterion used to discriminate resilient animals from vulnerable animals. In the study by Brodnik et al. (2017), rats were considered vulnerable when they met both criteria: less than 50 s in the open arms and less than 20 s in the odor-associated compartment. In this way, it can be assumed that rats showing an anxiety/fear response to stress are more vulnerable to the effects of cocaine. Conversely, in the present study, mice that spent a lower percentage of time in the open arms of the EPM were resilient to the long-term effects of RSD and did not develop cocaine-induced CPP. It is not logical to assume that the mice with higher anxiety levels were less vulnerable to cocaine; thus, we propose other interpretations of the results obtained. The EPM test not only reveals an anxious state but might also suggest behavioral disinhibition. In this sense, the longer time spent in the open arms by vulnerable mice that developed CPP might indicate a pre-existing impulsive phenotype (Gass et al., 2014) that predisposes them to be more vulnerable to the effects of cocaine. Furthermore, it is important to consider that the EPM entails a conflict between two natural tendencies: the motivation to stay in the protected closed arms, naturally associated with safety, and the motivation to explore the non-protected open arms, which may be associated with a potential danger or a threat (Ennaceur and Chazot, 2016). There is no objective evidence as to the real significance of a reduction in the open arms measures: i.e., whether it represents anxiety or a sense of security. From our point of view, the mice that were resilient to the long-term effects of RSD on cocaine reward were those that, after experiencing an attack from an opponent, actively avoided the open arms to stay safe from other potential threats.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Related With the Novelty-Seeking Profile of Defeated Mice in the Hole Board Test

Exposure to RSD induced a reduction in the number of dips in the hole board test in some defeated mice, since a tendency to such reduction was observed only in the group of defeated mice as a whole ($n = 25$, $p = 0.06$ with respect to controls). In rodents, novelty-seeking behavior has been defined as a "preference for" or a tendency to increase the exploration of novel objects and environments (Nadal-Alemany, 2008; Belin et al., 2011; Vidal-Infer et al., 2012). A very limited number of studies have evaluated the influence of stress exposure on novelty-seeking behavior, and the few data reported are controversial. In male rats, RSD did not modify their behavior in the hole board test 24 h after the last defeat (Albonetti and Farabollini, 1994), but chronic RSD reduced directed exploration in mice (Erhardt et al., 2009). Conversely, rats chronically exposed to predator odor before and during puberty showed increased novelty-seeking during late adolescence (Toledo-Rodriguez and Sandi, 2011). Such discrepant results are probably due to the different developmental periods in which the animals were exposed to stress. From our point of view, the lower number of dips in the hole board test in the subgroup of defeated mice could have been due to the fact that RSD induced an emotional arousing state that motivated a reduced exploration of a novel, potentially dangerous environment.

The influence that the novelty-seeking trait exerts on vulnerability to stress and drug use has been repeatedly demonstrated (Kabbaj et al., 2001; Duclot et al., 2011; Vidal-Infer et al., 2012; Duclot and Kabbaj, 2013; Clinton et al., 2014; Hodges et al., 2018). In particular, novelty-seeking behavior is one of the personality factors that may explain individual differences in vulnerability to drug abuse (Dellu et al., 1996). Higher novelty-seeking has been identified as a risk factor for the initiation of drug use and transition to abuse (Kelley et al., 2004; Staiger et al., 2007; Milivojevic et al., 2012; Mateos-García et al., 2015). In line with this idea, we observed that the subgroup of mice showing greater novelty-seeking after RSD was more vulnerable to the rewarding effects of cocaine. Conversely, mice performing a significantly lower number of dips (that is, mice that responded to RSD with emotionality or avoidance of a novel environment) remained resilient to the long-term effects of RSD on cocaine reward and did not develop CPP. These results, together with those observed in the EPM, lead us to assume that defeated mice that avoid potential risk are protected from the subsequent consequences of social stress on the rewarding effects of cocaine.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With Resilience to the Social Avoidance Induced by RSD in the Social Interaction Test

Exposure to RSD produced a short-term deficit of social interaction. This reduction of the ISI in defeated mice has been associated with the social avoidance that characterizes affective disorders (Golden et al., 2011), and has been repeatedly observed after RSD or social instability (Krishnan et al., 2007; Golden et al., 2011; Henriques-Alves and Queiroz, 2016; Browne et al., 2018; Dong et al., 2018; Hodges et al., 2018). Furthermore, the ISI is the most used measure to distinguish between mice that are resilient or vulnerable to the effects of different models of social defeat (Krishnan et al., 2007; Chaudhury et al., 2013; Donahue et al., 2014; Friedman et al., 2014; Hodes et al., 2014; Isingrini et al., 2016; Sun et al., 2016; Nelson et al., 2018; Prabhu et al., 2018; Gururajan et al., 2019). In this line, we have also observed a subgroup of resilient mice (with similar ISI to that of control mice) and another subgroup of vulnerable mice that displayed social avoidance. It must be taken into account that the type of opponent used in the social interaction test has an influence on the results observed. In the present study, the use of the OF1 strain instead of the strain employed as experimental animals probably induced a more pronounced social avoidance in defeated mice. In fact, it has been reported that, when the target in the social interaction test was a C57BL/6J mouse, both susceptible and resilient mice spent more time in the interaction zone than when the opponent was an aggressive CD1 mouse (Han et al., 2014). Notwithstanding, even when the opponent was of the same strain, the social interaction was significantly higher in resilient than in susceptible mice (Han et al., 2014).

Defeated mice resilient to social avoidance were also resilient to the long-term effects of RSD on cocaine reward. Only the subgroup of defeated mice with a deficit of social interaction developed CPP after conditioning with a low dose of cocaine that was ineffective in non-stressed control mice and in resilient defeated mice. Similar results have been observed by Krishnan et al. (2007), who reported that only mice with a deficit of social interaction (susceptible) developed CPP after conditioning with 5 mg/kg of cocaine 24 h after social defeat, while unsusceptible mice without social avoidance did not develop CPP. Similarly, vulnerable mice with lower levels of social interaction showed reduced alcohol self-administration in comparison to control mice not exposed to stress and to resilient animals without a social interaction deficit (Nelson et al., 2018).

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With the Resilience to Hyperreactivity Induced by RSD in the Tail Suspension Test

Exposure to RSD reduced the amount of time spent immobile in the TST, an unexpected result taking into account that immobility in this test has been considered to be depression-like behavior (Katz, 1982; Cryan et al., 2005; Pollak et al., 2010). However, other studies have shown that stressed mice spent less time being immobile than control mice in the tail suspension (Brockhurst et al., 2015) or in the forced swim tests (Suo et al., 2013; Sadler and Bailey, 2016). In contrast, other researchers have reported that RSD did not affect immobility 24 h after the last episode of defeat (Kinsey et al., 2007; Krishnan et al., 2007), or even increased it in defeated mice identified as vulnerable in a social interaction test (Dong et al., 2017). As Commons et al. (2017) stated, the behavioral alterations observed in the TST must be interpreted with caution, since this paradigm may model the stress-coping strategy from which depressive-like behavior is inferred. Besides, the use of the TST can be problematic in the case of C57BL/6 mice, as they have a propensity to climb using their tails (Can et al., 2012). In the present study, the decrease in immobility in defeated mice could be attributed to inoculation against stress; however, we suspect that such an effect is related to an enhanced reactivity of defeated mice to the situation of moderate inescapable stress that the TST represents. In contraposition to the conventional interpretation of immobility in the forced swim and TSTs as behavioral despair (Katz, 1982), it has been understood by some to represent enhanced anxiety (van Dijken et al., 1992). In support of this idea, a subgroup of defeated mice exhibiting less immobility in the TST reduced their consumption of sucrose, a behavior associated with the lack of interest in pleasurable activities that characterizes depression (Bowens et al., 2012). In the same line, we observed that RSD decreased the frequency of grooming in the splash test, an effect interpreted as depressive-like symptomatology (see the following section). Considered together, these results suggest that the decreased immobility of defeated mice in the TST should be interpreted as an enhanced reactivity to this stressful situation, rather than a reduction of depressive-like behavior.

In addition, our results indicate that vulnerable mice that are more immobile in the TST are more sensitive to the rewarding effects of cocaine and CPP acquired with a low dose of this drug. Conversely, resilient mice with immobility values similar to controls and not exposed to stress did not develop CPP. Thus, mice that were resilient to RSD-induced hyperreactivity were also resilient to the long-term effects of RSD on cocaine reward.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With Resilience to Depressive-Like Behavior Induced by RSD in the Splash Test

Exposure to RSD decreased the duration and frequency of grooming in the splash test, considered a relevant measure of the motivational state of animals (Butelman et al., 2019). A reduction of grooming behavior has been observed after exposure to different stressors (Jolles et al., 1979; Spruijt et al., 1992; Charney, 2004; Smolinsky et al., 2009; Heaney et al., 2011; Veloso et al., 2016; Szewczyk et al., 2019), and has been interpreted as anhedonia, as it is reversed by antidepressant drugs (Brachman et al., 2016; de Souza et al., 2019).

In addition, we have observed that some defeated mice remained resilient to the depressive-like behavior induced by RSD. Although there are no studies with the splash test, Krishnan et al. (2007) demonstrated that mice that were resilient to the effects of RSD in the social interaction test were also resilient to depression-like behavior evaluated with the sucrose preference test. Conversely, a recent study that segregated mice into resilient and vulnerable subjects according to their immobility values in the TST showed that vulnerable mice with higher immobility spent more time engaged in grooming and exhibited this behavior more frequently in an unfamiliar cage (Reis-Silva et al., 2019). A possible explanation for these divergent results is the different type of stressor used (RSD vs. tail suspension) and the controversial interpretation of the results obtained in the TST (as commented before, greater immobility has been interpreted as depression-like behavior and as lower reactivity to moderate inescapable stress). In the present study, the resilience to the short-term effects of RSD on the frequency of grooming predicted subsequent resilience to cocaine reward; only vulnerable mice with reduced grooming behavior acquired cocaine-induced CPP 3 weeks after RSD. Similar results were reported by Krishnan et al. (2007) 1 day after the last episode of defeat, as only mice with anhedonia (indicated by a lower sucrose preference) showed cocaine CPP.

Correlation Between Behavioral Markers of Resilience to the Long-Term Effects of RSD on the CPP Induced by Cocaine

As discussed in previous sections, the segregation of experimental animals into vulnerable or resilient subpopulations with respect to the effects of stress on cocaine reward has been the subject of only two studies. Brodnik et al. (2017) observed that the reinforcing efficacy of cocaine in the self-administration paradigm was lower in mice that were resilient to the effects of stress (predator odor exposure) on EPM behavior and context

avoidance. Previously, Krishnan et al. (2007) had reported that mice resilient to the effects of RSD on social interaction were also resilient to developing anhedonia and cocaine-induced CPP a short time after defeat. They also observed that the resilient phenotype regarding social interaction (but not regarding depressive-like behavior) persisted 4 weeks after defeat; however, the potential long-term enhanced vulnerability to the rewarding effects of cocaine was not evaluated (Krishnan et al., 2007). The results of the present work are in accordance with and extend those obtained in the aforementioned studies. Our main contribution is to demonstrate that some behavioral profiles of the short-term response to social stress predict the subsequent resilience of defeated mice to the rewarding effects of cocaine. Resilient mice that did not develop cocaine CPP were less submissive during defeat episodes, a behavioral profile associated with an active coping with stress (Finnell et al., 2017; Pearson-Leary et al., 2017; Grafe et al., 2018), which in turn is associated with resilience to developing mental disorders. Furthermore, resilient mice avoided the open arms of the EPM and showed less novelty-seeking in the hole board test, which can be interpreted as active avoidance-risk behavior (in concordance with the higher avoidance/flee behavior observed during the first defeat episode). Mice resilient to developing cocaine CPP were also resilient to social avoidance in the social interaction test, hyperreactivity in the TST and depressive-like behavior in the splash test.

We have attempted to establish a potential association of the different resilient phenotypes by means of correlations between the variables shown to be indicative of resilience to the long-term effects of RSD on cocaine reward (lower defense/submission, lower percentage of time in open arms, lower novelty-seeking, higher ISI, higher immobility in the TST and higher frequency of grooming). Furthermore, the contribution of each individual variable to cocaine resilience was determined by correlating these variables with the CPP score. There was a correlation between the time spent in submission and the ISI: the mice that showed less submission during the defeat episodes were resilient to developing a deficit of social interaction. The percentage of time in the open arms of the EPM correlated with the time spent immobile in the TST; thus, the behavior in both tests seemed to be associated in some way. In light of these results, we hypothesize that mice that are less reactive to stress (i.e., those that show more immobility) feel less of a need for safety in the EPM. The number of dips negatively correlated (although non-significantly, $p < 0.073$) with the frequency of grooming, which may indicate that mice that respond to social defeat with lower novelty-seeking are also more resilient to developing anhedonia. With respect to the CPP scores, only two correlations were statistically significant. First, the correlation between CPP score and the number of dips indicated that the novelty-seeking profile was a strong predictor of resilience or vulnerability to the rewarding effects of cocaine. Second, the correlation between CPP score and ISI indicated that social avoidance induced by RSD was associated with enhanced vulnerability to the rewarding effects of cocaine. These correlations suggest that resilience to the effects of social defeat on cocaine reward may be a result of particular behavioral traits or the combination of several behavioral traits. An important fact is that most of the behavioral tests used in the present study measure unrelated behaviors. However, even in the absence of a correlation with the CPP score, the response of defeated mice in each one of these behavioral tests was predictive of its subsequent resilience or vulnerability to cocaine reward. The main relevance of these results is that they show that cocaine use disorders should be considered from a multi-dimensional perspective. Such disorders result from the interaction of biological and behavioral processes that are altered by environmental factors, such as stress exposure. Some individual behavioral traits, such as the level of novelty-seeking or the degree of social interaction, may confer, by themselves, an enhanced or reduced responsivity to cocaine reward. However, more frequently, a complex neurobehavioral profile resulting from the combination of two or more behavioral traits contributes in a cumulative way to resilience or vulnerability to developing a drug addiction.

CONCLUSION

In the present study, we demonstrate that resilience to the long-term potentiation of the rewarding effects of cocaine-induced RSD is associated with different behavioral profiles. Resilient mice are characterized by less submission during defeat episodes, less interest in the open arms in the EPM, lower novelty-seeking, less reactivity in the TST, and an absence of RSD-induced deficits such as social avoidance and anhedonia (see **Table 1**). A limitation of the present work is the use of the median to discriminate between vulnerable and resilient mice in the behavioral procedures. With this approach, we defined as resilient any mouse below or above the median depending on the test and variable used. However, it is certainly improbable that 50% of the subjects were constantly resilient to the different effects of social defeat stress. In future studies, we will employ larger samples of defeated mice and quartiles (rather than the median) to divide them into resilient and non-resilient subjects, in order to give a more substantiality to the notion of resilience.

The general conclusion of this study, based on the data from all the tests performed, is that several individual traits, including an active coping response, and avoidance of potential dangers in unknown environments, and reduced acute stress reactivity, contribute to a subject's resilience to the negative consequences of social stress (deficit of social interaction, anhedonia and enhanced drug sensitivity). From a translational point of view, our results support the real-world observation that not all individuals exposed to social stress during late adolescence subsequently suffer from mental disorders. For example, not all adolescents exposed to bullying develop cocaine use disorders in adulthood. Resilient subjects have less probability of showing symptoms of post-traumatic stress disorder after a traumatic event (Tugade and Fredrickson, 2004; Wrenn et al., 2011; Lee et al., 2014), while more vulnerable subjects can suffer from mental disorders and addictive behaviors in response to this level of stress. In this context, it is necessary to promote in vulnerable individuals attitudes and personality traits that are characteristic of resilience. According to our results, and to evidence in humans, an active coping strategy (Feder et al.,

2009) and a search for social support (Wu et al., 2013) should be encouraged. Individuals with an active coping response attempt to change their perception of the stressful stimulus (Wu et al., 2013) by means of cognitive reevaluation, which may increase positive thinking, another individual factor associated with resilience (Meredith et al., 2011; Holz et al., 2019). In addition, it is necessary to decrease reactivity to stressful events and increase awareness of dangers, as well as to promote the self-control function and sense of safety. These can be achieved by means of problem-solving tasks, relaxation training and cognitive restructuration (Thompson et al., 2018).

Future works should address ways to increase resilience in vulnerable animals. The negative consequences of stress can be reduced through environmental manipulations (Greenwood and Fleshner, 2008; Schloesser et al., 2010; MacKay et al., 2017) and by allowing mastication during stress exposure, a model of active behavioral coping in rodents (Hennessy and Foy, 1987; Hori et al., 2004; Kubo et al., 2009; Stalnaker et al., 2009; Helmreich et al., 2012). Finally, it is important to study the neurobiological substrates of resilience, which underly the behavioral phenotypes observed in our study. There are recent reviews about the causes of resilience that highlight the importance of neuroplasticity in several brain networks, changes at the blood-brain barrier, genetic factors, and the role of the immune system, the metabolism and the gut microbiota (Cathomas et al., 2019; Feder et al., 2019; Holz et al., 2019; Tsyglakova et al., 2019; Turkson et al., 2019). Based on previous studies in our laboratory, we propose that a reduced inflammatory response, epigenetic changes (lower histone acetylation activity), reduced permeability of the BBB, and lower glutamate activity in the brain reward system may mediate the phenotype of resilience to the effects of RSD on cocaine reward (Montagud-Romero et al., 2016a, 2017; Rodríguez-Arias et al., 2017; García-Pardo et al., 2019). Understanding the individual traits and the neurobiological mechanisms that promote resilience may give rise to multiple new approaches to prevention and the development of pharmacological or behavioral interventions that can increase resilience to the negative sequelae of stress and their influence on drug addiction and other mental disorders.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

AUTHOR CONTRIBUTIONS

MA and MG-P contributed to the conception and design of the study. MA, CC-L, MM-C, and AS-O performed the experiments, organized the databases and performed the statistical analyses. CC-L, MM-C, and AS-O wrote sections of the manuscript. MG-P wrote the complete first draft of the manuscript. MA wrote the final version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00278/full#supplementary-material.

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Dopamine D_3 Receptor Antagonism Reverses the Escalation of Oxycodone Self-administration and Decreases Withdrawal-Induced Hyperalgesia and Irritability-Like Behavior in Oxycodone-Dependent Heterogeneous Stock Rats

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Prescription opioids, such as oxycodone, are highly effective analgesics for clinical pain management, but approximately 25% of patients who are prescribed opioids misuse them, and 5%–10% develop an opioid use disorder (OUD). Effective therapies for the prevention and treatment of opioid abuse and addiction need to be developed. The present study evaluated the effects of the highly selective dopamine D_3 receptor antagonist VK4-116 ([R]-*N*-[4-(4-[3-chloro-5-ethyl-2-methoxyphenyl]piperazin-1-yl)-3-hydroxybutyl]-1*H*-indole-2-carboxamide) on oxycodone addictive-like behaviors. We used a model of extended access to oxycodone self-administration and tested the effects of VK4-116 on the escalation of oxycodone self-administration and withdrawal-induced hyperalgesia and irritability-like behavior in male and female rats. Pretreatment with VK4-116 (5–25 mg/kg, i.p.) dose-dependently decreased the escalation of oxycodone self-administration and reduced withdrawal-induced hyperalgesia and irritability-like behavior in opioid-dependent rats. These findings demonstrate a key role for D_3 receptors in both the motivation to take opioids and negative emotional states that are associated with opioid withdrawal and suggest that D_3 receptor antagonism may be a viable therapeutic approach for the treatment of OUD.

Keywords: VK4-116, escalation, opioid, dependance, withdrawal

INTRODUCTION

More than 2 million Americans currently suffer from substance use disorders that are related to prescription opioid pain relievers, including oxycodone (Oxycontin®, Roxycodone®, Oxecta®), and 500,000 are addicted to heroin (Substance Abuse and Mental Health Services Administration, 2013). Over the past 20 years, the consumption of oxycodone increased by ∼500%, and

opioid-related overdose deaths quadrupled (Kolodny et al., 2015; Compton et al., 2016). Although opioid medications effectively treat acute pain and help relieve chronic pain for some patients (Moore et al., 2013), their use presents a dilemma for healthcare providers because of the risk of addiction. Only three treatments have been approved by the United States Food and Drug Administration for the treatment of opioid use disorder (OUD): methadone, buprenorphine, naltrexone, and their combination (e.g., Suboxone) or extended-release formulations (e.g., Vivitrol). Methadone is a synthetic opioid compound that binds primarily to the μ-opioid receptor (MOR), and buprenorphine is a MOR partial agonist. Methadone and buprenorphine are particularly effective in reducing opioid-induced mortality and maintaining patients in treatment, but important safety concerns and strict regulations because of their agonist properties at MORs have limited their use (National Academies of Sciences, Engineering, and Medicine, 2018). Naltrexone has lower efficacy and poor treatment adherence that have limited its real-world effectiveness (Ndegwa et al., 2016). The recent introduction of extended-release naltrexone (Vivitrol) has slightly improved the use of naltrexone compared with previous formulations (Ndegwa et al., 2016). New treatments for OUD with better efficacy and without agonistic effects at the MOR need to be developed.

Compelling evidence from animal studies suggests the involvement of dopamine D_3 receptors in the development of addictive behaviors that are caused by many drugs of abuse, including opioids (Heidbreder and Newman, 2010; Sokoloff and Le Foll, 2017). D_3 receptor antagonism has been shown to attenuate nicotine (Ross et al., 2007), oxycodone (You et al., 2019), and cocaine (Xi et al., 2005) self-administration, conditioned place preference (CPP) that is induced by drugs of abuse (Ashby et al., 2003; Song et al., 2013), and the reinstatement of drug-seeking behavior that is triggered by drug priming (Vorel et al., 2002; Andreoli et al., 2003; Xi and Gardner, 2007), stress (Xi and Gardner, 2007), and drug-associated cues (Aujla and Beninger, 2005; Higley et al., 2011; Galaj et al., 2015). Moreover, D_3 receptor antagonism reduces locomotor sensitization and reverses the lowering of brain stimulation reward thresholds that is induced by drugs of abuse (Sonntag et al., 1992; Pak et al., 2006; Spiller et al., 2008; Higley et al., 2011). In contrast, D_3 receptor antagonism does not affect the seeking of natural rewards, such as sucrose self-administration and sexual activity (Sonntag et al., 1992; Clément et al., 2009; Higley et al., 2011; You et al., 2017). However, all of these studies were conducted in animals that were subjected to limited-access opioid self-administration (e.g., 3 h/day). The efficacy of D_3 receptor antagonism in animals that exhibit the escalation of opioid intake and opioid dependance remains to be determined. Therefore, the present study tested the effects of the highly selective D_3 receptor antagonist VK4-116([R]-*N*-[4-(4-[3-chloro-5-ethyl-2-methoxyphenyl]piperazin-1-yl)-3-hydroxybutyl]-1*H*-indole-2-carboxamide; *in vitro* profile: D_3 receptor K_i = 6.8 nM, 1,700-fold greater selectivity for D_3 receptors vs. D_2 receptors, and metabolic stability in mouse microsomes; Kumar et al., 2016; You et al., 2019) on oxycodone self-administration and withdrawal-induced hyperalgesia and irritability-like behavior in rats. The rats were given extended access to oxycodone self-administration, a model with high face validity, predictive validity, and construct validity for OUD (Zhang et al., 2014; Wade et al., 2015). This model is highly relevant to substance use disorders (Edwards and Koob, 2013; George et al., 2014) and associated with neuroadaptations that are also observed in humans with substance use disorders (Adinoff et al., 1990; Briand et al., 2008; George et al., 2008, 2012; Vendruscolo et al., 2012). The escalation model has been shown to exhibit seven of the 11 criteria of the *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition (DSM-5; American Psychiatric Association, 2013), including most of the criteria that are required for severe use disorder: (1) tolerance (Ben-Shahar et al., 2006); (2) withdrawal (Ahmed et al., 2002; Vendruscolo et al., 2011); (3) substance taken in larger amount than intended (Ahmed and Koob, 1998); (4) unsuccessful efforts to quit (Ahmed and Cador, 2006; Lenoir et al., 2007); (5) considerable time spent to obtain the drug (Wee et al., 2008); (6) important social, work, or recreational activities given up because of use (George et al., 2008; Lenoir et al., 2013); and (7) continued use despite adverse consequences (Vanderschuren and Everitt, 2004; Vendruscolo et al., 2012; Xue et al., 2012; Seif et al., 2013). The use of extended-access oxycodone self-administration in animal strains that exhibit large individual differences, such as heterogeneous stock (HS) rats (Hansen and Spuhler, 1984; Woods and Mott, 2017), may provide new insights into the development of VK4-116 as a potential treatment for OUD.

The effect of VK4-116 on oxycodone intake (i.e., the primary endpoint in the present study to evaluate treatment efficacy) was tested in two independent cohorts of rats using different experimental designs (between-subjects and within-subjects) to confirm reproducibility. The effects of VK4-116 on hyperalgesia and irritability-like behavior that were associated with oxycodone withdrawal were tested using a within-subjects design.

MATERIALS AND METHODS

Animals

Male and female HS rats were generated at the National Institutes of Health in the 1980s to encompass as much genetic diversity as possible by outbreeding eight inbred rat strains (ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, and WN/N; Hansen and Spuhler, 1984). The HS rats (n = 60, n = 50 for the between-subjects experiment, n = 10 for the within-subjects experiment) were provided by Dr. Leah Solberg Woods (Medical College of Wisconsin, now at Wake Forest University School of Medicine) and housed two per cage on a reverse 12 h/12 h light/dark cycle (lights off at 8:00 AM) in a temperature (20–22°C) and humidity (45%–55%) controlled vivarium with *ad libitum* access to tap water and food pellets (PJ Noyes Company, Lancaster, NH, USA). All of the procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. At the time of testing, the rats' body weights ranged between 350 and 400 g.

Intravenous Catheterization

The animals were anesthetized by isoflurane inhalation, and intravenous catheters were aseptically inserted in the right jugular vein using a modified version of a procedure that was described previously (de Guglielmo et al., 2015, 2017). The vein was punctured with a 22-gauge needle, and the tubing was inserted and secured inside the vein by tying the vein with suture thread. The catheter assembly consisted of an 18 cm length of Micro-Renathane tubing (0.023-inch inner diameter, 0.037-inch outer diameter; Braintree Scientific, Braintree, MA, USA) that was attached to a guide cannula (Plastics One, Roanoke, VA, USA). The guide cannula was bent at a near right angle, embedded in dental acrylic, and anchored with mesh (2 cm square). The catheter exited through a small incision on the back, and the base was sealed with a small plastic cap and metal cover cap. This design helped to keep the catheter base sterile and protected. The catheters were flushed daily with heparinized saline (10 U/ml of heparin sodium; American Pharmaceutical Partners, Schaumburg, IL, USA) in 0.9% bacteriostatic sodium chloride (Hospira, Lake Forest, IL, USA) that contained 20 mg/0.2 ml of the antibiotic Timetin (GlaxoSmithKline, Brentford, UK).

Operant Training

Self-administration was performed in operant conditioning chambers (29 cm × 24 cm × 19.5 cm; Med Associates, St. Albans, VT, USA) that were enclosed in sound-attenuating, ventilated environmental cubicles. The front door and back wall of the chambers were constructed of transparent plastic, and the other walls were opaque metal. Each chamber was equipped with two retractable levers that were located on the front panel. Oxycodone was delivered through plastic catheter tubing that was connected to an infusion pump. The infusion pump and a cue light were activated by responses on the right (active) lever. Responses on the left (inactive) lever were recorded but did not have any scheduled consequences. Activation of the pump resulted in the delivery of 0.1 ml of the fluid. A computer controlled fluid delivery and behavioral data recording.

Oxycodone Self-administration

Each session was initiated by the extension of two retractable levers into the operant chamber. Responses on the right active lever were reinforced on a fixed-ratio 1 (FR1) schedule by intravenous oxycodone (150 μg/0.1 ml/kg/infusion) that was infused over 6 s, followed by a 20 s timeout period that was signaled by the illumination of a cue light above the active lever. Daily 12-h sessions were conducted for 15 days (five sessions/week). Responses on the left inactive lever were recorded but had no scheduled consequences. The animals had access to food but not water during the 12-h session.

Drugs

Oxycodone (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sodium chloride (Hospira, Lake Forest, IL, USA) and administered at a dose of 150 μg/0.1 ml/kg. The dose of oxycodone was selected based on previous studies (Wade et al., 2015; Nguyen et al., 2019) and because it produces significant plasma oxycodone concentrations (40 ng/ml; Mavrikaki et al., 2017). VK4-116 was synthesized by Kumar et al. (2016) in the Newman laboratory at the National Institute on Drug Abuse, based on a published procedure. VK4-116 was dissolved in 25% 2-hydroxypropyl-β-cyclodextrin and intraperitoneally injected at doses of 0, 5, 15, and 25 mg/kg as previously reported (You et al., 2019).

Mechanical Nociceptive Von Frey Testing

Mechanical nociception, reflected by hind paw withdrawal thresholds, was determined by an observer who was blind to the experimental conditions using von Frey filaments, ranging from 8.511 to 281.838 g. The test was performed similarly to previous studies (Kallupi et al., 2018; Kononoff et al., 2018). The test began after 10 min of habituation to the testing environment. A series of von Frey filaments were applied from below the wire mesh to the central region of the plantar surface of the left hind paw in ascending order of force, beginning with the smallest filament (8.511 g). The filament was applied until buckling of the hair occurred, and the filament remained in place for 2 s. Rapid withdrawal of the hind paw was considered a positive response. The stimulus was incrementally increased until a positive response was observed and then decreased until a negative response was observed to determine a pattern of responses to apply to previously described statistical methods (Dixon, 1980). Once the threshold was determined for the left hind paw, the same testing procedure was applied to the right hind paw after 5 min. The 50% paw withdrawal threshold was determined by the formula $Xf + k\delta$, where Xf is the last von Frey filament applied, k is the Dixon value that corresponded to the response pattern, and δ is the mean difference between stimuli. Paw withdrawal thresholds were determined for rats before self-administration (baseline) and 12 h after the last self-administration session (12-h withdrawal).

Irritability-Like Behavior

To test irritability-like behavior during oxycodone withdrawal, we used the bottle-brush test, based on the methods of Lagerspetz and Portin (1968) and Riittinen et al. (1986) and modified slightly for rats (Kimbrough et al., 2017). Irritability-like behavior was tested 12 h after the last self-administration session (12-h withdrawal). Irritability-like behavior was examined by measuring aggressive and defensive responses during the bottle-brush test. Irritability-like behavior sessions were conducted in a randomized order for each animal. Testing consisted of 10 trials per rat in plastic cages (10.5 inches × 19 inches × 8 inches; Ancare, Bellmore, NY, USA) with fresh bedding. During each trial, the rat started at the back of the cage. A bottle brush was rotated toward the animal's whiskers (from the front of the cage) by a treatment-naive experimenter. The brush was rotated around the whiskers of the rat for approximately 1 s. The brush was then rotated back to the front of the cage where it was allowed to hang vertically for approximately 2 s, during which behavioral responses were recorded. A 10-s intertrial interval was used. Three observers who were blind to treatment scored the behaviors in real-time. For each rat, separate sums of aggressive and defensive responses across all trials were determined for each

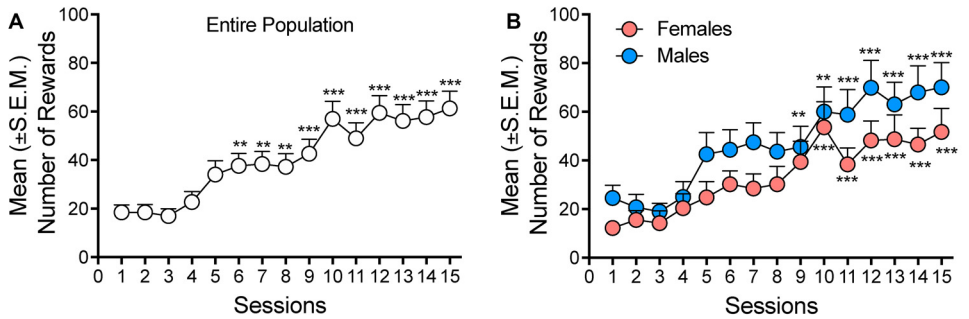

FIGURE 1 | (A) Escalation of oxycodone intake in heterogeneous stock (HS) rats. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. day 1. **(B)** Escalation of oxycodone intake in male (blue) and female (salmon) HS rats. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. day 1.

observer. Aggressive and defensive response scores for each rat were then calculated by averaging the observers' sums. This was then used to calculate a group mean and SEM. The following were scored as aggressive responses: smelling the target, biting the target (during the initial phase of rotating the brush forward and back to the starting position), boxing the target, following the target, exploring the target (using paws or mouth to manipulate the brush without biting or boxing), mounting the target, and delayed biting (during the 2 s that the brush hung at the starting position). The following were scored as defensive responses: escaping from the target, digging, burying, defecation, jumping, climbing, vocalization, and grooming. Grooming and digging were additionally recorded during the 10-s intertrial intervals.

Effect of VK4-116 on the Escalation of Oxycodone Self-administration (Between-Subjects)

Rats (n = 50, 25 males and 25 females) were trained to self-administer oxycodone under an FR1 schedule of reinforcement in daily 12-h sessions. Each active lever press resulted in the delivery of one oxycodone infusion (150 μg/kg/0.1 ml infusion). A 20-s timeout period followed each oxycodone infusion. During the timeout period, responses on the active lever did not have scheduled consequences. This timeout period occurred concurrently with the illumination of a cue light that was located above the active lever to signal delivery of the positive reinforcement. The rats were trained to self-administer oxycodone in fourteen 12-h sessions (5 days/week). At this point, the rats were divided into four groups (n = 12/13 group with similar intake) and intraperitoneally injected with VK4-116 (0, 5, 15, and 25 mg/kg) 30 min before beginning the session.

Effect of VK4-116 on the Escalation of Oxycodone Self-administration and Withdrawal-Induced Hyperalgesia and Irritability-Like Behavior (Within-Subjects)

A separate group of rats (n = 10, four males and six females) were trained to self-administer oxycodone as described above. After 14 sessions of oxycodone self-administration, the animals were intraperitoneally injected with VK4-116 (0, 15, and 25 mg/kg) in a counterbalanced order to evaluate the effects of VK4-116 on withdrawal-induced hyperalgesia using the von Frey test. At the end of the hyperalgesia test, the animals were allowed to self-administer oxycodone so that the effects of VK4-116 on self-administration were assessed again using a Latin-square design. The animals were subjected to oxycodone self-administration at 2-day intervals between drug tests. At the end of the Latin square, the rats were baselined again for oxycodone self-administration and intraperitoneally injected with VK4-116 (0 and 25 mg/kg) in a counterbalanced order to evaluate the effects of VK4-116 on withdrawal-induced irritability-like behavior.

Statistical Analysis

The self-administration data were analyzed using repeated-measures analysis of variance (ANOVA) of the number of infusions that were earned during the escalation interval. The effects of VK4-116 on self-administration and hyperalgesia were analyzed using appropriate one- or two-way ANOVAs (between- or within-subjects) according to the experimental design. The irritability-like behavior data were analyzed using Student's t-test. Significant effects in the ANOVA were followed by the Newman–Keuls *post hoc* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of VK4-116 on the Escalation of Oxycodone Self-administration

Between-Subjects Study

After 3 weeks of oxycodone self-administration, the rats gradually escalated their oxycodone intake (one-way ANOVA, $F_{(14,658)}$ = 15.46, $p < 0.001$). The Newman–Keuls *post hoc* test revealed the significant escalation of oxycodone self-administration that began from session 6 until session 15 compared with the first day of extended access ($p < 0.01$ for sessions 6–8; $p < 0.001$ for sessions 10–15; **Figure 1A**). The two-way ANOVA that also incorporated sex as a variable

indicated that both males and females escalated their oxycodone intake, with a significant main effect of time ($F_{(14,644)} = 15.15$, $p < 0.001$). However, no differences in oxycodone intake were observed between males and females, reflected by the lack of a main effect of sex ($F_{(1,46)} = 2.738$, $p > 0.05$) and no sex × time interaction ($F_{(1,46)} = 0.614$, $p > 0.05$; **Figure 1B**). Treatment with VK4-116 significantly decreased operant responding for oxycodone ($F_{(3,44)} = 3.454$, $p < 0.05$). The Newman–Keuls *post hoc* test revealed that VK4-116 reduced oxycodone self-administration at the dose of 25 mg/kg ($p < 0.05$, 25 mg/kg vs. 0 mg/kg; **Figure 2A**). The two-way ANOVA that also incorporated sex as a variable indicated that VK4-116 significantly reduced oxycodone intake in males and females, reflected by a significant main effect of treatment ($F_{(3,39)} = 4.203$, $p < 0.05$), with no main effect of sex ($F_{(1,39)} = 0.703$, $p > 0.05$) and no sex × treatment interaction ($F_{(3,39)} = 0.004$, $p > 0.05$; **Figures 2B,C**). Inactive lever responding was low and unaltered by VK4-116 treatment (**Figures 2A–C**, bottom panels).

Further analysis of the data allowed us to identify two different subpopulations of rats. Using a median split, we were able to divide the rats into High and Low responders based on their level of oxycodone self-administration (unpaired *t*-test, $t_{46} = 8.897$, $p < 0.001$; **Figure 3A**). The data on the effects of VK4-116 on oxycodone self-administration were then re-analyzed based on the effects of VK4-116 on these two subpopulations, the results of which showed that VK4-116 reduced oxycodone intake selectively in High-responder rats. The two-way ANOVA, with group (High and Low) and treatment (0, 5, 15, and 25 mg/kg) as between-subjects factors, showed significant effects of group ($F_{(1,40)} = 36.3$, $p < 0.001$) and treatment ($F_{(3,40)} = 5.143$, $p < 0.01$)

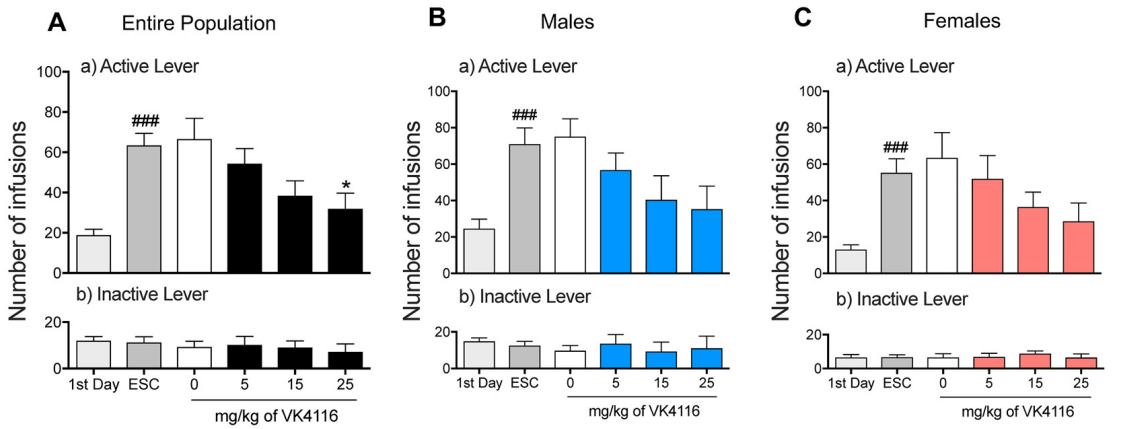

FIGURE 2 | (A) Effect of VK4116 on the escalation of oxycodone self-administration in HS rats. **(B)** Effect of VK4116 on the escalation of oxycodone self-administration in male HS rats. **(C)** Effect of VK4116 on the escalation of oxycodone self-administration in female HS rats. The upper panels (a) represent responses at the active lever. The lower panels (b) represent responses at the inactive lever. $^{*}p < 0.05$, vs. 0 mg/kg; $^{###}p < 0.001$, vs. first day of oxycodone self-administration.

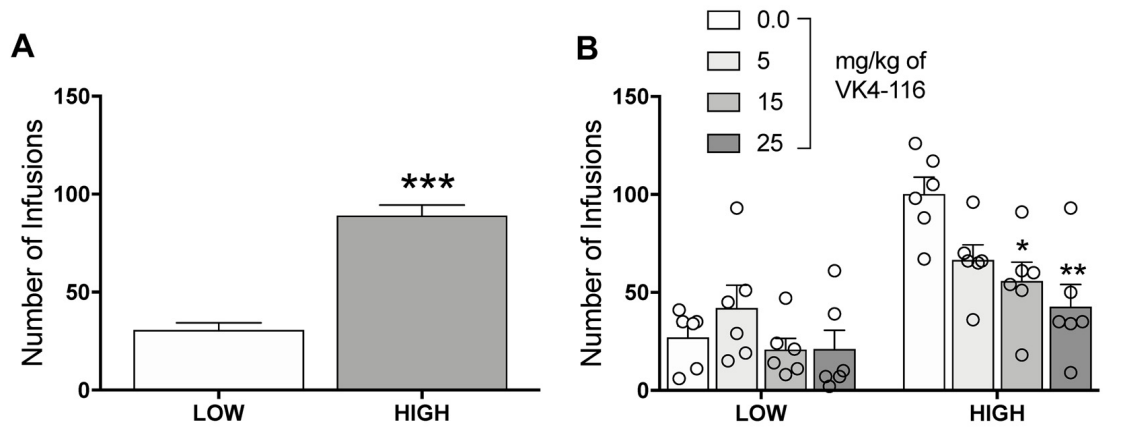

FIGURE 3 | (A) Oxycodone self-administration in HS rats with high vs. low intake. $^{***}p < 0.001$. **(B)** Effect of VK4116 in HS rats with high vs. low oxycodone intake. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. 0 mg/kg.

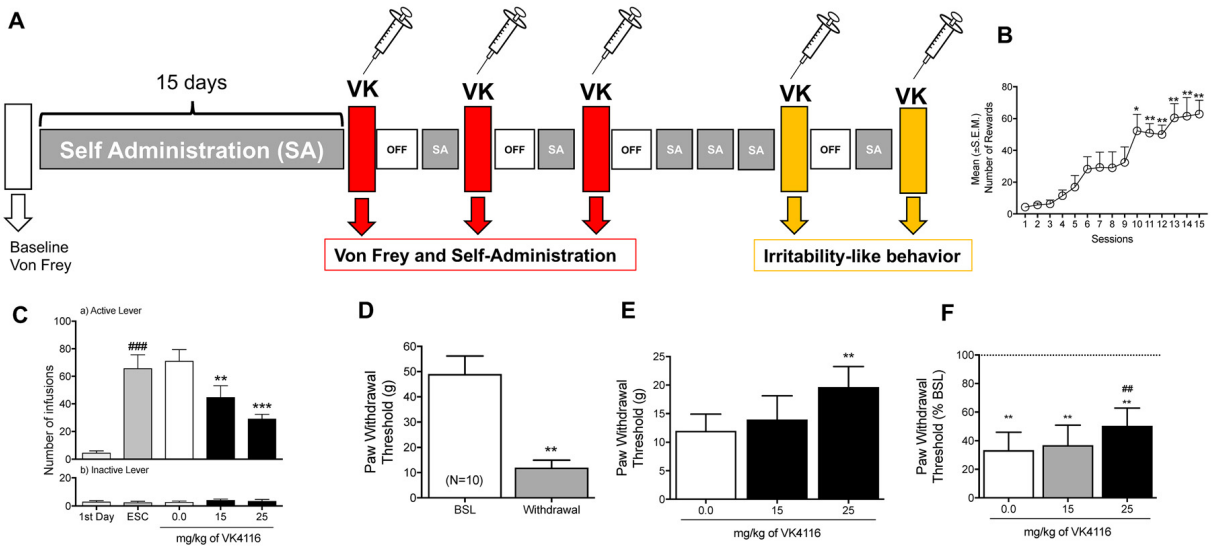

FIGURE 4 | (A) Schematic diagram of the experiment. **(B)** Escalation of oxycodone intake in HS rats. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. day 1. **(C)** Effect of VK4116 in HS rats (within-subjects design). The upper panel (a) represents responses at the active lever. The lower panel (b) represents responses at the inactive lever. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. 0 mg/kg; $^{###}p < 0.001$, vs. first day of oxycodone self-administration. **(D)** Development of mechanical hyperalgesia after the escalation of oxycodone self-administration in HS rats. $^{**}p < 0.01$, vs. baseline (BSL). **(E,F)** Effect of VK4116 on oxycodone withdrawal-induced hyperalgesia. The data are presented as grams of force applied. **(E)** $^{**}p < 0.01$, vs. 0 mg/kg and as a percent change from baseline pre-oxycodone. **(F)** $^{**}p < 0.01$, vs. BSL; $^{##}p < 0.01$, vs. 0 mg/kg.

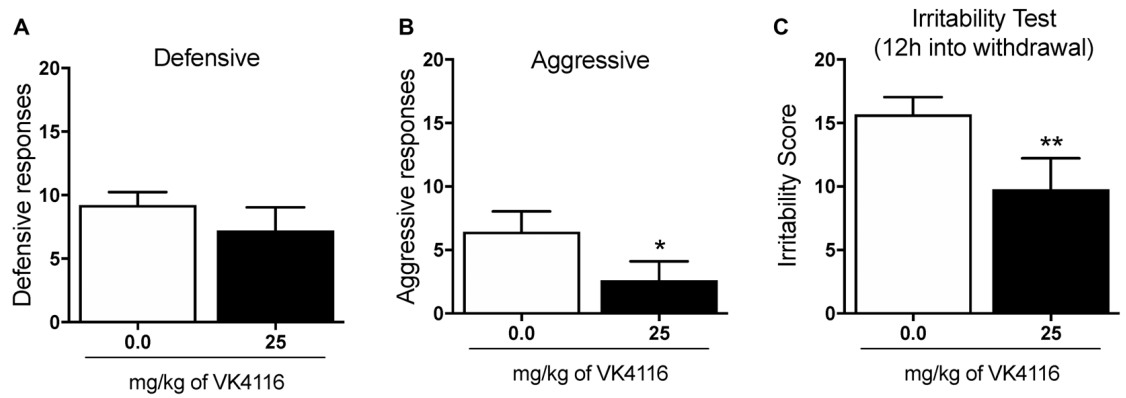

FIGURE 5 | Effect of VK4116 on oxycodone withdrawal-induced irritability-like behavior. **(A)** Number of defensive responses. **(B)** Number of aggressive responses. **(C)** Total irritability score (defensive + aggressive). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. 0 mg/kg.

and a significant group × treatment interaction ($F_{(3,40)} = 3.441$, $p < 0.05$). The Newman–Keuls *post hoc* test revealed that VK4-116 reduced oxycodone self-administration at doses of 15 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.01$) selectively in High-responder rats (**Figure 3B**).

Within-Subjects Study

After 3 weeks of oxycodone self-administration, the rats gradually escalated their oxycodone intake (one-way ANOVA, $F_{(14,126)} = 14.25$, $p < 0.0001$). The Newman–Keuls *post hoc* test revealed the significant escalation of oxycodone self-administration that began from session 10 until session 15 compared with the first day of extended access ($p < 0.05$ for session 10; $p < 0.001$ for sessions 11–15; **Figures 4A,B**). The two-way ANOVA that also incorporated sex as a variable indicated that both males and females escalated their oxycodone intake, reflected by a significant main effect of time ($F_{(14,112)} = 12.53$, $p < 0.001$, data not shown), with no main effect of sex ($F_{(1,8)} = 0.3883$, $p > 0.05$) and no sex × time interaction ($F_{(14,112)} = 0.3832$, $p > 0.05$, data not shown). These results confirmed the observations in the between-subjects experiment that treatment with VK4-116 significantly decreased operant

responding for oxycodone ($F_{(2,18)}$ = 13.68, $p < 0.001$). The Newman–Keuls *post hoc* test revealed that VK4-116 reduced oxycodone self-administration at both doses tested ($p < 0.001$, 25 mg/kg vs. 0 mg/kg; $p < 0.0001$, 25 mg/kg vs. 0 mg/kg; **Figure 4C**). Inactive lever responding was low and unaltered by VK4-116 treatment (**Figure 4C**, bottom panel).

Effect of VK4-116 on the Negative Emotional State That Is Induced by Oxycodone Withdrawal

At 12 h of withdrawal after 15 sessions of extended-access oxycodone self-administration, the rats exhibited the development of hyperalgesia, reflected by lower paw withdrawal thresholds in the von Frey test compared with baseline pre-oxycodone thresholds ($t_{(9)}$ = 4.825, $p < 0.01$; **Figure 4D**). The one-way ANOVA indicated that treatment with VK4-116 significantly reduced withdrawal-induced hyperalgesia ($F_{(2,18)}$ = 7.017, $p < 0.01$). The Newman–Keuls *post hoc* test revealed that VK4-116 reduced withdrawal-induced hyperalgesia at the dose of 25 mg/kg ($p < 0.01$, 25 mg/kg vs. 0 mg/kg; **Figure 4E**). **Figure 4F** shows the data plotted as a percentage of baseline before oxycodone exposure, indicating that VK4-116 reduced withdrawal-induced hyperalgesia but not to levels of the baseline response.

At the end of this experiment, the animals were baselined again to determine their level of oxycodone self-administration and tested for the effects of VK4-116 (25 mg/kg) on irritability-like behavior 12 h into withdrawal. Treatment with VK4-116 did not alter defensive responses ($t_{(9)}$ = 1.212, $p < 0.01$; **Figure 5A**) but significantly reduced aggressive responses ($t_{(9)}$ = 2.759, $p < 0.01$; **Figure 5B**) and the total irritability score ($t_{(9)}$ = 3.484, $p < 0.01$; **Figure 5C**).

DISCUSSION

The present study used two different experimental designs (between- and within-subjects) and found that D_3 receptor antagonism with VK4-116 dose-dependently reduced oxycodone self-administration in oxycodone-dependent male and female rats with a history of escalated oxycodone self-administration. The reduction of oxycodone intake was mainly driven by the effect of VK4-116 in a subpopulation of rats that exhibited a high intake phenotype (i.e., High responders). We then found that the effect of VK4-116 was not limited to drug intake and also extended to a reduction of withdrawal-induced hyperalgesia and irritability-like behavior in oxycodone-dependent rats.

The effects of VK-116 were similar in male and female rats and replicated using two different experimental designs (between- and within-subjects). VK4-116 partially reversed the escalation of oxycodone self-administration, which is consistent with previous studies that reported that D_3 receptor antagonism decreased opioid self-administration in rats that were given limited access to oxycodone or heroin self-administration (Boateng et al., 2015; You et al., 2017; Jordan et al., 2019a) and decreased opioid-induced CPP in rats (Ashby et al., 2003). The present results replicated these findings and further demonstrated that VK4-116 was also effective in an animal model of opioid dependance.

The escalation of intake in opioid-dependent animals has been hypothesized to be driven by the development of a negative emotional state during opioid withdrawal (Koob, 2019). Opioid withdrawal is characterized by hyperalgesia, irritability-like behavior, and anxiety-like behavior (Edwards et al., 2012; Koob, 2019). We hypothesized that the effects that were observed in self-administration studies might be mediated by the ability of VK4-116 to alleviate the negative emotional state during opioid withdrawal. As expected, pretreatment with VK4-116 attenuated oxycodone withdrawal-induced hyperalgesia and irritability-like behavior. These results are consistent with previous studies that reported that VK4-116 dose-dependently attenuated naloxone-precipitated conditioned place aversion in chronic oxycodone-treated rats (You et al., 2019) and that the D_3 receptor antagonist SB-277011A blocked naloxone-precipitated conditioned place aversion in chronic morphine-treated rats (Rice et al., 2012).

The inhibitory effects of VK4-116 on the escalation of oxycodone self-administration and withdrawal-induced hyperalgesia and irritability-like behavior are unlikely to be attributable to nonspecific motor impairment because a previous study that tested VK4-116 reported that it did not affect locomotor activity (Kumar et al., 2016; Jordan et al., 2019b). Moreover, treatment with VK4-116 and other D_3 receptor antagonists did not alter operant sucrose self-administration (Vorel et al., 2002; Clément et al., 2009; Higley et al., 2011; You et al., 2017).

In summary, the present study found that VK4-116 decreased opioid self-administration and attenuated aversive states that were induced by oxycodone withdrawal. The negative emotional states that arise from drug withdrawal are considered to be major factors that drive compulsive drug taking and seeking in drug-dependent individuals (Koob and Mason, 2016; Koob and Volkow, 2016). VK4-116 reduced the reinstatement of drug-seeking behavior that was triggered by oxycodone priming (Jordan et al., 2019a). Despite evidence of the involvement of D_3 receptors in drug addiction, the clinical translation of these findings has been challenging because of insufficient absorption, distribution, metabolism, and excretion properties of D_3 antagonists and possible cardiotoxicity when they are administered in the presence of cocaine (Appel et al., 2015; Keck et al., 2015). The only highly selective D_3 receptor antagonist that has been tested in humans is GSK598809, which was evaluated for the treatment of obesity, nicotine dependence, and alcohol dependence (Dodds et al., 2012; Mugnaini et al., 2013; Murphy et al., 2017). Acute GSK598809 administration produced a significant short-term reduction of nicotine craving (Mugnaini et al., 2013), providing the first clinical evidence that D_3 receptor antagonism may be effective for the treatment of substance use disorders.

VK4-116 has been shown to be highly selective and have a stable metabolic profile across species (Kumar et al., 2016; Jordan et al., 2019a). This metabolic stability distinguishes VK4-116 from many previously characterized D_3 receptor ligands and suggests better translational potential (Jordan et al., 2019a). Altogether, the present results and previous findings

suggest that VK4-116 may be a promising pharmacotherapeutic agent for the treatment of OUD given its ability to reduce the motivation to take opioids and attenuate opioid withdrawal symptoms.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC University of California San Diego.

AUTHOR CONTRIBUTIONS

GG designed and performed the research and wrote the manuscript. MK and SS performed the research. AN provided the compound. OG designed the research and wrote the manuscript.

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REVIEW
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Mechanisms of Shared Vulnerability to Post-traumatic Stress Disorder and Substance Use Disorders

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Psychoactive substance use is a nearly universal human behavior, but a significant minority of people who use addictive substances will go on to develop an addictive disorder. Similarly, though ∼90% of people experience traumatic events in their lifetime, only ∼10% ever develop post-traumatic stress disorder (PTSD). Substance use disorders (SUD) and PTSD are highly comorbid, occurring in the same individual far more often than would be predicted by chance given the respective prevalence of each disorder. Some possible reasons that have been proposed for the relationship between PTSD and SUD are self-medication of anxiety with drugs or alcohol, increased exposure to traumatic events due to activities involved in acquiring illegal substances, or addictive substances altering the brain's stress response systems to make users more vulnerable to PTSD. Yet another possibility is that some people have an intrinsic vulnerability that predisposes them to both PTSD and SUD. In this review, we integrate clinical and animal data to explore these possible etiological links between SUD and PTSD, with an emphasis on interactions between dopaminergic, adrenocorticotropic, GABAergic, and glutamatergic neurobehavioral mechanisms that underlie different emotional learning styles.

Keywords: comorbidity, self-medication, sensitization, individual differences, dual-diagnosis

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INTRODUCTION

Most people will experience a traumatic event in their lifetime. It is normal to exhibit fear during a traumatic situation and to have strong reactions afterward, such as flashbacks and nightmares. Perceived threats induce stereotyped reactions in the mind and body that are meant to cause individuals to respond appropriately and protect themselves from harmful situations. Even though these fear reactions during and after the traumatic experience are not unusual, it is vital that they subside with time. Out of the nearly 90% of adults in the United States that experience a traumatic event, about 10% cannot recover naturally from the trauma and continue to feel in danger and exhibit high levels of stress even when they are not in a dangerous situation (Kilpatrick et al., 2013). This persistent fear is characteristic of post-traumatic stress disorder (PTSD), a debilitating neuropsychiatric illness that causes

individuals to continually suffer from emotional distress even years after experiencing the trauma. While PTSD and substance use disorders (SUD) are phenomenologically distinct in many obvious ways, this review will highlight similar neuropsychiatric processes that can lead to the pathologically intense emotional and motivational reactions that characterize both these disorders.

PTSD most commonly presents in people who have experienced natural disasters, terrorist attacks, war, violent and sexual assaults, and other life-threatening incidents (Kessler et al., 1995; Creamer et al., 2001). Both women and men can develop PTSD, but it is twice as common in women (Dell'Osso et al., 2011). The first studies on PTSD came mostly from male war veterans (especially Vietnam), but with time researchers started noticing that women who experienced sexual assault showed very similar symptoms to male veterans (Kardiner, 1941; Figley, 1978; American Psychiatric Association, 1980; Herman, 1997). This led to increased interest in studying PTSD in both males and females, and to expanding the categories of traumatic experiences considered capable of causing PTSD (Lasiuk and Hegadoren, 2006). As it is currently defined, patients with PTSD must fit several criteria. The person should have experienced a traumatic event (Criterion A) and must be experiencing symptoms in each of four different clusters. The first cluster (Criterion B) is experiencing intrusive memories or re-experiencing the traumatic event, including nightmares, flashbacks, and both psychological and physiological reactions to reminders of the event. The second set of symptoms (Criterion C) are of avoidance, which includes avoiding the thoughts and feelings associated with the event as well as the people tied to it. The third group of symptoms (Criterion D) is negative alterations in mood and cognition, which encompass memory problems exclusive to the event, negative thoughts and sense of blame for one's self and others, reduced interest in engaging in activities, and detachment and isolation from other people. The last set of symptoms (Criterion E) are increased arousal, described as irritability and anger, hypervigilance, difficulty sleeping and, in general, feeling "on edge"(American Psychiatric Association, 2013).

Epidemiological evidence suggests a close relationship between PTSD and SUD. As many as 50–75% of combat veterans with PTSD also have drug or alcohol use disorders (Kulka et al., 1990), and structured interviews detect PTSD in up to 42.5% of patients in inpatient substance abuse programs (Cottler et al., 1992). As devastating as PTSD can be, its clinical course often seems to be worsened by its relationship with SUD. Studies have consistently shown that the co-occurrence of PTSD and SUD makes each individual condition more severe and difficult to treat (Saladin et al., 1995; Ouimette et al., 1996, 1998; Clark et al., 2001). Patients with comorbid PTSD and SUD have poorer mental health functioning, poorer treatment adherence and response, more inpatient hospitalizations, worse physical health, and more interpersonal problems (Brown et al., 1995; Stevens et al., 2003; Ouimette et al., 2006; Norman et al., 2007; Driessen et al., 2008). Patients tend to believe their own PTSD and SUD are functionally related and prefer concurrent, integrated treatment (Brown et al., 1998). Clinicians view these dual-diagnosis patients as particularly challenging, in part because they feel uncertain how best to prioritize and integrate treatment of the two disorders (Najavits, 2002; Back et al., 2009).

In this review article, data from both clinical populations and animal models are presented to highlight the high prevalence of PTSD and SUD comorbidity and propose possible etiological factors that might explain their co-occurrence. Many possible explanations have been proposed for the relationship between PTSD and SUD, and several of these will be considered in turn (**Figure 1**). First, evidence is presented suggesting that the negative consequences of seeking and using addictive drugs may increase exposure to traumatic events, thereby raising the risk of developing PTSD. An analogous idea is then explored that PTSD may increase exposure to addictive drugs through attempts to self-medicate psychiatric symptoms with drugs or alcohol. Next, some overlapping mechanisms of trauma and abuse substances that alter neural and endocrine signals and increase vulnerability to both PTSD and SUD are highlighted. Finally, the focus of the review turns toward intrinsic vulnerability factors that may predispose certain individuals to both PTSD and SUD, including both genetic factors and early life events. The potential role of different emotional learning styles in predisposing some individuals to develop neuropsychiatric disorders is also explored. It is important to point out that these different explanations are not mutually exclusive, and there is evidence to support each of them. Most cases of comorbid PTSD and SUD are likely due to a combination of several of these processes acting simultaneously on the same individual.

SUBSTANCE USE AS A RISK FACTOR FOR TRAUMA EXPOSURE

The first possibility to consider is that SUD in effect causes PTSD by exposing the individual to traumatic stressors resulting from the pursuit and use of addictive substances. One commonality between SUD and PTSD is their incontrovertible dependence on instigating environmental factors. Just as the development of SUD requires exposure to addictive substances, PTSD requires exposure to traumatic events (American Psychiatric Association, 2013). Patients with SUD necessarily are involved in risky substance use, and this kind of use can substantially increase the chances of encountering a wide range of traumatic experiences. Overdose deaths are common in this population, and high rates of rape, physical assaults, and other forms of interpersonal violence have been documented as well (Clark et al., 2001; Johnson et al., 2003, 2006; Kingston and Raghavan, 2009; Lee et al., 2018). One study found that almost a third of traumas resulting in PTSD among SUD patients occurred as a direct result of the use or procurement of illicit substances (Brady et al., 1998). Data from the St. Louis Catchment Area study indicated that users of cocaine and opioids were more than three times as likely as the general population to report a history of trauma, most commonly interpersonal violence (Cottler et al., 1992). An earlier cross-sectional population study of 3,132 adults suggested a more complex relationship between substance use and trauma, with a history of sexual assault emerging as a risk factor for the development of SUD, and then the SUD, in turn, was a risk factor

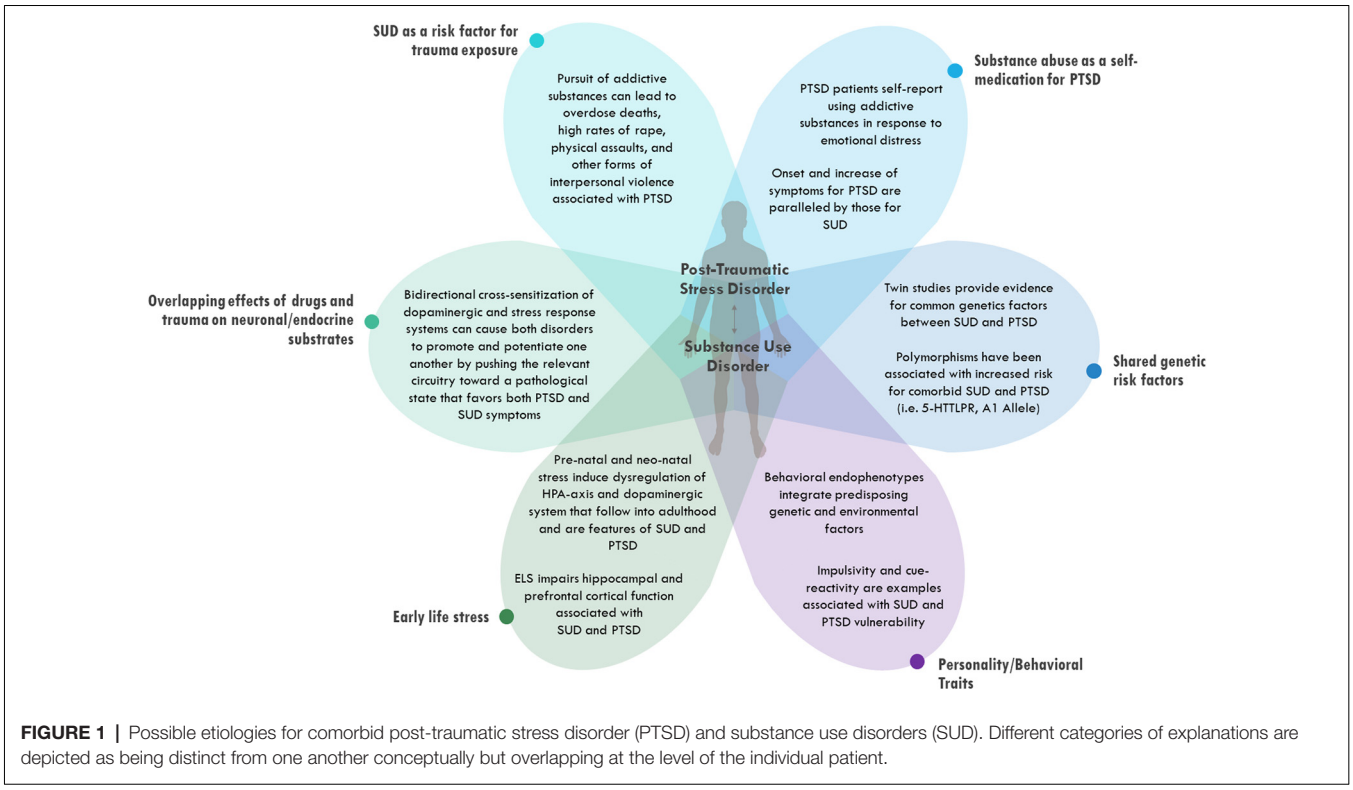

FIGURE 1 | Possible etiologies for comorbid post-traumatic stress disorder (PTSD) and substance use disorders (SUD). Different categories of explanations are depicted as being distinct from one another conceptually but overlapping at the level of the individual patient.

for subsequent assaults (Burnam et al., 1988). As we will see, however, this is but one of several possible connections between PTSD and SUD.

SUBSTANCE ABUSE AS SELF-MEDICATION FOR PTSD SYMPTOMS

Perhaps the most widely accepted explanation for the relationship between PTSD and SUD is the self-medication hypothesis which essentially posits that high rates of SUD are the result of patients using addictive substances to self-medicate their PTSD symptoms (Khantzian, 1985, 1997). For example, a longitudinal study in 1996 on 61 Vietnam veterans investigated the course of illness for both PTSD and SUD symptoms and reported the effects of abused substances on the symptoms of PTSD. They found that most patients developed symptoms like re-experiencing, hyperarousal, and avoidance within 2 years of exposure to combat, with a smaller percentage developing them during the combat tour, and others not meeting full PTSD criteria until 10 years after the combat. Interestingly, they found that the course of alcohol and substance abuse followed the same pattern as the PTSD symptoms. In comparison to 2 years before the war, there was a significant increase at every time point evaluated after the war for the use of alcohol, heroin, cocaine, and marijuana that lasted up to 24 years after the trauma. Overall, these findings suggest that the onset and increase of symptoms for PTSD are closely paralleled by those for alcohol and substance abuse. Additionally, most patients reported that the use of alcohol, marijuana, benzodiazepines, and heroin reduced their PTSD symptoms, supporting the hypothesis that patients are using these substances in order to self-medicate (Bremner et al., 1996). Similar studies in both military and civilian populations have identified PTSD as a prospective risk factor for SUD, and have found that PTSD patients self-report using addictive substances in response to emotional distress (Shipherd et al., 2005; Ullman et al., 2005; Reed et al., 2007; Waldrop et al., 2007; Haller and Chassin, 2014; McDevitt-Murphy et al., 2015).

One important clinical implication of the self-medication hypothesis is that, because PTSD symptoms are the primary drivers of substance use in these patients, effective treatment for comorbid PTSD and SUD should focus primarily on the PTSD symptoms. A "sequential treatment" strategy resulting first in significant improvement of PTSD symptoms should subsequently reduce the need for self-medication and lead to an improvement in SUD outcomes that would otherwise be difficult to achieve. Several clinical trials have attempted to identify effective strategies for the treatment of comorbid PTSD and SUD, but as noted in a recent Cochrane review, these experiments have generally been plagued by high attrition rates and suboptimal study designs (Roberts et al., 2015). Exposure therapy is a highly effective treatment for PTSD involving exposure to trauma-related stimuli that is continued until the fear/anxiety response subsides (Foa et al., 2005, 2008; Wood et al., 2009; Cusack et al., 2016). While several studies have found exposure therapy to improve PTSD outcomes in patients with comorbid PTSD and SUD, none has reported significant improvements in SUD outcomes relative to controls (Simpson

et al., 2017). Few studies have focused on the effects of SUD-specific treatments on PTSD symptoms in this population. One Australian randomized controlled trial tested the efficacy of the Flinders Program of SUD-focused care management, which includes individualized delivery of self-management skills, medication adherence strategies, motivational enhancement, problem-solving, and health-care system navigation, for a sample of 77 Vietnam veterans with alcohol use disorder, almost all of whom also had comorbid PTSD. The group that received the Flinders Program intervention showed greater improvement in SUD outcomes than controls, but there were no group differences in PTSD outcomes (Battersby et al., 2013). The preponderance of evidence suggests that integrated treatments designed to address both SUD and PTSD simultaneously may be associated with better outcomes than sequential treatment (McGovern et al., 2011, 2015; Boden et al., 2012). Though the potential clinical utility of this information is clear, it does little to shed light on the etiological links between SUD and PTSD.

Patients with PTSD often cite their psychiatric symptoms as the reason they use addictive drugs. However, a strong argument can be made that if a patient truly is primarily using substances as part of a conscious strategy to reduce his or her PTSD symptoms, that person does not really have an SUD. SUDs fundamentally involve a loss of control over substance use such that conscious plans, strategies, and explanations for substance use no longer match up with the behavior [National Institute on Alcohol Abuse and Alcoholism (NIAAA) (2019); National Institute on Drug Abuse (NIDA) (2019)]. This does not negate the potential importance of self-medication in the etiology of comorbid PTSD and SUD; what starts out as self-treatment of PTSD symptoms can expose individuals to high levels of substance use, thereby greatly increasing their risk of developing a SUD. However, as the addictive process takes hold the substance use will gradually begin to take on a life of its own and may, therefore, be expected to continue even after the instigating psychiatric symptoms are under good control.

A somewhat different version of the self-medication hypothesis was proposed by Volpicelli et al. (1999). The model went beyond the initial concept that during times of stress, alcohol is used to reduce anxiety levels. Based on the observation that rats tend to increase their alcohol preference days after the stress and not during the days of stress exposure (Volpicelli et al., 1990), they hypothesize that the increase in alcohol consumption seen after a traumatic experience—like that observed on PTSD patients—is more related to post-trauma changes to the stress response system rather than the exposure to the stress itself. Thus, in order to understand this relationship, it is necessary to examine biochemical processes and changes that take place both during and after a traumatic event. The model proposes that during the traumatic event, as part of the "fight or flight" response, there is an increase in the level of endorphins in the brain (Kavushansky et al., 2013). Neuroimaging studies have suggested that, in addition to their well-known role in ameliorating physical pain, endorphins also serve to reduce distressing emotional responses (Liberzon et al., 2002; Zubieta et al., 2003). After trauma, the endorphin system habituates with a reduction in available opioid receptors (Liberzon et al., 2007; Pietrzak et al., 2014), producing a period of withdrawal and symptoms of emotional distress that may contribute to PTSD. Since alcohol can increase endorphin levels, PTSD patients will find that alcohol makes up for that lack of endorphin signaling and compensates for the endorphin withdrawal, leading to the use of alcohol as a way to self-medicate and avoid emotional distress (Volpicelli et al., 1999).

A similar hypothesis centers on the dysregulation of both the glutamatergic and GABAergic systems of PTSD patients, as revealed primarily by proton magnetic resonance spectroscopy studies. Glutamatergic abnormalities such as increases in glutamate in the temporal cortex and reductions in the anterior cingulate are thought to occur due to stress and trauma-induced overflow of glutamate that results in excitotoxicity and inflammatory processes, contributing to long-term problems with regulating stress responses in the central nervous system (Meyerhoff et al., 2014). In conjunction with the glutamate abnormalities seen in PTSD patients, there appears to be a reduction of cortical GABA levels in the parieto-occipital region (Meyerhoff et al., 2014; Rosso et al., 2014). Reduced GABA levels in this region correlate with the severity of PTSD symptoms, particularly insomnia (Meyerhoff et al., 2014). Interestingly, in a study of PTSD patients with alcohol use disorder, it was found that cortical glutamate and GABA levels in the parieto-occipital and temporal cortices were normalized when compared to PTSD patients without alcohol abuse disorder. GABA and glutamate levels in these regions were no longer correlated with PTSD symptom severity or sleep quality in the comorbid population, though the correlation was significant among PTSD patients without an alcohol use disorder (Pennington et al., 2014). These findings suggest that self-medication with alcohol among PTSD patients may help to stabilize glutamate and GABA levels, which could result at least initially in improved PTSD symptoms. However, the comorbid population also showed significant abnormalities suggesting structural and functional damage to the anterior cingulate cortex, all of which strongly correlated with increased PTSD symptom severity that would ultimately lead to worse outcomes in this group (Pennington et al., 2014).

OVERLAPPING EFFECTS OF TRAUMA AND DRUGS ON NEURONAL AND ENDOCRINE SUBSTRATES

As the previously described model indicates, some hypotheses invoke shared neural mechanisms to explain the frequent co-occurrence of PTSD and SUD, as opposed to just increased exposure to trauma and/or drugs of abuse. As will be reviewed in this section, several studies have found evidence of similarly dysregulated brain circuitry in both disorders, particularly in circuits involved in reward and cognitive processes. Therefore, it is possible that at the neural level both disorders can promote and potentiate one another by pushing the relevant circuitry toward a pathological state that favors both PTSD and SUD symptoms. Many of these mechanisms have been espoused as unidirectional, meaning either that PTSD predisposes toward SUD or vice versa, but in almost all cases the reverse causality would logically follow

since both disorders are proposed to act on the same biological systems. This section will highlight some examples of biological systems thought to be affected similarly by both PTSD and SUD.

The first such example is the dopaminergic system, and more specifically dopaminergic projections from the ventral tegmental area in the midbrain to the striatum and prefrontal cortex. These are the mesolimbic and mesocortical systems respectively, and both are highly involved in regulating behavioral responses to rewarding stimuli (Schultz, 2002; Olsen, 2011). Not only is the mesolimbic system involved in mediating responses to natural rewards (e.g., eating, sexual behavior, and exercising), but it has also been proposed as the final common pathway for the rewarding properties of substances of abuse (Pierce and Kumaresan, 2006). These include psychostimulants (e.g., cocaine and amphetamine), ethanol, opiates, cannabinoids, and nicotine with all exerting pharmacological and physiological effects primarily by increasing dopamine transmission in the mesolimbic system either directly or indirectly (Pierce and Kumaresan, 2006). This reward-induced dopaminergic activity promotes motivated behaviors and links those behaviors to cues associated with the reward (Wyvell and Berridge, 2000; Sotak et al., 2005; Hamid et al., 2016).

Aversive and stressful experiences affect the dopaminergic reward pathway in ways that largely mimic the effects of addictive drugs. Both human and animal studies have shown that acute exposure to stress causes increased dopamine release in the nucleus accumbens (Abercrombie et al., 1989; Rougé-Pont et al., 1993; Kalivas and Duffy, 1995; Pruessner et al., 2004; Scott et al., 2006; Wood et al., 2007). Though the mechanisms are not entirely clear, animal studies have shown that this effect is at least partially mediated by activation of the hypothalamic-pituitary-adrenal (HPA) axis, components of which promote dopamine release (Piazza et al., 1996; Rougé-Pont et al., 1998). Stress enhances the effects of drug-related cues on the dopaminergic system, leading to increased cue-induced craving and reinstatement of drug self-administration (Liu and Weiss, 2002; Buffalari and See, 2009; Fox et al., 2014; Moran-Santa Maria et al., 2014). Clinical studies have also found that acute stress is strongly associated with an increased acute risk for relapse to drug use (Khantzian, 1985; Sinha, 2001).

Chronic exposure to alcohol and other drugs of abuse causes long-term changes in reward processing that are thought to promote a continued escalation of substance use. Even though positive hedonic feelings occur shortly after the drug intake, negative hedonic responses follow—especially after repeated exposures—due to alterations in the brain reward system and stress-related structures such as the extended amygdala, resulting in a withdrawal syndrome including dysphoria, irritability, anxiety, and other negative emotional states (Zhang and Schulteis, 2008; Leventhal et al., 2013; Su et al., 2017; Fleming et al., 2019). Some hypothesize that over time the desire to avoid the negative feelings associated with withdrawal becomes the primary motivational factor for compulsive drug-seeking behavior (Solomon and Corbit, 1974; Koob and Volkow, 2010). A key tenet of this opponent-process theory is that circuitry involved in producing the reinforcing effects of drugs of abuse eventually undergoes tolerance, resulting in long-term reductions in dopaminergic activity, an increased reward threshold, and a decreased desire to pursue natural rewarding stimuli (Volkow et al., 1997, 2007, 2014; Martinez et al., 2007). In what amounts to a more intricate version of the self-medication hypothesis, the experienced drug user is described as engaging in ever-increasing levels of drug use in an effort to overcome a chronic and deepening reward deficit.

A similar reward deficit is thought to be a central feature of PTSD, in which case it would be classified as a depressive-like anhedonia syndrome as described in Criterion D. It has been shown that PTSD patients are less likely to expend effort to gain access to a rewarding stimulus (Elman et al., 2005), and they report less reward expectancy and satisfaction if a reward is delivered (Hopper et al., 2008). Some of the underlying brain mechanisms are thought to include reduced activation of mesolimbic structures like the nucleus accumbens in response to positive gains as well as other regions crucial for reward processing including the medial prefrontal cortex (Sailer et al., 2008). PTSD patients have also been found to have an increased density of dopamine transporters in the striatum, which is thought to be a sign of greater dopamine turnover and perhaps reduced dopaminergic tone as dopamine is cleared more efficiently from the synapses (Hoexter et al., 2012). This is similar to the decreased striatal D2 receptor density observed during abstinence in patients with SUD that is thought to mediate withdrawal-related drug craving (Volkow et al., 1993, 2001). Thus, it is possible that alterations in reward circuits produced by PTSD and SUD complement and reinforce one another, resulting in anhedonic states that perpetuate both disorders.

In addition to the above-described anhedonia and overall decrease in dopaminergic activity, chronic drug use is also characterized by a sensitized, hyperdopaminergic response to drug-related cues, with associated increases in motor activity and motivated behaviors including drug self-administration (Kalivas and Stewart, 1991; Robinson and Berridge, 1993; Vezina, 2004). Though most of the original evidence for sensitization was derived from animal research (Robinson and Becker, 1986), behavioral and dopaminergic sensitization to drug cues has now been reported in several human studies as well (Boileau et al., 2006; O'Daly et al., 2011; Booij et al., 2016). This dopaminergic incentive-sensitization effect has often been portrayed as being in conflict with opponent-process, but incentive-sensitization can also be seen as a necessary complement to a theory that is attempting to explain both a general loss of interest in motivated behaviors and a simultaneous increase in one specific type of motivated behavior, namely substance use. The sensitization effect appears very specific to drug-related cues, because evidence of tolerance, rather than sensitization, is generally observed when such cues are absent (Leyton and Vezina, 2013). Sensitization is thought to occur because drugs of abuse directly or indirectly increase dopaminergic transmission in the nucleus accumbens (Hyman et al., 2006). Glutamatergic synapses that are involved in linking drug-related stimuli to drug-taking behavioral responses are active at the time of this dopamine release and are therefore strengthened every time the drug is used due to activation of relatively low-affinity dopamine type 1 receptors. In contrast, synapses representing non-drug related stimuli and actions are

preferentially active in the presence of lower concentrations of dopamine that are more likely to activate high-affinity dopamine type 2 receptors, which will progressively weaken the synaptic strength in those circuits (Grace et al., 2007; Surmeier et al., 2007; Lovinger, 2010). Over time, the drug user's thoughts and behaviors become increasingly funneled toward the drug and its related stimuli, at the expense of all other non-drug rewards regardless of how motivating they may have been in the past (Leyton and Vezina, 2014; Berridge and Robinson, 2016).

Repeated or prolonged exposure to stress can also recapitulate some of the core pathophysiology of SUD. Sensitization of the dopaminergic response to stress has been extensively documented with repeated stress exposure (Jordan et al., 1994; Tidey and Miczek, 1996; Naef et al., 2013), and the behavioral and neurochemical effects of repeated stress cross-sensitize with those of repeated drug exposure (Prasad et al., 1995; Piazza and Le Moal, 1996; Booij et al., 2016). Sensitization of the stress response has been documented in PTSD patients and is thought to be a core feature of the disorder (Dykman et al., 1997; Yehuda, 1997; Elzinga and Bremner, 2002). For example, when subjected to cognitive stress, male veterans suffering from PTSD have increased stress responses and adrenocorticotrophic hormone (ACTH) levels compared to controls, which reflects their higher distress level (de Kloet et al., 2012). It has also been reported that patients who are less responsive to PTSD therapy have salivary cortisol responses to trauma-related imagery that actually strengthens over the course of treatment rather than decreasing or remaining constant (Rauch et al., 2017). Animal studies suggest that, especially with repeated re-exposure to trauma-related cues, these conditioned stress responses can become progressively stronger and expand to other central neurochemical systems such as norepinephrine (Anisman and Sklar, 1979; Jedema et al., 2008; Chen et al., 2012) and serotonin (Adell et al., 1988; Zhang et al., 2014; Hasegawa et al., 2018).

Heightened stress responses lead to increased activity of norepinephrine neurons within the locus coeruleus due to stimulation by corticotropin-releasing factor (Curtis et al., 1997; Reyes et al., 2008). Activity in these norepinephrine neurons triggers a range of aversive and anxiety-like emotional responses (McCall et al., 2015; Hirschberg et al., 2017). Hyperactive norepinephrine signaling is thought to be a core feature of the pathophysiology of PTSD (Bremner et al., 1997; Yehuda et al., 1998; Geracioti et al., 2001; Pietrzak et al., 2013; Steuwe et al., 2014). It may also be involved in SUD, as human and animal studies have found elevations in both central and peripheral noradrenergic activity during all phases of substance use including acute intoxication, chronic use, withdrawal, and relapse (Hawley et al., 1981; Kovács et al., 2002; Patkar et al., 2003; Lanteri et al., 2008; Fitzgerald, 2013). This might suggest that blockade of excessive noradrenergic activity would be helpful for both SUD and PTSD. Indeed, the alpha-1 adrenergic antagonist prazosin has well-established efficacy for reducing PTSD nightmares (Raskind et al., 2003, 2007, 2013; Germain et al., 2012) and prazosin reduced drug self-administration in several animal studies (Walker et al., 2008; Greenwell et al., 2009; Rasmussen et al., 2009; Forget et al., 2010; Lê et al., 2011; Froehlich et al., 2015). Clinical trials of alpha-1 antagonists have also shown promise for the treatment of alcohol use disorder (Simpson et al., 2018; Wilcox et al., 2018). Despite these promising results for each individual disorder, so far prazosin has not been shown to improve outcomes for patients with comorbid PTSD and SUD (Petrakis et al., 2016; Verplaetse et al., 2019). Other noradrenergic agents have been tested with more mixed results for PTSD and SUD, but overall manipulation of the noradrenergic system remains a promising avenue for treatment of this difficult comorbidity.

The relationship between the serotonergic system and comorbid SUD and PTSD is less clear than for the other monoamines. The main evidence for serotonin playing an important role in the pathophysiology of PTSD comes from clinical responses to manipulations of the serotonergic system. Currently, selective serotonin reuptake inhibitors (SSRIs) are the only medications with an FDA approval for the treatment of PTSD (Brady et al., 2000; Davidson et al., 2001; Marshall et al., 2001). Acute reduction of serotoninergic activity using tryptophan depletion exacerbates PTSD symptoms (Corchs et al., 2015). However, administration of the serotonin agonist meta-chlorophenyl-piperamine also causes an acute exacerbation of PTSD symptoms (Southwick et al., 1995), and SSRIs have limited to no efficacy for many PTSD patients (Hertzberg et al., 2000; Zohar et al., 2002). There is some indirect evidence of serotonergic involvement in the development of SUD as well. As with dopamine and norepinephrine, animal studies have shown that most drugs of abuse acutely increase serotonin in both cortical and subcortical areas (Tao and Auerbach, 1995; Selim and Bradberry, 1996; Teneud et al., 1996; Singer et al., 2004; Pum et al., 2007), though cannabis is a notable exception that actually decreases serotonergic activity (Sano et al., 2008). The effects of chronic drug use on serotonin are also fairly consistent across classes, with chronic cocaine, alcohol and morphine causing long-term decreases (Parsons et al., 1995; McBride et al., 2004; Goeldner et al., 2011), but no clear effect of either chronic amphetamine or nicotine (Touiki et al., 2008; Barr et al., 2013). Selective serotonin reuptake inhibitors are generally not effective for SUDs (Kranzler et al., 1995; Lima et al., 2003; Hughes et al., 2014), though some studies indicate that antidepressants for alcohol use disorder may improve outcomes for some patients and worsen outcomes for others depending on family history and pattern of alcohol use (Pettinati et al., 2000; Chick et al., 2004; Kranzler et al., 2012). Clinical trials of SSRIs for comorbid SUD and PTSD have thus far not been promising (Brady et al., 2005; Petrakis et al., 2012).

PERSONALITY/BEHAVIORAL TRAITS

Because of the often complex interactions between relevant genetic and environmental factors, it can be difficult to recognize individual factors that affect vulnerability to PTSD and SUD. Behavioral endophenotypes are more closely related to the abnormalities that characterize these disorders and have the potential to integrate many different underlying genetic and environmental factors, in effect providing a valuable summary of data that might otherwise be prohibitively difficult or impossible to get (Gottesman and Gould, 2003). One such endophenotype

that has been consistently associated with both PTSD and SUD is impulsivity (Weiss et al., 2013; James et al., 2014; Walker et al., 2018; Grubbs and Chapman, 2019). Impulsivity is a multifaceted concept in research but can be broadly defined as a tendency to engage in risky, premature, or situationally inappropriate actions that are characterized by a lack of planning or forethought (Robbins et al., 2012; Jentsch et al., 2014; Dalley and Robbins, 2017). As has been found in patients with PTSD and SUD, impulsivity is associated with lower dopaminergic activity in the NAc at baseline and in response to neutral cues, but exaggerated striatal responses to more salient cues (Forbes et al., 2009; Hahn et al., 2009; Lee et al., 2009; Colzato et al., 2010; O'Sullivan et al., 2011; Reeves et al., 2012). Impulsivity is also thought to result from impaired prefrontal cortical control over motivationally relevant signals from the NAc and other subcortical structures (Rolls et al., 1994; Aron et al., 2004; Schmaal et al., 2012; Davis et al., 2013). This same pattern of prefrontal hypoactivity that is insufficient to restrain subcortical impulses has been identified in functional neuroanatomical studies as a key etiologic factor for both SUD and PTSD in preclinical studies (Peters et al., 2009; Goode and Maren, 2019).

Another related behavioral trait is "cue reactivity," or a tendency towards exaggerated neuronal, emotional and motivational responses to stimuli that have been associated with emotionally salient events. The relevance of cue reactivity to PTSD is clear because excessive fear responses triggered by trauma cues is a core diagnostic feature of PTSD, and the intensity of trauma cue reactions correlates well with PTSD symptom severity (Shin et al., 2004; Rabellino et al., 2016; Rauch et al., 2017). Cue reactivity to drug-related stimuli also predicts relapse in patients with SUD (Rohsenow et al., 1990; Carter and Tiffany, 1999; Janes et al., 2010). Reactions to drug- and trauma-cues seem to intensify and reinforce one another among patients with PTSD and SUD. For example, one study measured visual, physiological, and behavioral responses of patients with comorbid PTSD and cocaine use disorder to cues associated with both their trauma and preferred drug. Compared to those of patients with a single diagnosis of cocaine use disorder and age- and gender-matched controls, patients with dual-diagnosis had excessive cue-reactivity to both the trauma- and drug-related visual cues (Sokhadze et al., 2008). Trauma cues elicit higher levels of distress and negative emotion in patients with comorbid PTSD and SUD when they are accompanied by drug-related imagery (Coffey et al., 2002). Patients with comorbid PTSD and SUD also show more intense drug cue reactivity, including increased cravings to use drugs, when exposed to personalized trauma cues (Coffey et al., 2010; Tull et al., 2011; Read et al., 2017). Both PTSD and SUD patients have a tendency to act impulsively in response to emotionally charged stimuli, a trait that is known as "emotional urgency" (Whiteside and Lynam, 2001; Cyders and Smith, 2008), and this tendency correlates with symptom severity and functional impairment (Ehring and Quack, 2010; Smith and Cyders, 2016). These findings suggest that symptoms of emotional urgency, impulsivity and cue reactivity are interrelated and may cross-sensitize in PTSD and SUD, thereby exacerbating the severity of both illnesses.

In addition to its role in the pathophysiology of PTSD and SUD, cue reactivity may also be a pre-existing behavioral trait that predisposes individuals to develop these disorders. This possibility has mainly been explored in preclinical studies by comparing animals that exhibit individual variation in their reactivity to conditioned cues. An example of this is "sign-trackers" (STs) and "goal-trackers" (GTs) which can be identified using a Pavlovian conditioned approach procedure. When a food reward is paired with a localizable cue such as a retractable lever, STs approach and are attracted to the cue itself, whereas GTs direct their attention away from the cue and towards the location of impending reward delivery (Flagel et al., 2009; Tomie and Morrow, 2018). Sign-tracking is thought to indicate vulnerability to SUD because STs show increased psychomotor sensitization to cocaine (Flagel et al., 2008), have higher preference for cocaine over food (Tunstall and Kearns, 2015) and show increased cue-induced reinstatement of nicotine (Versaggi et al., 2016) and cocaine (Saunders and Robinson, 2011). In addition, it has been shown that STs, identified by the high levels of incentive salience they attribute to reward-related cues, also show elevated fear responses to a tone that has been paired to a foot-shock (Morrow et al., 2011). This indicates that the sign-tracking trait may represent a more general tendency to attribute excessive motivational salience to cues paired with biologically relevant events, regardless of emotional valence. Sign-tracking may therefore also be a risk factor for PTSD, as suggested by evidence that repeated exposure of STs to aversive stimuli results in a fear response that increases over time, instead of decreasing or remaining stable as is the case for GTs (Morrow et al., 2015). Sign-tracking has not yet been studied in human PTSD patients, but as described in the previous paragraph the related trait of cue-reactivity is elevated in subjects with PTSD.

It is important to note that the exaggerated emotional and motivational cue reactivity of STs is specifically tied to discrete, localizable cues. There are no differences between STs and GTs in learning instrumental tasks, so general associative learning and memory processes appear to be intact in both phenotypes (Ahrens et al., 2016; Fitzpatrick et al., 2019). However, STs show lower levels of contextual fear than GTs, as well as decreased context-induced reinstatement of drug self-administration (Morrow et al., 2011; Saunders et al., 2014). Thus, STs tend to react strongly to conditioned cues regardless of the circumstances under which they are encountered, whereas GTs use contextual cues to modulate their conditioned emotional responses (Pitchers et al., 2017). Patients with PTSD show exactly these kinds of deficits; their learned fear responses are insensitive to contextual shifts, safety signals, or other indicators of whether the present circumstances are "safe" or "unsafe" (Maren et al., 2013; Garfinkel et al., 2014; Liberzon and Abelson, 2016). For example, extinction learning is impaired in PTSD patients such that they show relatively high levels of fear in "safe" contexts (Milad et al., 2009; Wicking et al., 2016), but in renewal tests, PTSD patients also fail to show increased fear in the "unsafe" context (Garfinkel et al., 2014). Again this does not appear to be due to a general learning deficit, as PTSD patients do not differ from controls in explicitly or "cognitively" differentiating "safe" and "unsafe" contexts (Steiger et al., 2015).

Rather, the difficulty appears to be specifically in using contextual information to modulate the conditioned emotional response to cues. According to this conceptualization, the problem with fear in PTSD is not that the fear response is too strong. After all, intense fear in life-threatening situations is perfectly normal. It is the expression of fear in inappropriate circumstances that makes these reactions pathological. Drug use is also a normal human behavior, as evidenced by lifetime use estimates in the United States of 48% for illicit drugs, 63% for tobacco products, and 80% for alcohol (Substance Abuse and Mental Health Services Administration, 2018). However over the course of addiction substance use occurs in increasingly inappropriate contexts, such that it comes to interfere with work, relationships, and other important responsibilities. Though there is substantial evidence that physiological and behavioral consequences of drug use can be highly context-dependent (Crombag et al., 2001; Badiani, 2013), there is also evidence of decreased contextual modulation of responses to drug cues among SUD patients as compared to subjects who used drugs but do not have SUD (Garland et al., 2018). Thus, a failure to use contextual information in order to appropriately modify conditioned responses to emotionally salient cues may be a common feature of both PTSD and SUD.

SHARED GENETIC FACTORS

The high rates of comorbidity between neuropsychiatric disorders have suggested that many of them might share common genetic risk factors. It has been proposed that genetic overlap may help to explain the frequent co-occurrence of externalizing disorders like SUD with internalizing disorders such as PTSD, generalized anxiety disorder, and major depressive disorder (Kendler et al., 2003). Twin studies have provided some evidence for genetic commonalities between PTSD and SUD (Xian et al., 2000; McLeod et al., 2001; Koenen et al., 2003; Wolf et al., 2010). For example, in the year 2000, Xian et al. (2000), conducted a study on 3,304 male-male twin pairs from the Vietnam Era Twin Registry (VETR) to examine the genetic overlap of PTSD with alcohol dependence (AD) and drug dependence (DD). According to their study, the risk for PTSD was due to 15.3% common genetics with AD and DD, while risk for AD was accounted for by 55.7% common genetics with PTSD and DD (Xian et al., 2000). Similarly to that found in males, a study of 3,768 female-female twin pairs found that trauma exposure and PTSD had a significant genetic correlation with AD accounting for 28% of its genetic variance (Sartor et al., 2011). Interestingly, another study with twins registered in the VETR focused on how anxiety and mood disorders loaded on externalizing and internalizing factors. They showed that PTSD was unique in the sense that it loaded on both externalizing and internalizing factors while none of the other anxiety/mood disorders loaded on externalizing factors (Wolf et al., 2010). They concluded that the high comorbidity between these internalizing and externalizing disorders can be attributed to genetic factors that predispose to both types of disorders.

Several studies have also focused on specific genetic variants. Of particular interest has been the D2 dopamine receptor (D2DR) *Taq*I A1 allele which has been previously implicated in alcohol (Neiswanger et al., 1995; Lawford et al., 1997; Dahlgren et al., 2011) and DD (Noble et al., 1993; Comings et al., 1996; Lawford et al., 2000; Li et al., 2019). A study of military veterans found that this polymorphism was more frequent in PTSD patients, but only in those who were also harmful drinkers. PTSD patients that were not harmful drinkers did not differ in their A1 allele frequency when compared to controls with a low-risk level of alcohol consumption. In addition, they found that PTSD patients with the A1 allele drank more than twice the amount of alcohol compared to PTSD patients lacking the allele (Young, 2002). In humans, the D2DR A1 allele has been previously linked to reduced density of D2 receptors in the striatum (Noble et al., 1993; Pohjalainen et al., 1998; Jönsson et al., 1999), which is thought to contribute to a hypodopaminergic state and the reward deficiency syndrome associated with SUD and PTSD (Blum et al., 2000, 2012; Elman et al., 2005, 2018; Hopper et al., 2008). In preclinical models, reduced baseline levels of striatal D2 receptors has served as a predictor of an increased rate of cocaine self-administration in both rats (Dalley et al., 2007) and non-human primates (Nader et al., 2006). Interestingly, in rats, this reduced D2 receptor levels in the nucleus accumbens are correlated with a trait of impulsivity as measured by the five-choice serial reaction time task (Dalley et al., 2007). As described in the previous section, impulsivity is thought to be a behavioral endophenotype associated with increased vulnerability to both SUD and PTSD. Selectively bred alcohol-preferring P rats also show reduced D2 receptor levels in the nucleus accumbens (McBride et al., 1993), and upregulation of the D2 receptor in this region decreases both ethanol preference and intake in these rats (Thanos et al., 2001). Similarly, in rats that have been trained to self-administer cocaine, treatment with a D2R vector to increase expression in the nucleus accumbens attenuated the amount of cocaine infusions and lever presses for cocaine, an effect that lasted 6 days (Thanos et al., 2008). In mice, exposure to chronic mild stress induces increased ethanol intake and preference in heterozygous *Drd2*+/− mice compared to wild-type (Delis et al., 2013). *Drd2*+/− mice exposed to chronic mild stress also show increased immobility during the forced swim test, but this is reversed by ethanol intake, supporting the link between SUD and stress regulation (Delis et al., 2013). In preclinical models of PTSD-like symptoms, modulation of the D2 receptor has proven promising in attenuating negative symptoms. For example, in rats subjected to a single prolonged stress (SPS) procedure that mimics psychological trauma, administration of the D2 partial agonist aripiprazole corrects the context- and cue-induced extinction retrieval impairment produced by SPS (Lin et al., 2019). In a similar manner, the D2/D3 agonist rotigotine reduced exaggerated conditioned auditory fear responses and also reduced immobility in the forced swim test in mice that had been subjected to SPS (Malikowska-Racia et al., 2019).

Another gene of interest is the 5-HTTLPR polymorphism of the serotonin transporter, which has been associated with stress reactivity (Gunthert et al., 2007; Gotlib et al., 2008; Miller et al., 2013; Alexander et al., 2014). A study of

environmental and genetic factors in children found that the early use of alcohol could be predicted by an interaction between history of maltreatment and the 5-HTTLPR polymorphism (Kaufman et al., 2007). Another study conducted in adults found that reported experiences of childhood adversity and adult traumatic events predicted PTSD. Again, the 5-HTTLPR polymorphism did not predict PTSD alone, but it did increase risk when combined with childhood and adult traumas (Xie et al., 2009, 2012). This polymorphism is thought to reduce expression levels of the serotonin transporter by impairing the transcriptional efficiency of the gene promoter (Lesch et al., 1996). Thus, translational value has been the SERT knockout and knockdown rodent models. Studies have shown that these mice do not differ in their baseline levels of stress hormones, but in response to a physical stressor, SERT+/− and SERT−/− mice show exaggerated responses of plasma ACTH levels (Murphy et al., 2001) as well as increased plasma epinephrine in SERT−/− (Tjurmina et al., 2002) suggesting alterations in the HPA axis stress-induced response. In addition, exposure to a predator odor induces long-lasting anxiogenic effects in SERT−/− mice as measured by increased anxiety-like behaviors in the plus maze and light/dark box test when compared to wild-type, which may be relevant as a model of increased vulnerability to PTSD (Adamec et al., 2006). SERT−/− mice also show enhanced cocaine-conditioned place preference when compared to wild-type mice (Sora et al., 1998), and SERT−/− rats also show enhanced cocaine-conditioned place preference in addition to an increased psychomotor response to cocaine and intravenous self-administration (Homberg et al., 2008). Altogether, both clinical and preclinical data suggest that alterations in the expression of the serotonin transporter can affect both stress responsivity and the rewarding properties of addictive substances which can influence vulnerability to both SUD and PTSD.

EARLY LIFE STRESS

The impact of early life stress (ELS) on predisposition to neuropsychiatric disorders has been widely studied, particularly with regard to SUD and PTSD (Bremner et al., 1993; Heim and Nemeroff, 2002; Enoch, 2011; Rodrigues et al., 2011; Syed and Nemeroff, 2017; Walters and Kosten, 2019). The HPA-axis plays a central role in mediating stress responses and restoring basal states following a stressor (Habib et al., 2001; Smith and Vale, 2006; Lightman and Conway-Campbell, 2010). Early life stressors, which include neglect as well as physical, emotional, and sexual abuse (Bernstein et al., 1994), can have long-lasting effects on the HPA-axis and manifest as maladaptive behaviors later in adulthood due to both cognitive and emotional impairments. The developing brain is particularly sensitive to external influences, which can alter gene expression, neurochemical balance, neuronal maturation, and synaptic function both basally and in response to stress (Weinstock, 2005, 2008; Glover et al., 2010; van Bodegom et al., 2017; Matthews and McGowan, 2019).

As previously described, disruptions of the stress response due to dysregulation of the HPA-axis as well as altered reward processing due to imbalances in mesolimbic activity can increase susceptibility to both SUD (Piazza et al., 1996; Volkow et al., 1997, 2014; Rougé-Pont et al., 1998; Martinez et al., 2007) and PTSD (Yehuda, 1997; Sailer et al., 2008). In rats, ELS is associated with anxiety-like behaviors such as impaired fear extinction (Judo et al., 2010; Bingham et al., 2013; Wilson et al., 2013), and enhanced psychomotor responses to alcohol (Kawakami et al., 2007), opiates (Kalinichev et al., 2002), amphetamine (Henry et al., 1995; Kehoe et al., 1996; Brake et al., 2004), and cocaine (Kehoe and Boylan, 1992; Brake et al., 2004; Thomas et al., 2009; Anier et al., 2014) as well as enhanced acquisition of cocaine (Kosten et al., 2000; Flagel et al., 2003), methamphetamine (Lewis et al., 2013), and alcohol (Gondré-Lewis et al., 2016) self-administration. In addition, human data shows that maltreated children report alcohol use seven times higher than that of control children as well as an earlier age of drinking initiation, which are predictors of future AD (Kaufman et al., 2007). Furthermore, later in adulthood, the level of substance use positively correlates with both PTSD symptoms and the level of sexual, physical, and emotional childhood trauma (Khoury et al., 2010).

In rats, many studies have shown that pre- and neo-natal stress (e.g., restraint, hypoxia, foot-shocks, etc. to the mother or maternal separation, social deprivation, etc. to the pups) can alter both basal and stressed-induced CRH, ACTH, and corticosterone levels through activation of the HPA-axis, changes which persist into adulthood. As is also the case with the human literature, there has been variability in the results of animal studies due to influences of sex, developmental stage during the stress exposure, the nature of the stressor and its duration, and the age at testing (Weinstock, 2008; van Bodegom et al., 2017). Nonetheless, most studies have been consistent with showing some type of alteration in HPA reactivity. These changes are accompanied by adaptations in the limbic and cortical system, such as increases in the expression of CRHR1 in regions like the PVN (Bravo et al., 2011; Fan et al., 2013; Wang et al., 2013), the amygdala (Bravo et al., 2011; Brunton et al., 2011), the hippocampus (O'Malley et al., 2011), and the prefrontal cortex (Vázquez et al., 2003; O'Malley et al., 2011), which is thought to be crucial for initiation of the stress response (Bale and Vale, 2004; Henckens et al., 2016). In addition, decreased expression of the glucocorticoid receptor in the hippocampus (Henry et al., 1994; Levitt et al., 1996; Green et al., 2011; Bingham et al., 2013) and prefrontal cortex (Green et al., 2011; Bingham et al., 2013) is thought to disrupt the ability of the system to respond appropriately to feedback loops and control stress responses (Jacobson and Sapolsky, 1991; de Kloet et al., 1993; Herman and Cullinan, 1997; Mizoguchi et al., 2003; Herman et al., 2012). ELS results in exaggerated responses to psychological stressors as seen in adult animals exposed to pre- and post-natal stress (Engelmann et al., 1996; Vallée et al., 1997; Tazumi et al., 2005; Brunton and Russell, 2010). As previously described, PTSD patients show dysregulation of the HPA-axis (de Kloet et al., 2006; Dunlop and Wong, 2019). Although basal levels of cortisol seem to be decreased in many studies (Mason et al., 1986; Yehuda et al., 1990; Yehuda and Seckl, 2011), the stress response to trauma-related cues is exaggerated and perpetuated due to the

inability of the system to restore homeostasis (Yehuda, 1997; Elzinga and Bremner, 2002). Importantly for the relationship between ELS, PTSD and SUD, the interaction between the stress and dopaminergic systems seems to be carefully coordinated, suggesting reciprocal modulation between the two systems (Härfstrand et al., 1986; Piazza and Le Moal, 1996; Piazza et al., 1996; Marinelli and Piazza, 2002; Rougé-Pont et al., 1998). In the mesolimbic circuit, particularly within the VTA-NAc projection, ELS causes long-term changes in dopaminergic activity. Both animal and human studies have reported an increase in stress-induced dopamine release during adulthood (Hall et al., 1999; Brake et al., 2004; Pruessner et al., 2004; Yorgason et al., 2013), and animal studies have shown long-lasting changes in dopamine receptors including decreased NAc D2R expression in prenatally stressed rats treated with nicotine (Said et al., 2015) as well as in rats subjected to maternal separation (Majcher-Ma ślanka et al., 2017). As previously mentioned, decreased density of the D2R in the NAc is thought to be an important contributor to drug craving (Volkow et al., 1993, 2001).

Another target of ELS is the hippocampus, which expresses a high density of glucocorticoid receptors (Jacobson and Sapolsky, 1991; Maras and Baram, 2012). ELS can impair hippocampal development by degrading its structure and function. The impairments in learning and memory associated with ELS are thought to be mainly mediated by significant reductions in hippocampal volume and synaptic activity. ELS causes decreased neurogenesis and cell proliferation, reductions in spine density, dendritic atrophy, and disruption of synaptic pruning (Lemaire et al., 2000; Andersen and Teicher, 2004; Brunson et al., 2005; Ivy et al., 2010; Oomen et al., 2010; Hulshof et al., 2011). These changes can directly impact synaptic plasticity in the form of impaired long-term potentiation and facilitated long-term depression (Brunson et al., 2005; Yang et al., 2006; Ivy et al., 2010). For example, in rats subjected to contextual fear conditioning, ELS causes synaptic inhibition between the hippocampus and the medial prefrontal cortex in response to the extinction trials, and this is accompanied by persistent freezing due to impaired extinction retrieval of the fear memory (Judo et al., 2010), a feature of PTSD in humans (Maren et al., 2013; Garfinkel et al., 2014). Decreased hippocampal volume as a result of ELS has been reported in both animal and human models (Uno et al., 1994; Andersen and Teicher, 2004; Humphreys et al., 2019) and this is consistent with significant hippocampal volume reductions in combat- and childhood-related PTSD (Bremner et al., 1995, 1997; Karl et al., 2006; Wang et al., 2010) that worsens over the duration of the disorder (Felmingham et al., 2009; Chao et al., 2014). These findings are not confined to PTSD; reduced hippocampal function has also been identified as a feature of AD (Agartz et al., 1999; Beresford et al., 2006), cannabis dependence (Chye et al., 2019), and methamphetamine psychosis (Orikabe et al., 2011).

The prefrontal cortex is another important player in the inhibition of stress responses and regulation of cognitive and emotional processing (Diorio et al., 1993; Ochsner and Gross, 2005; Herman et al., 2012). ELS has been linked to reduced prefrontal cortical volume and function in adulthood, most likely due to the permanent impact these neurophysiological changes can have on the region while it is still undergoing development (Chocyk et al., 2013). The prefrontal cortex normally regulates subcortical processing of both appetitive and aversive cues. Both decreased volume and hypoactivity of the prefrontal cortex have been linked to anxiety and addiction disorders. In rats, postnatal stress causes a volumetric reduction of the prefrontal cortex (Sarabdjitsingh et al., 2017) and this is consistent with human data showing that childhood emotional maltreatment reduces the volume of the medial prefrontal cortex (van Harmelen et al., 2010) and results in hypoactivity within this region in adulthood (van Harmelen et al., 2014). Patients with PTSD also show hypoactivity of the ventromedial prefrontal cortex (Hayes et al., 2012) and significant thinning (Geuze et al., 2008) that correlates with the severity of their PTSD symptoms (Wrocklage et al., 2017). Reduced prefrontal cortical volumes have also been observed in SUD patients (Liu et al., 1998; Fein et al., 2002; Schlaepfer et al., 2006; Tanabe et al., 2009; Becker et al., 2015), along with a range of deficits in executive control indicating functional impairment of the prefrontal cortex (Goldstein et al., 2004; Goldstein and Volkow, 2011; Ramey and Regier, 2019). In addition, ELS has been shown to blunt the increase in prefrontal D2R expression that normally occurs during adolescence (Brenhouse et al., 2013). This may suggest a link between ELS and subsequent SUD risk since rats exposed to a long access self-administration paradigm show a decrease in D2R mRNA and protein expression in the orbital prefrontal cortex in conjunction with impaired sustained attention (Briand et al., 2008).

Similar to the glutamatergic synaptic overflow reported in PTSD patients and reduced cortical levels of GABA, ELS by maternal separation in mice leads to increased basal levels of glutamate release in the somatosensory cortex as well as increased glutamate release after a nociceptive stressor. Maternally separated mice seem to show an inability to restore glutamatergic homeostasis which may, in turn, affect glucocorticoid secretion in response to stressors (Toya et al., 2014). In addition, in the hippocampus, maternal separation in rats seems to also affect the glutamatergic/GABAergic, excitatory/inhibitory ratio by affecting the expression of proteins responsible for the cycling of these neurotransmitters. This includes upregulation of EAAT1 and EAAT2, which is in accordance with a homeostatic response to increased synaptic glutamatergic levels, as well as downregulation of VGAT and GAD63 which may suggest reduced GABA levels (Martisova et al., 2012). These alterations are thought to be mainly mediated by stress-induced corticosterone release, as chronic treatment with corticosterone recapitulated most of the effects seen in maternally separated rats (Martisova et al., 2012).

Overall, these data suggest that exposure to ELS induces long-lasting effects in the HPA-axis and stress response system that in turn influence the activity of key neurotransmitters like dopamine, glutamate and GABA, and impair proper development and function of structures like the hippocampus and prefrontal cortex. As was described, many of the features that characterize ELS overlap with those previously linked with PTSD and SUD and may help explain why ELS is such a powerful risk factor for developing these disorders in adulthood.

CONCLUSION

Based on the data from patients and animal models, many neurobiological and biochemical factors could be implicated in the development of PTSD and SUD comorbidity. So far, the exact neurobiological underpinnings for this comorbidity remain unknown. There are some putative explanations, e.g., self-medication, that do not require any direct neurobiological relationship between PTSD and SUD. However, based on multiple overlapping psychological and physiological effects of trauma and abuse substances, there are likely several neurobiological mechanisms whereby the development of one disorder can impact the development of the other. Much of the evidence reviewed in this article suggest that chronic exposure to either stress or drugs of abuse can push the mesolimbic motivational system into a state that is poised to react to salient stimuli with a surge of emotional and motivational activity that may be difficult to restrain and result in a multitude pathological behaviors such as those seen in both PTSD and SUD. The study of individual differences in vulnerability to develop these disorders may provide further insight into the surprising prevalence of comorbid PTSD and SUD, especially since both disorders represent a relatively uncommon reaction to the nearly ubiquitous experiences of trauma and substance use. A better understanding of the neurobiology and basic psychological processes that can predispose toward both PTSD and SUD would assist in the rational design of more effective treatment strategies aimed specifically at patients vulnerable to comorbid psychiatric disorders.

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Both CM-R and JM contributed equally to the initial draft and subsequent revisions of this article.

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Prepulse Inhibition of the Startle Reflex as a Predictor of Vulnerability to Develop Locomotor Sensitization to Cocaine

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Prepulse inhibition (PPI) of the startle reflex is a measure of sensory-motor synchronization. A deficit in PPI has been observed in psychiatric patients, especially those with schizophrenia and vulnerable subjects, since the neural bases of this disorder are also involved in the regulation of PPI. Recently, we have reported that baseline PPI levels in mice can predict their sensitivity to the conditioned reinforcing effects of cocaine in the conditioned place preference (CPP) paradigm. Mice with a low PPI presented a lower sensitivity to the conditioned rewarding effects of cocaine; however, once they acquired conditioned preference with a higher dose of the drug, a more persistent associative effect of cocaine with respect to environmental cues was evident in these animals when compared with High-PPI mice. Therefore, we proposed that the PPI paradigm can determine subjects with a higher vulnerability to the effects of cocaine. Developing locomotor sensitization after pre-exposure to cocaine is considered an indicator of transitioning from recreational use to a compulsive consumption of the drug. Thus, the aim of the present study was to evaluate whether subjects with a low PPI display a higher locomotor sensitization induced by cocaine. First, male and female OF1 mice were classified as High- or Low-PPI according to their baseline PPI levels. Subsequently, the motor effects induced by an acute dose of cocaine (Experiments 1 and 2) and the development of locomotor sensitization induced by pre-exposure to this drug (Experiments 3 and 4) were recorded using two apparatuses (Ethovision and actimeter). Low-PPI mice presented low sensitivity to the motor effects of an acute dose of cocaine, but a high increase of activity after repeated administration of the drug, thus suggesting a great developed behavioral sensitization. Differences after pretreatment with cocaine vs. saline were more pronounced among Low-PPI subjects than among High-PPI animals. These results endorse our hypothesis that the PPI paradigm can detect subjects who are more likely to display behaviors induced by cocaine and which can increase the risk of developing a cocaine use disorder. Herein, we further discuss whether a PPI deficit can be considered an endophenotype for cocaine use disorder.

Keywords: prepulse inhibition, cocaine, male and female mice, endophenotype, behavioral sensitization, motor effects, biomarker

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INTRODUCTION

Cocaine use is a serious health problem with important social and economic consequences. Cocaine is the most widely consumed illegal stimulant drug in Europe and North America, mainly by the young adult population [European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2019)]. It is one of the drugs with the fastest transition from abuse to compulsive use (Flórez-Salamanca et al., 2013), and its addiction presents a persistent and high susceptibility to relapse (Volkow et al., 2016). Additionally, its compulsive use is often associated with multiple cardiovascular, neurological, and psychiatric disorders (Galicia et al., 2014; González-Llona et al., 2015; Kariisa et al., 2019). Unfortunately, there is as of yet no approved treatment deemed effective for dependence on this drug (Czoty et al., 2016; Volkow and Boyle, 2018). In this context, obtaining physiological markers, as endophenotypes, that identify the individuals that are most vulnerable to developing a cocaine use disorder is a priority for research in this area. An endophenotype is considered a biomarker of genetic vulnerability that can be measured reliably and indicates a possible risk of a psychiatric disorder with clinical state independency (Beauchaine, 2009). Moreover, it must meet certain criteria, such as distinction from illness in the general population, inheritability, and presence at a higher rate in non-affected family members than in the general population (Gottesman and Gould, 2003).

Deficiencies in the inhibitory mechanisms of the brain, of dopaminergic origin primarily, can result in low levels of self-control in individuals, related in turn to a greater predisposition to developing compulsive drug use (Belin et al., 2013). The prepulse inhibition (PPI) of the startle response is a neurophysiological measure of sensory-motor gating. A deficit in PPI may indicate alterations in the cerebral dopaminergic system; for example, in the mesolimbic pathway (Swerdlow et al., 2016). This deficit is considered an endophenotype of schizophrenia, and has been observed in many other psychiatric disorders (Swerdlow and Light, 2016).

Recently, given that neural structures regulating PPI and drug addiction have been shown to coincide (Volkow and Morales, 2015; Arenas et al., 2017), we have begun to evaluate the predictive ability of PPI in order to identify animals that are more sensitive to the effects of cocaine. In a previous study (Arenas et al., 2018), we showed that the baseline PPI level of mice predicts their sensitivity to the conditioned reinforcing effects of cocaine in the conditioned place preference (CPP) paradigm. Mice with a high PPI—both males and females—acquired CPP with a subthreshold dose of cocaine (1 mg/kg) and with an effective dose (6 mg/kg), while animals with a low PPI did not present conditioned preference with either of these two doses. Males and females with a lower PPI required higher doses of cocaine (12 mg/kg) to acquire conditioned preference than those with a high PPI; in other words, they were less sensitive to the conditioned reinforcing effects of the drug. Nevertheless, among the mice with a low PPI, it took longer to extinguish the conditioned preference in males, while preference was reinstated with lower doses than High-PPI animals in females. These behavioral differences could have been related to differences in the levels of D1 and D2 dopamine receptors in the striatum of these animals. For instance, mice with a low PPI, especially females, presented higher levels of D2 receptor expression (Arenas et al., 2018).

The increased motor response to a drug after repeated exposure is a phenomenon termed behavioral sensitization or motor sensitization (for review, see Steketee and Kalivas, 2011). Development of this sensitization is a possible indicator of the probability of transitioning from recreational use to a compulsive consumption of drugs (Robinson and Berridge, 2003, 2008) and a behavioral representation of drug-induced synaptic plasticity (Lüscher and Malenka, 2011; Steketee and Kalivas, 2011). Long-term sensitization may underlie drug craving and relapse to cocaine use (Steketee, 2005). It has recently been demonstrated that intermittent access to a drug is more effective in producing long-term changes in the brain and addiction-like behaviors, with intermittent patterns of use being especially pronounced during the transition to addiction, prior to regular use (Kawa et al., 2019). This neuroplasticity induced by cocaine use is observed specifically in the dopaminergic system, from the ventral tegmental area to the nucleus accumbens (NAc), which is also a central circuit for the regulation of PPI (Doherty et al., 2008; Rohleder et al., 2016). Moreover, the behavioral effects of cocaine are mediated by D1 and D2 receptors, which have also been associated with baseline PPI levels (Doherty et al., 2008; Arenas et al., 2018). If a PPI deficit indicates alterations in the mesolimbic dopaminergic system (Swerdlow et al., 2016), it is possible that a subject with low PPI is more vulnerable to the cerebral changes induced by cocaine use.

Furthermore, the aim of the present study was to evaluate whether PPI can also be considered an endophenotype for vulnerability to developing sensitization to cocaine-induced motor effects in male and female mice. For this purpose, male and female OF1 mice were classified as High- or Low-PPI according to their baseline PPI levels. The motor effects induced by an acute dose of cocaine (10 mg/kg) and the development of motor sensitization induced by pre-exposure to this drug (Ferrer-Pérez et al., 2018) were then recorded. There are many procedures that can be performed to measure the motor activity of mice. In our laboratory, we primarily use two automatic methods to record the activity of animals in an open field: a computerized video-tracking system named Ethovision and an apparatus with infrared lights, namely, an actimeter (Blanco-Gandía et al., 2017; Ferrer-Pérez et al., 2018; García-Pardo et al., 2019). These two automatic procedures for measuring the motor activity of mice assess the different aspects of the motor response; the Ethovision program measures the distance traveled in cm by the animal, while the actimeter records both the horizontal and vertical activity of the mice. According to previous experience, we hypothesized that the motor response of male and female mice induced by cocaine would not be the same in the two procedures, since they each evaluate different aspects of the motor response. Many authors suggest employing a battery of different tests that

measure the same behavioral parameters in order to obtain more reliable information about the behavior in question (Kalueff et al., 2007; Ramos, 2008). Given that our objective was to demonstrate the predictive capacity of the PPI paradigm to detect vulnerability to developing motor sensitization regardless of the procedure used, we decided to use both methods. Additionally, we considered it necessary to evaluate the motor effect of an acute dose of cocaine (10 mg/kg), as we expected that Low-PPI mice would display lower sensibility to the effects of cocaine than High-PPI mice, in accordance with the results in Arenas et al. (2018). Although mice received a single dose of cocaine in the sensitization procedure, those in the control group had previously received a regime of saline injections, which could have created a stressful context that increased the sensibility of mice to the effects of cocaine (Ferrer-Pérez et al., 2018, 2019).

MATERIALS AND METHODS

Subjects

A total of 253 (131 males and 122 females) mice of the OF1 strain (Charles River, Barcelona, Spain) were employed in the four experiments. The animals arrived at the laboratory at 6 weeks of age and were all housed in groups of four in plastic cages (28 cm length × 28 cm width × 14.5 cm height) under the following conditions according to RD 1201/2005: constant temperature (21 ± 2°C), a relative humidity of 60%, an inverted 12-h light cycle (white lights on 19:30–7:30), and food and water available ad libitum (except during behavioral tests).

Procedures involving mice and their care were conducted in conformity with national, regional, and local laws and regulations, which are in accordance with the Directive 2010/63/EU of the European Parliament and of the council of September 22, 2010, on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the present study: 2014/VSC/PEA/00118 and 2016/VSC/PEA/00132.

Drugs

Cocaine hydrochloride (Laboratorios Alcaliber, Spain) was diluted in physiological saline (0.9% NaCl) at a volume of 0.01 ml/g and injected intraperitoneally (IP) at a dose of 10 mg/kg for locomotor activity and 25 mg/kg to produce sensitization. Control mice were injected IP with the corresponding volume of physiological saline.

Apparatus

Prepulse Inhibition (PPI)

Two PPI devices were used, each consisting of a Plexiglas tube (28 ± 15 ± 17 cm) on top of a platform with a sensor at its base that measures the force applied by the animal to the platform after a sound stimulus. The value used in the study was the peak value of the startle response. This value was translated by an accelerometer and the signal was collected and digitized by a computer. The apparatus (mod startle response CERS) and the software were purchased from CIBERTEC, S.A, Madrid. Spain. The description of the unit is found in Arenas et al. (2018).

Open Field Recorded by Ethovision

The apparatus was composed of four Plexiglas open-field chambers (30 cm long ± 30 cm wide ± 35 cm high) in which locomotor activity was registered by a computerized video-tracking system (Ethovision, Noldus S.A., The Netherlands). The movements of the mouse inside the open-field chambers were recorded and translated automatically by the software to horizontal distance traveled (in cm) for every 10-min period.

Open Field Recorded by Actimeter

Locomotor activity was measured automatically by an actimeter (CIBERTEC S.A., Spain) consisting of eight cages (33 × 15 × 13 cm), each with eight infrared lights located in a frame around the cage (see Mateos-García et al., 2015). In this apparatus, beams were positioned on the horizontal axis 2 cm apart, at a height just above the bottom of the cage (body level of the mice). The different frames were separated from each other at a distance of 4 cm and, since they were opaque, prevented the animals from seeing conspecifics.

Procedures

In separate studies, after categorization according to PPI, the animals were evaluated with the Ethovision apparatus (Experiment 1) and actimeter (Experiment 2) to measure the motor response induced by 10 mg/kg of cocaine. The locomotor sensitization induced by 25 mg/kg of cocaine was also measured using Ethovision (Experiment 3) and the actimeter (Experiment 4). Locomotor activity tests were performed when mice had entered adulthood (Experiment 1: PND 71–88; Experiment 2: PND 55–74; Experiment 3: PND 58–75; Experiment 4: PND 68–70).

PPI Procedure

After an adaptation period of 5 days, all the mice about to be included in an experiment performed the pre-pulse inhibition test (PPI), on PND 46–65. The procedure, similar to that used in previous studies (Arenas et al., 2018), was carried out in two phases (acclimation and test) over 2 days. On the first day, mice were placed in the animal holder for 5 min with a constant 65-dB white noise as background noise, but without startle stimuli. On the second day, the PPI test was performed. This phase also began with 5 min of 65-dB white noise, but was followed by a program of stimuli in which the white noise continued to be present. The program consisted of two phases: the first was a series of 50 trials of 120-dB pulses to establish the baseline—this pulse was chosen as it was the maximum value reached in the startle response in pilot studies—and the PPI was evaluated in the second. In order to obtain a more stable value of the PPI level, two different prepulse intensities were employed (75 and 85 dB during 4 ms each), along with two different inter-stimulus intervals (30 ms and 100 ms) and one single pulse at an intensity of 120 dB, for 20 ms each. Thus, four types of prepulse-pulse trials were performed—75 dB/30 ms, 75 dB/100 ms, 85 dB/30 ms and 85 dB/100 ms—all of them followed by a 120-dB pulse. To determine the value of the PPI, we ran the four types of prepulse-pulse trials alongside single instances of the 75, 85, and 120 dB tones, 10 times each, in pseudorandom order, giving a total of 70 trials separated by a 20-s interval. The prepulses (75/85 dB)

were introduced to verify that they were not acting as pulses and that the 120-dB pulse was the only stimulus inducing the startle response in the animal. The total duration of phase 2 was 45 min.

PPI was calculated as a percentage score: PPI (%) = 100 − (startle response for pulse with pre-pulse ± 100/startle response for pulse alone), and the mean of the four PPI obtained (75 dB/30 ms, 75 dB/100 ms, 85 dB/30 ms, and 85 dB/100 ms) was used to divide the animals into high or low PPI by means of a two-cluster analysis of K mean. Cluster centers were determined separately by sex to allow for equal distribution of males and females in each PPI group. Mice (10 males and eight females) with a PPI level near the cutoff point between High- and Low-PPI were removed from the experiment to emphasize the difference between High- and Low-PPI animals, and to avoid fluctuation of the cut off point among the samples in each Experiment, which can lead to errors. The final experimental groups are shown in **Table 1**.

Cocaine-Induced Behavioral Sensitization

The protocol for motor sensitization induced by cocaine involved three phases: the sensitization induction phase, in which three administrations of physiological saline or cocaine 25 mg/kg were performed on consecutive days, once per day; the sensitization development phase, an interval of 5 days during which no injections were administered; and the test phase, in which locomotor activity induced by 10 mg/kg of cocaine (challenger) was evaluated. This procedure was selected based on previous reports showing that it evokes locomotor sensitization induced by cocaine in mice (Ferrer-Pérez et al., 2018).

To assess the locomotor response or locomotor sensitization induced by cocaine, we recorded the first 60 min before administering the drug (phase of habituation to the environment) and the 30 min after (phase of test). The activity data were grouped in 10-min intervals. During the 90-min recording period, males and females were not kept together in the same room.

Statistical Analysis

Statistical analyses were performed using the IBM SPSS Statistics v24.0 software (Systat Software Inc., Chicago, IL, USA). In order to obtain a more stable value of PPI level, a mean of the four PPI percentage obtained (75 dB/30 ms, 75 dB/100 ms, 85 dB/30 ms and 85 dB/100 ms) was used to classify the animals according to their PPI levels. A cluster analysis of K means was performed for the distribution of the animals within each sex according to their higher/lower PPI level. A one-way ANOVA with a dependent variable (PPI) was performed in each experiment to check whether there were differences in the PPI between sexes. For the locomotor activity data, a mixed ANOVA was performed with two between variables—Sex (males and females) and PPI (High and Low)—and one within variable, Minutes (six 10-min periods to analyze locomotor activity in the habituation phase, and four 10-min periods to analyze locomotor activity in the test phase). For the behavioral sensitization data, a mixed ANOVA was performed with three between variables—Sex (males and females), PPI (High and Low) and Pre-Treatment (Saline and Cocaine)—and one within variable, Minutes (six 10-min periods to analyze locomotor activity in the habituation phase, and four 10-min periods to analyze locomotor activity in the test phase), using Bonferroni's pairwise *post hoc* comparisons. In order to test our hypothesis about varying vulnerability to developing behavioral sensitization in Low- and High-PPI mice, we also performed the Tukey's honest significant difference (HSD) test, which does not require previous ANOVA with a significant interaction between factors and is highly conservative against type I error (Wilcox and Rousselet, 2018). All results are expressed as mean ± SEM. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Experiment 1: Locomotor Activity Induced by Cocaine (10 mg/kg) Recorded by Ethovision

Male and female mice were classified as High-PPI or Low-PPI animals using a two-cluster analysis ($F_{(1,21)} = 62.552$; $p < 0.0001$, in males; $F_{(1,19)} = 71.962$; $p < 0.0001$, in females). The ANOVA performed for the data obtained during the whole period

TABLE 1 | Classification as High-Prepulse inhibition (PPI) or Low-PPI mice using a two-cluster analysis (mean ± SEM).

		Low-PPI		High-PPI	
		Mean ± SEM	***n***	**Mean ± SEM**	***n***
Experiment 1	Males	4.05 ± 3.5	10	34.6 ± 1.9	11
	Females	5.5 ± 3.2	9	36.8 ± 1.9	10
Experiment 2	Males	2.5 ± 2.5	9	24.6 ± 2.6	10
	Females	2.8 ± 2.3	9	28.1 ± 2.9	10
Experiment 3	Males saline	9 ± 2.9	10	35.3 ± 2.9	12
	Females saline	−4.8 ± 3.4	9	25.9 ± 2.3	9
	Males cocaine	8.3 ± 3.7	9	33.3 ± 1.9	11
	Females cocaine	−6.8 ± 5	9	26.8 ± 2.1	9
Experiment 4	Males saline	−4.1 ± 4.3	10	29.5 ± 3.5	10
	Females saline	−12.1 ± 4.1	8	36.5 ± 2.6	12
	Males cocaine	−6.8 ± 3.8	9	30.3 ± 3.5	10
	Females cocaine	−8.9 ± 4.7	8	37.2 ± 3.7	12

Percentage of PPI (mean of the four PPI obtained at 75 dB/30 ms, 75 dB/100 ms, 85 dB/30 ms, and 85 dB/100 ms) was used to classify the animals according to their PPI levels. A two-cluster analysis of K means was performed for the distribution of the animals within each sex according to their higher/lower PPI level.

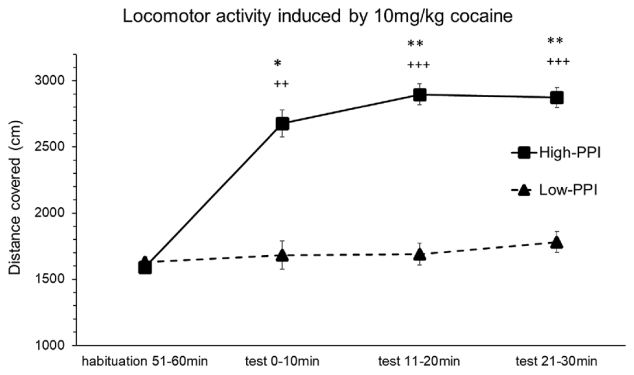

FIGURE 1 | Locomotor response induced by an acute dose of cocaine (10 mg/kg) recorded by Ethovision. Locomotor response induced by 10 mg/kg of cocaine in mice (both males and females) categorized as high and low prepulse inhibition (PPI). Data presented as mean values ± SEM during the 30-min period of distance covered in centimeter. $^{*}p < 0.05$, $^{**}p < 0.01$ Low-PPI vs. High-PPI; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. habituation.

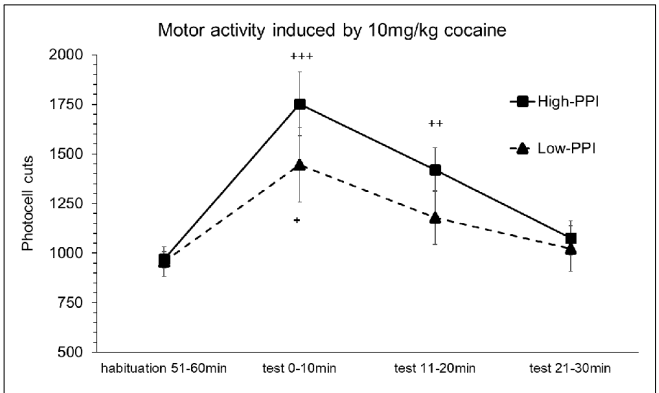

FIGURE 2 | Motor response induced by an acute dose of cocaine (10 mg/kg) recorded by actimeter. Motor response induced by 10 mg/kg of cocaine in mice (both males and females) categorized as High- and Low-PPI. Data presented as mean values ± SEM of photocell cuts during the 30 min period. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. habituation.

(60 min) revealed an effect of habituation to the environment ($F_{(5,32)} = 33.841$; $p < 0.0001$), showing that motor activity in all the animals decreased after the first 10 min until 60 min (ps < 0.01). This analysis also revealed a significant effect of the variable Sex ($F_{(1,36)} = 13.387$; $p < 0.001$), with females presenting higher locomotor activity than males (data not shown). The results of the motor activity of male and female mice together in response to 10 mg/kg of cocaine are presented in **Figure 1**. The ANOVA showed a significant effect of the variable Minutes ($F_{(3,34)} = 8.006$; $p < 0.0001$), as all animals together significantly increased their activity at 20 min ($p < 0.002$) and at 30 min ($p < 0.0001$), and displayed a tendency towards an increase at 10 min ($p = 0.056$). A significant effect of the variable PPI ($F_{(1,36)} = 8.148$; $p < 0.007$) and the interaction Minutes*PPI ($F_{(3,34)} = 5.487$; $p < 0.003$) was also observed. The animals with a high PPI displayed a higher locomotor activity than Low-PPI mice: High-PPI mice increased their locomotor activity at 10 min ($p < 0.005$), at 20 min ($p < 0.0001$), and at 30 min ($p < 0.0001$) with respect to habituation. High-PPI mice also exhibited more activity than those with a low PPI at 10 min ($p < 0.041$), at 20 min ($p < 0.001$), and at 30 min ($p < 0.002$). The interaction Sex*Minutes*PPI was not significant.

Experiment 2: Locomotor Activity Induced by Cocaine (10 mg/kg) Recorded by Actimeter

Male and female mice were classified as High-PPI or Low-PPI animals using a two-cluster analysis: male ($F_{(1,17)} = 43.313$; $p < 0.0001$) and female ($F_{(1,17)} = 42.490$; $p < 0.0001$). The ANOVA performed for the data obtained during the whole period (60 min) revealed an effect of habituation to the environment ($F_{(5,30)} = 70.451$; $p < 0.0001$), demonstrating that all the animals decreased their motor activity after the first 10 min until 60 min (ps < 0.01). This analysis also revealed a significant effect of the variable Sex ($F_{(1,34)} = 14.825$; $p < 0.0001$), with males presenting a higher locomotor activity than females (data not shown). The results of motor activity induced by 10 mg/kg of cocaine are presented in **Figure 2**. The analysis revealed a significant effect of the variable Minutes ($F_{(3,32)} = 9.977$; $p < 0.0001$), with all the animals displaying higher activity with respect to habituation at 10 min ($p < 0.0001$) and at 20 min ($p < 0.001$). The interactions Minutes*PPI and Sex*Minutes*PPI were not significant. However, the Tukey HSD test indicated that cocaine induced hyperactivity at 10 min ($p < 0.042$) in all Low-PPI mice, and at 10 min ($p < 0.0001$) and 20 min ($p < 0.001$) in all High-PPI mice.

Experiment 3: Locomotor Sensitization Induced by Cocaine Recorded by Ethovision

Male and female mice were classified as High-PPI or Low-PPI animals using a two-cluster analysis: male ($F_{(1,43)} = 75.968$; $p < 0.0001$) and female ($F_{(1,36)} = 67.667$; $p < 0.0001$). The ANOVA performed for the data obtained during the whole period (60 min) revealed an effect of habituation to the environment ($F_{(5,66)} = 39.709$; $p < 0.0001$), with a decrease in motor activity after the first 10 min until 60 min observed in all animals (data not shown). However, the interaction Minutes*Sex was significant ($F_{(5,66)} = 3.487$; $p < 0.007$), with males displaying higher activity in the first 10 min ($p < 0.04$) and lower activity in the last 60 min ($p < 0.04$) in comparison with females. The results of motor activity in response to 10 mg/kg of cocaine (the challenger) after pre-treatment with physiological saline or cocaine 25 mg/kg are presented in **Figures 3A,B**. The analysis revealed a significant effect of the variable Minutes ($F_{(3,68)} = 53.674$; $p < 0.0001$), with all animals displaying higher activity induced by the cocaine challenge for all time points with respect to the habituation phase (ps < 0.001), and a higher activity to the first 10 min than 20 min ($p < 0.02$) and 30 min ($p < 0.05$). The variable Pre-Treatment ($F_{(1,70)} = 14.394$; $p < 0.0001$) and the interaction Minutes*Pre-Treatment ($F_{(3,68)} = 10.323$; $p < 0.0001$) were significant, revealing higher activity in mice pretreated with cocaine than those pretreated with saline to the first 10 min

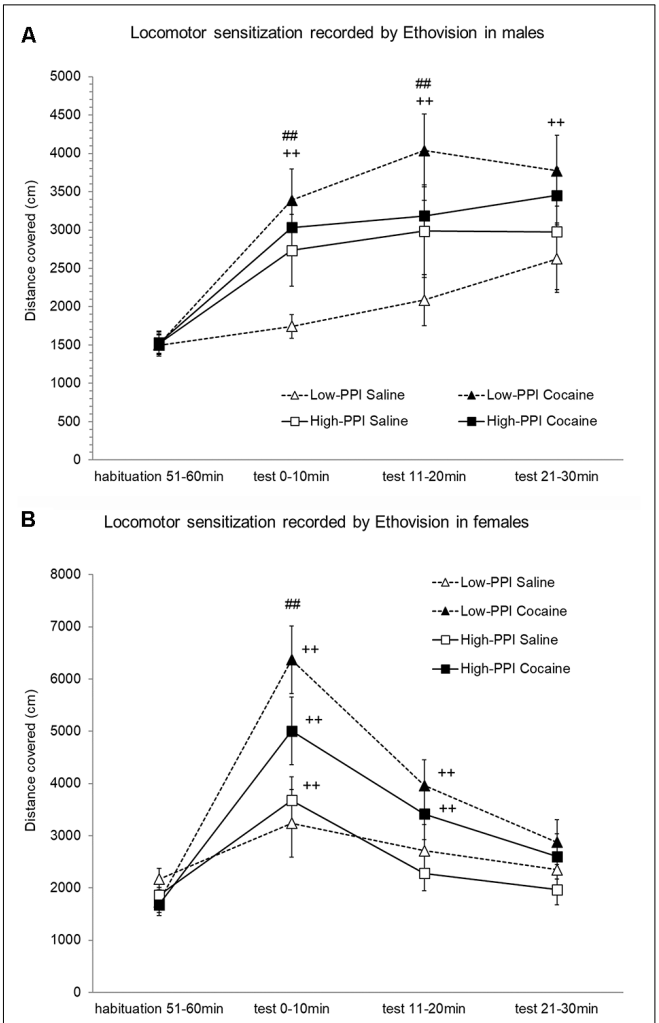

FIGURE 3 | (A) Cocaine sensitization recorded by Ethovision in males. During the induction phase, the animals received a pre-treatment with saline or 25 mg/kg cocaine per day on three consecutive days. Five days later, all the animals received a 10 mg/kg challenge. Data presented as mean values ± SEM during the 30-min period of distance covered in centimeter. $^{\#\#}p < 0.01$ Low-PPI cocaine vs. saline; $^{++}p < 0.01$ Low-PPI cocaine, High-PPI cocaine and saline vs. habituation. **(B)** Cocaine sensitization recorded by Ethovision in females. During the induction phase, the animals received a pre-treatment with saline or 25 mg/kg cocaine per day on three consecutive days. Five days later, all the animals received a 10 mg/kg challenge. Data presented as mean values ± SEM during the 30-min period of distance covered in centimeter. $^{\#\#}p < 0.01$ Low-PPI cocaine vs. saline; $^{++}p < 0.01$ vs. habituation.

($p < 0.0001$), 20 min ($p < 0.001$), and 30 min ($p < 0.05$). The interaction Minutes*PPI*Pre-Treatment almost reached significance ($F_{(3,68)} = 2.262$; $p < 0.08$). The Tukey HSD test indicated higher hyperactivity induced by the cocaine challenge in mice pretreated with cocaine with respect to those pretreated with saline only in Low-PPI mice—females at 10 min ($p < 0.0001$) and males at 10 min ($p < 0.029$) and at 20 min ($p < 0.004$)—with no significant differences observed between the High-PPI mice pretreated with cocaine vs. saline. Moreover, with respect to the habituation phase, a significant increase of activity was observed in Low-PPI males pretreated with cocaine at 10 min ($p < 0.002$), 20 min ($p < 0.0001$), and 30 min ($p < 0.0001$); in High-PPI males pretreated with saline at 10 min ($p < 0.037$), 20 min ($p < 0.004$), and 30 min ($p < 0.01$); in High-PPI males pretreated with cocaine at 10 min ($p < 0.008$), 20 min ($p < 0.001$), and 30 min ($p < 0.001$); in Low-PPI females pretreated with cocaine at 10 min ($p < 0.0001$) and 20 min ($p < 0.0001$); in High-PPI females pretreated with saline at 10 min ($p < 0.003$); and in High-PPI females pretreated with cocaine at 10 min ($p < 0.0001$) and 20 min ($p < 0.003$).

Experiment 4: Locomotor Sensitization Induced by Cocaine Recorded by Actimeter

Male and female mice were classified as High-PPI or Low-PPI using a two-cluster analysis: male ($F_{(1,42)} = 79.646$; $p < 0.0001$) and female ($F_{(1,42)} = 102.673$; $p < 0.0001$; see **Figure 4A**). The ANOVA for the data obtained during the whole period (60 min) revealed an effect of habituation to the environment ($F_{(5,67)} = 35.594$; $p < 0.0001$), with all the animals decreasing their motor activity after the first 10 min until 60 min (data not shown). The results of motor activity in response to 10 mg/kg of cocaine challenge after pre-treatment with physiological saline or 25 mg/kg of cocaine are presented in **Figures 4A,B**. The analysis revealed a significant effect of the variable Sex ($F_{(1,71)} = 5.306$; $p < 0.024$), with higher activity in males than in females; the variable Pre-Treatment ($F_{(1,71)} = 10.527$; $p < 0.002$), with higher activity among the mice pretreated with cocaine vs. saline; and the variable Minutes ($F_{(3,69)} = 46.105$; $p < 0.0001$), as the cocaine challenge increased activity with respect to the habituation phase in all the animals together at all the time points measured ($ps < 0.001$). Furthermore, the interaction Sex*Minutes ($F_{(3,69)} = 3.939$; $p < 0.012$) was significant, with males displaying more hyperactivity induced by cocaine than females at 10 min ($p < 0.023$), 20 min ($p < 0.022$), and 30 min ($p < 0.016$). The interaction Minutes*Pre-Treatment ($F_{(3,69)} = 11.301$; $p < 0.0001$) was also significant; mice pretreated with saline showed a significant increase of activity at 10 min ($p < 0.0001$), while those pre-treated with cocaine showed a significant increase of activity at 10 min ($p < 0.0001$) and 20 min ($p < 0.0001$) in comparison to the habituation phase. In this way, the mice pre-treated with cocaine exhibited higher hyperactivity than those pre-treated with saline at 10 min ($p < 0.0001$) and 20 min ($p < 0.0001$). No other significant interactions were observed. However, the Tukey HSD test indicated higher hyperactivity induced by the cocaine challenge in mice pretreated with cocaine vs. saline: in High-PPI males at 20 min ($p < 0.041$), in Low-PPI males at 10 min ($p < 0.0001$) and 20 min ($p < 0.001$), and in Low-PPI females at 10 min ($p < 0.032$). Moreover, with respect to the habituation phase, a significant increase of activity was observed in High-PPI males pretreated with saline at 10 min ($p < 0.01$); in High-PPI males pretreated with cocaine at 10 min ($p < 0.0001$), 20 min ($p < 0.0001$), and 30 min ($p < 0.009$); in Low-PPI males pretreated with cocaine at 10 min ($p < 0.0001$) and 20 min ($p < 0.0001$); in High-PPI females

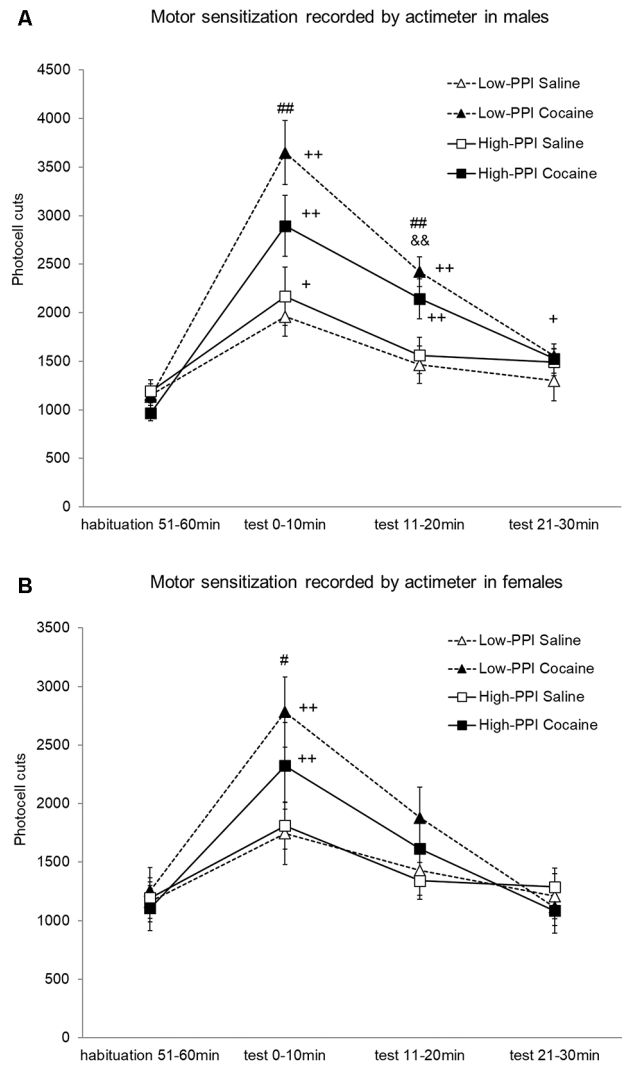

FIGURE 4 | (A) Cocaine sensitization recorded by the actimeter in males. During the induction phase, the animals received a pre-treatment with saline or 25 mg/kg cocaine per day on three consecutive days. Five days later, all the animals received a 10 mg/kg challenge. Data presented as mean values ± SEM of photocell cuts during the 30-min period. $^{\#\#}p < 0.01$ Low-PPI cocaine vs. saline; $^{\&\&}p < 0.01$ High-PPI cocaine vs. saline; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. habituation. **(B)** Cocaine sensitization recorded by the actimeter in females. During the induction phase, the animals received a pre-treatment with saline or 25 mg/kg cocaine per day on three consecutive days. Five days later, all the animals received a 10 mg/kg challenge. Data presented as mean values ± SEM of photocell cuts during the 30-min period. $^{\#}p < 0.05$ Low-PPI cocaine vs. saline; $^{++}p < 0.01$ vs. habituation.

pretreated with cocaine at 10 min ($p < 0.0001$); and in Low-PPI females pretreated with cocaine at 10 min ($p < 0.0001$).

DISCUSSION

The main objective of the present study was to evaluate the predictive capacity of the PPI paradigm to identify subjects among male and female mice that are more vulnerable to the motor effects of cocaine and the development of behavioral sensitization induced by the drug. The results reveal for the first time that male and female mice with a low PPI display lower hyperactivity when administered an acute dose of cocaine (10 mg/kg), but that they seem to develop a higher motor sensitization to the drug after intermittent exposure. Low-PPI mice pretreated with cocaine presented more hyperactivity induced by cocaine than those pretreated with saline, while High-PPI animals were less affected by pretreatment with cocaine. In a previous study, we provided the first evidence that both male and female mice with a low PPI are more vulnerable to the conditioned rewarding and reinstating effects of cocaine (Arenas et al., 2018). We observed how Low-PPI mice displayed less sensitivity to the conditioned rewarding effects of cocaine, but a stronger and more persistent conditioned preference when conditioned with a higher dose. Therefore, we consider that Low-PPI mice seem to be more vulnerable to the rewarding and motor effects of cocaine.

In accordance with previous studies in male rodents (Abizaid et al., 2011; Blanco-Gandía et al., 2017), a dose of 10 mg/kg of cocaine induced hyperactivity in all mice together; however, we have now observed this motor effect of cocaine in both male and female mice when measured with two different procedures. Nonetheless, this dose did not increase the motor activity of animals with a low PPI in the open field when measured with the Ethovision and actimeter apparatuses. This lower sensibility to the motor effects of an effective dose of cocaine in Low-PPI mice is in line with the lower sensitivity of these subjects to the rewarding effects of drug with respect to High-PPI mice seen in our previous study (Arenas et al., 2018).

Furthermore, as expected, all the groups that received pretreatment with cocaine developed cocaine motor sensitization, as reported in other studies using the same (Ferrer-Pérez et al., 2018; García-Pardo et al., 2019) and other (Gracia-Rubio et al., 2016; Luján et al., 2018) experimental protocols. Thus, when all the mice pretreated with cocaine were compared with those pretreated with saline, it was evident that the drug had induced higher hyperactivity. Nonetheless, when subjects were separated into High- and Low-PPI groups, higher hyperactivity was induced by cocaine principally in latter, in both males and females, suggesting a higher motor sensitization in mice with a lower PPI, a result obtained with both Ethovision and actimeter protocols. Other authors have also reported a higher behavioral sensitization to the effects of other DA agonists, such as amphetamine, in Low-PPI vs. High-PPI mice, suggesting that males characterized by low basal PPI are more susceptible to the development of dopamine sensitization (Peleg-Raibstein et al., 2013). Motor sensitization, also called behavioral sensitization, is the increased motor activity induced by cocaine after intermittent drug administration (Steketee and Kalivas, 2011). It is considered an indicator of neural changes caused by cocaine consumption (Lüscher and Malenka, 2011), which can induce compulsive consumption of abuse drugs, a characteristic behavior of addiction (Robinson and Berridge, 2003, 2008). Intermittent patterns of use are especially pronounced during the transition to addiction, prior to regular use. Recently, it has been demonstrated that intermittent access

to drugs is more effective than continued use in producing long-term changes in the brain and addiction-like behaviors (Kawa et al., 2019). The results of the present study highlight more pronounced behavioral changes after pretreatment with cocaine in mice with a lower PPI than in those with a higher PPI, a result that was once again observed in both males and females. In this way, Low-PPI mice pretreated with the drug presented higher hyperactivity when they received a cocaine challenge than when they were pretreated with saline, an increase of motor activity that was less marked among High-PPI mice pretreated with cocaine vs. saline. The marked behavioral sensitization of Low-PPI mice in the motor response induced by cocaine is also in accordance with results obtained previously in our laboratory (Arenas et al., 2018) regarding the reward response of these mice to cocaine. In the study in question, animals with low PPI also presented more pronounced addictive-like behavior in the CPP induced by cocaine than High-PPI mice, since the former mice required a higher dose of cocaine to acquire CPP (12 mg/kg). Once they were conditioned, preference was not extinguished in males and was reinstated in females with lower doses of cocaine than in their control counterparts (Arenas et al., 2018). It is known that both male and female rats develop addiction-like behaviors with intermittent administration experience, but that this occurs more rapidly and more robustly in females (Kawa et al., 2019). This greater effect in females is due to the "telescoping effect" described in the clinical literature, consisting of an accelerated progression from the initiation of substance use to the onset of dependence (Griffin et al., 1989; Kosten et al., 1993; Greenfield et al., 2010). Hence, our results seem to support the idea that alterations in the brain that underlie psychomotor sensitization also underlie sensitization to the incentive motivational effects of drugs (Robinson and Berridge, 1993; De Vries et al., 1998; Lorrain et al., 2000; Allain et al., 2017).

As a whole, these results suggest that the long-term consequences of use of psychostimulants such as cocaine or amphetamine can differ depending on basal levels of PPI. Other studies have reported that amphetamine has varying effects depending on the basal PPI level of subjects (Swerdlow et al., 2003; Talledo et al., 2009). In this context, amphetamine was shown to largely decrease PPI in subjects with the highest basal levels of PPI, in both men (Swerdlow et al., 2003) and women (Talledo et al., 2009). Other DA agonists, such as pergolide and amantadine, have been shown to reduce PPI in men with basal PPI above the median of the normal distribution, whereas they slightly amplified PPI in men with basal levels below the median (Bitsios et al., 2005). These seemingly opposite effects could be explained by the role of DA in the NAc in the modulation of PPI (Doherty et al., 2008; Rohleder et al., 2016). Amphetamine decreases PPI in a strain of rats with a relatively low COMT gene expression in the NAc, while it increases PPI in another strain of rats with relatively high COMT gene expression in the NAc (Talledo et al., 2009). In light of this evidence, DA availability in the NAc seems to be critical in determining the level of PPI observed; i.e., a higher DA level will induce a PPI deficit, while a lower DA level will increase PPI (Arenas et al., 2017). The role of DA in determining PPI levels has been bolstered since activation of midbrain cholinergic neurons has been shown not to mediate PPI levels (Azzopardi et al., 2018). One of the main brain nuclei that modulate PPI is the pedunculopontine tegmentum (PPTg; Arenas et al., 2017), which is one of the major cholinergic centers of the brain (Gut and Winn, 2016). However, it has recently been demonstrated that cholinergic PPTg neurons do not mediate PPI, in contrast to a longstanding hypothesis maintaining that they do (Azzopardi et al., 2018). Furthermore, the lack of motor and rewarding effects of cocaine observed in the mice with a low PPI level may reflect an altered DA system. Hence, these animals, especially females, show higher levels of D2R expression than those with a higher PPI level (Arenas et al., 2018), and high D2 receptor levels in the striatum have been related to a lack of pleasant effects perceived from psychostimulants (Volkow et al., 1999). Indeed, clinical studies have reported that schizophrenic patients display higher amounts of D2 receptors (Seeman, 2013) in addition to a PPI deficit (Braff, 2010). Some individuals at familial risk of addictions exhibit higher striatal D2R availability (Volkow et al., 2006), although it is still under debate whether these elevated D2R densities are protective against or a risk for addiction (Leyton, 2017).

We also observed sex differences in the spontaneous activity of mice in the habituation sessions. In general, it is considered that female rodents are more active than males (Blizard et al., 1975), though this difference depends on the age, environment, and procedure used (Tamás et al., 2005; Alstott and Timberlake, 2009; Bogdanova et al., 2013). In the present study, females presented a higher locomotor activity when measured with the Ethovision apparatus, while males showed a higher motor activity when measured with the actimeter. These differences between males and females indicate that the procedures for evaluating animal activity measure different behaviors. In this context, the Ethovision protocol for measuring the motor activity of mice records ambulatory activity; in other words, it provides the distance in cm traveled by the animal. Our female animals displayed a higher ambulatory activity in this protocol, in line with a previous study with a different strain of mice (Sershen et al., 2002) and another with rats (Blizard et al., 1975). In contrast, the actimeter apparatus recorded all the motor activity of the animal, since it did not distinguish locomotion from stereotypic or grooming movements, with males showing a higher activity than females in this case.

In summary, the results of our study are in accordance with those of our previous study (Arenas et al., 2018), and endorse the predictive capacity of the PPI paradigm to identify individuals who are more likely to display behaviors induced by cocaine and which can increase the risk of developing a cocaine use disorder. Bearing all the discussed results in mind, subjects with a lower PPI present a cocaine response that entails a higher risk of transitioning from abuse to dependence. First, they present lower sensitivity to the motor and reinforcing effects of cocaine, which may drive them to higher consumption rates. Second, when Low PPI mice are exposed to higher doses of cocaine, they present stronger

associative cocaine effects with environmental cues than High PPI mice, which leads to a more persistent drug-seeking behavior. Third, mice with a lower PPI exposed to intermittent administration of cocaine present more pronounced behavioral changes; that is, they develop great behavioral sensitization induced by the drug, which is considered an indicator of the possible transition to a compulsive consumption of drugs, a characteristic behavior of addiction (Robinson and Berridge, 2003, 2008; Lüscher and Malenka, 2011). Furthermore, we demonstrate this enhanced motor sensitization induced by cocaine using two different protocols for evaluating motor activity. It is important to point out that a deficit in PPI has been observed in many psychiatric disorders characterized by alterations in the cerebral dopaminergic system, and is thus already considered an endophenotype of schizophrenia (Swerdlow and Light, 2016). Additionally, the dopaminergic system, from the ventral tegmental area to the NAc, is considered a central circuitry for the regulation of PPI (Doherty et al., 2008; Rohleder et al., 2016) and motivation for reward and drug-seeking (Bergamini et al., 2016; Scofield et al., 2016). Therefore, we endorse PPI paradigm as a useful indicator of subjects with a higher vulnerability to the effects of cocaine. Notwithstanding, future studies should be performed to confirm whether a deficit in PPI is a possible endophenotype of cocaine use disorder.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Use and Care Committee of the University of Valencia.

AUTHOR CONTRIBUTIONS

MA, MB-G, JM, and CM contributed to the conception and design of the study. MB-G and CM performed the experiments and collected and analyzed the data. MA and CM performed the statistical analyses. All authors interpreted the data. MA and MB-G wrote the manuscript.

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Comparing ABA, AAB, and ABC Renewal of Appetitive Pavlovian Conditioned Responding in Alcohol- and Sucrose-Trained Male Rats

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Conditioned responding can be renewed by re-exposure to the conditioning context following extinction in a different context (ABA renewal) or by removal from the extinction context (AAB or ABC renewal). ABA renewal is robust in Pavlovian and operant conditioning paradigms. However, fewer studies have investigated AAB and ABC renewal of appetitive conditioning, and those that did predominantly used operant conditioning tasks. Renewal has theoretical relevance for extinction and for exposure-based treatments for substance use disorders that aim to extinguish reactivity to drug-predictive cues. We therefore investigated ABA, AAB, and ABC renewal of Pavlovian conditioned responding to cues that predicted either alcohol or sucrose. Male, Long-Evans rats (Charles River) were exposed to either 15% ethanol (Study 1: "alcohol") or 10% sucrose (Study 2: "sucrose") in their home cages. Next, they were trained to discriminate between two auditory stimuli (white noise and clicker; 10 s) in conditioning chambers equipped with distinct olfactory, visual, and tactile contextual stimuli (context A). One conditioned stimulus (CS+) was paired with fluid delivery (0.2 ml/CS+; 3.2 ml/session; alcohol or sucrose in separate experiments), and the second CS (CS−) was not. In all sessions (conditioning, extinction, and test), each CS was presented 16 times/session on a variable-time 67-s schedule, and entries into the fluid port were recorded. CS+ port entries were then extinguished by withholding fluid delivery either in context A or in a second, different context (context B). Next, we assessed ABA, AAB, and ABC renewal in the absence of fluid delivery. During extinction, CS+ port entries were initially elevated in context A relative to context B. ABA renewal of CS+ port entries occurred in both alcohol- and sucrose-trained rats. ABC renewal approached statistical significance when data from both experiments were combined. No AAB renewal was observed, and, in fact, alcohol-trained rats showed AAB suppression. These results corroborate the reliability of ABA renewal and suggest that ABC renewal is a modest effect that may require greater statistical power to detect. From a treatment perspective, the lack of AAB renewal suggests that exposure-based treatments for substance use disorders might benefit from implementation in real-world, drug-use contexts.

Keywords: renewal, context, reinstatement, relapse, alcohol, sucrose, reward, Pavlovian conditioning

INTRODUCTION

Substance use disorders represent a global health crisis, and effective treatments are still needed (Degenhardt et al., 2013; Whiteford et al., 2013). One form of behavioral treatment involves repeated, systematic exposure to drug-predictive cues in the absence of drug use, which extinguishes cue-elicited conditioned reactivity (Bouton and Bolles, 1979; Bouton and King, 1983). Through extinction, exposure-based treatment aims to reduce the capacity of drug-predictive cues to impact relapse. However, gains from this approach may be transient and confined to the treatment context (Monti et al., 1993; Drummond and Glautier, 1994; Carter and Tiffany, 1999; Miller et al., 2001; Conklin and Tiffany, 2002; Bouton et al., 2012; Mellentin et al., 2017).

This idea comes from research that has identified processes that threaten long-term extinction. One such process, called "renewal," refers to "the recovery of an extinguished conditioned response when testing occurs in a context different from that in which extinction treatment took place" (Polack et al., 2013). One of the first reports of renewal used a task in which a tone conditioned stimulus (CS) was paired with a shock unconditioned stimulus (US) in a specific context called "context A" (Bouton and Bolles, 1979). Conditioned responding to the CS was then extinguished in a different context (context B) by presenting the CS without shock. At test, responding to the CS without shock was renewed in context A. This experimental design is called "ABA renewal," where sequential letters represent the conditioning, extinction, and test contexts. ABA renewal has been widely reported in aversive Pavlovian conditioning paradigms (Bouton and Bolles, 1979; Bouton and King, 1983; Ji and Maren, 2005; Fujiwara et al., 2012) and has important implications for the nature of extinction (Rescorla, 1993; Bouton et al., 2011). First, it suggests that extinction does not permanently erase the original CS–US association that was acquired during conditioning. Second, it suggests that extinction may produce a new, inhibitory CS–no US association that is specific to the extinction context. The latter hypothesis is based on studies showing that removal from the extinction context was sufficient to trigger renewal. In those studies, renewal was tested in a new context following training and extinction in either the same (AAB renewal) or different (ABC renewal) contexts (Gunther et al., 1998; Harris et al., 2000; Corcoran and Maren, 2004; Thomas et al., 2004).

The renewal effect has clear implications for exposure-based treatments for substance use disorders that are conducted in treatment settings bearing little resemblance to real-world, drug-use contexts. Indeed, research conducted in rodents using operant conditioning procedures has found reliable evidence of ABA renewal for drug (Crombag and Shaham, 2002; Fuchs et al., 2005; Diergaarde et al., 2008; Hamlin et al., 2008; Wing and Shoaib, 2008; Chaudhri et al., 2009; Marinelli et al., 2009; Bossert et al., 2012, 2019; Palombo et al., 2017) and non-drug reinforcers (Hamlin et al., 2006; Zironi et al., 2006; Marchant et al., 2009; Todd et al., 2012). Interestingly, AAB renewal of operant responding was not detected in two studies using drug reinforcers (Crombag and Shaham, 2002; Fuchs et al., 2005), whereas in studies using food pellets, AAB renewal was either absent (Nakajima et al., 2000) or detected but modest compared to ABA renewal (Bouton et al., 2011). Finally, ABC renewal of operant responding was observed for food pellets (Bouton et al., 2011) and liquid sucrose (Zironi et al., 2006), but not for ethanol (Zironi et al., 2006). Thus, while there is extensive support for ABA renewal, the evidence for AAB and ABC renewal of appetitive operant behavior is inconsistent or sparse.

In appetitive Pavlovian conditioning studies, ABA renewal is also a reliable effect (Chaudhri et al., 2008b; Anderson and Petrovich, 2015); however, AAB and ABC renewal are understudied. One laboratory reported ABC renewal using a discriminative conditioning task, and just as in operant conditioning, ABC renewal appeared to be a numerically smaller effect than ABA renewal (Campese and Delamater, 2013). AAB renewal of appetitive Pavlovian conditioned responding for food pellets was observed in one study (Bouton and Ricker, 1994), but another study failed to detect AAB renewal (Goddard, 1999). ABC and AAB renewal have also been observed following overexpectation (Rescorla, 2007) or using an autoshaping procedure (Rescorla, 2008). A summary of studies that tested ABA renewal in conjunction with AAB and/or ABC renewal is presented in **Table 1**.

Given the theoretical and clinical implications of renewal, we sought to develop a comprehensive picture of the renewal of appetitive Pavlovian conditioned responding. Consequently, we investigated ABA, AAB, and ABC renewal in rats that were trained to associate a discrete, auditory CS (CS+) with either alcohol or sucrose delivery in separate experiments. A second, control CS (CS−) was also present during conditioning sessions but was not explicitly paired with fluid delivery. Using this task, we previously reported selective ABA renewal of responding to an alcohol-predictive CS+ (Chaudhri et al., 2008b) that was dependent on dopaminergic (Sciascia et al., 2014) and cholinergic neurotransmission (Lacroix et al., 2017). We also reported ABA renewal of responding to a CS+ that predicted sucrose (Chaudhri et al., 2008b) and a reduction of this effect by optogenetic activation of the infralimbic prefrontal cortex during the CS (Villaruel et al., 2018). Given that ABA renewal is a reliable phenomenon across learning paradigms, we predicted that a return to the conditioning context following extinction in a different context would selectively renew CS+ responding in both alcohol- and sucrose-trained rats. By comparison, we anticipated that AAB and ABC renewal would be detectable, but modest effects.

We examined ABA, AAB, and ABC renewal concurrently in separate studies using rats trained with either alcohol or sucrose. This experimental design enabled us to compare the extinction of CS+ responding in the same context as conditioning (context A) and in a different context (context B). We conducted these comparisons in order to inform theoretical explanations of the renewal effects that we observed.

TABLE 1 | Studies that examined ABA renewal in conjunction with either AAB or ABC renewal of appetitive conditioning.

Year	Author(s) and Journal	Subjects	CS	Reinforcer/US	Context modalities	ABA	AAB	ABC
Operant conditioning								
2000	Nakajima et al., 2000 *Learn Motiv*	Male Wistar	Lever	Food pellet	V, T, A, Chamber size, Transport	Yes	No	Not tested
2002	Crombag and Shaham, 2002 *Behav Neurosci*	Male LE	Light	Heroin–cocaine	V, A	Yes	No	Not tested
2004	Bossert et al., 2004 *J Neurosci*	Male LE	Tone-light	Heroin	V, T, A Circadian	Yes	No	Not tested
2005	Fuchs et al., 2005 *Neuropsycho-pharmacology*	Male SD	No CS	Cocaine	V, T, A, O	Yes	No	Not tested
2006	Zironi et al., 2006 *Behav Brain Res*	Male LE	No CS	Alcohol or sucrose	V, T, O	Yes	Not tested	Yes sucrose; No alcohol
2011	Bouton et al., 2011 *Learn Behav*	Female Wistar	No CS	Food pellet	V, T, O	Yes	Yes	Yes
2012	Todd et al., 2012 *Learn Behav*	Female Wistar	No CS	Food pellet	V, T, O, Chamber size	Yes	Not tested	Yes
2013	Todd, 2013 *JEP: Anim Behav Process*	Female Wistar	No CS	Food pellet	V, T, O, A, Room	Yes	Yes	Yes
2015	Bouton and Schepers, 2015 *JEP: Anim Learn Cogn*	Female Wistar	No CS	Food pellet	V, T, O, A, Room	Yes	Not tested	Yes
2017	Schepers and Bouton, 2017 *Psychol Sci*	Female Wistar	No CS	Food or sucrose pellet	Interoceptive (satiated vs. hungry)	Yes	No	Not tested
2017	Trask et al., 2017 *J Neurosci*	Male Wistar	No CS	Sucrose pellet	V, T, O, Room	Yes	Not tested	Yes
Pavlovian conditioning								
1994	Bouton and Ricker, 1994 *Anim Learn Behav*	Male and Female Wistar	Tone-light	Food pellet	V, T, O	Not tested	Yes	Not tested
1999	Goddard, 1999 *Learn Motiv*	Female SD	No CS	Food pellet	V, T, O	Yes	No	Not tested
2013	Campese and Delamater, 2013 *Behav Brain Res*	Male and Female LE	Tone-light	Food pellet	V, T, Chamber size, Chamber shape, Room	Yes	Not tested	Yes
2007	Rescorla, 2007 *Anim Learn Behav*	Male SD	Noise and Light	Food pellet	T, O	Yes	Yes	Yes
2008	Rescorla, 2008 *Q J Exp Psychol*	Female Carneau Pigeon	Light	Grain	V	Yes	Yes	Yes

Column 3 contains the subjects used (LE: Long-Evans; SD: Sprague Dawley). Column 6 contains the context modalities used (V: visual, T: tactile, O: olfactory, A: auditory).

MATERIALS AND METHODS

Animals

Male, Long-Evans rats (220–240 g on arrival; n = 75) were obtained from Charles River Laboratories (Saint-Constant, QC, Canada). Upon arrival, rats were individually housed in polycarbonate home cages (44.5 cm × 25.8 cm × 21.7 cm) in a climate-controlled vivarium that was maintained on a 12-h light/dark cycle (lights on at 07:00). Behavioral procedures were conducted during the light cycle. Food (Charles River Rodent Diet, Saint-Hubert, QC, Canada) and water were always available in the home cage. Acclimation to the vivarium as well as regular weighing and handling occurred for 6 days before experiments began. The Animal Research Ethics Committee at Concordia University approved all procedures, which concurred with guidelines from the Canadian Council on Animal Care.

Apparatus

Behavioral procedures were conducted in conditioning chambers (ENV-009A; 32.8 cm × 32.8 cm × 32.8 cm; Med Associates, Inc., St Albans, VT, United States) that were housed within custom-made, ventilated, sound-attenuating melamine cubicles (53.6 cm × 68.2 cm × 62.8 cm) located in a behavioral testing room that was distinct from the vivarium. The side walls of

each chamber were made of stainless-steel panels, and the rear wall, ceiling, and front wall were made of clear acrylic glass. The floors were made of metal bars that extended from the rear wall to the front wall (ENV-009A-GF). A fluid receptacle (ENV-200R3AM) was located 2 cm above the floor, near the center of the right wall, and infrared sensors (ENV-254-CB) measured fluid port entries. Fluid was delivered into the receptacle via a 20-ml syringe that was mounted onto a pump (PHM-100, 3.33 RPM) located outside the sound-attenuating cubicle. A white house light (75 W, 100 mA, ENV-215M) was located near the ceiling on the left side of the chamber. The left wall also featured a white noise amplifier with cage speaker (ENV-225SM, calibrated to 8 dB above background, approximately 80–85 dB) and a clicker stimulus (ENV-135M, 75–80 dB). A computer running Med-PC IV controlled fluid delivery and auditory stimulus presentations and recorded port entries.

Drugs and Solutions

A 15% (v/v) ethanol solution was prepared by diluting 95% ethanol in tap water. Sucrose was dissolved in tap water to obtain a final concentration of 10% (w/v). Lemon, almond, and cedar wood odors were prepared by suspending lemon oil (Cat#: W262528, CAS#: 8008-56-8, Sigma-Aldrich, Oakville, ON, Canada), benzaldehyde (Cat#: B6259, CAS#: 100-52-7, Sigma-Aldrich), and cedar wood oil (Cat#: W522406, CAS#: 68990-83-0, Sigma-Aldrich) in tap water (10% v/v), respectively.

General Procedures

Home Cage Fluid Exposure

One week after arrival, rats (initial n = 37, final n = 36 with 1 rat dropped due to aggressive behavior) were acclimated to the taste and pharmacological effects of ethanol in the home cage using a 24-h, intermittent-access, two-bottle choice procedure that induces high levels of ethanol consumption in rats (Wise, 1973; Simms et al., 2008; Sparks et al., 2014). Rats had access to water via a 400-ml plastic bottle for 7 days/week. However, on Mondays, Wednesdays, and Fridays, a 100-ml graduated cylinder containing 15% ethanol ("alcohol") was placed onto the lid of the home cage for 24-h, for a total of 23 sessions. Before each session, alcohol cylinders, water bottles, and rats were weighed, and 24-h later, alcohol cylinders and water bottles were reweighed to record consumption. To mitigate the effects of side preference on intake, the placement of alcohol and water on the left and right sides of the cage lid was alternated across sessions. Spillage was accounted for by subtracting alcohol and water lost from bottles that were placed on empty cages from consumption during the corresponding session.

One week after arrival, a separate group of rats (initial n = 38, final n = 33 with 3 rats dropped due to aggressive behavior and 2 rats dropped for self-injuries over grooming) received intermittent access to 10% sucrose ("sucrose") in an identical manner. Two 24-h sessions of sucrose exposure separated by 24-h were conducted, because unlike ethanol, rats do not require extensive acclimation to sucrose.

Consumption of ethanol and sucrose solutions in the first and last sessions of this phase for each experiment is shown in **Table 2**.

TABLE 2 | Home cage consumption of 15% ethanol or 10% sucrose.

		ABA	AAB	ABC
15% ethanol	**ml**			
	First session	1.03 ± 0.34	1.82 ± 0.91	1.04 ± 0.58
	Final session	12.78 ± 2.2*	14.61 ± 2.91*	12.87 ± 2.72*
	g/kg			
	First session	0.41 ± 0.13	0.7 ± 0.3	0.41 ± 0.23
	Final session	3.06 ± 0.56*	3.37 ± 0.65*	3.1 ± 0.64*
10% sucrose	**ml**			
	First session	68.4 ± 3.69	63.23 ± 3.32	53.55 ± 5.8
	Final session	81.02 ± 3.05*	78.72 ± 3.16*	75.15 ± 7.77*
	g/kg			
	First session	22.17 ± 1.17	20.61 ± 1.15	17.19 ± 1.83
	Final session	23.77 ± 0.89	23.47 ± 1.1*	21.87 ± 2.28*

*Data are presented as means ± SEM. *p < 0.05 compared to the first session (paired t-test).*

Habituation to Conditioning Chambers and Context Familiarization

Following home cage fluid exposure, rats were transported on a cart from the vivarium to the behavioral testing room and handled individually for 1 min. The next day, they were placed into a designated conditioning chamber within the behavioral testing room for 20 min, during which time the house light was illuminated and entries into the fluid port were recorded. Chambers were set up as one of three contexts. Context 1 consisted of a smooth acrylic glass floor, black walls, and lemon odor applied to the waste pan. Context 2 had a wire mesh floor, clear walls, and almond odor applied to the waste pan. Context 3 had a perforated metal floor, striped walls, and cedar wood odor applied to the waste pan. Chambers were set up as context 1 on the first day of habituation, context 2 on the second day, and context 3 on the third day. Context pre-exposure is standard practice in renewal protocols (Nakajima et al., 2000; Bouton et al., 2011; Campese and Delamater, 2013; Todd, 2013).

Pavlovian Discrimination Training

After habituation, rats received Pavlovian discrimination training in 19 daily (Monday–Friday) sessions each lasting approximately 1 h. Session onset was indicated by illumination of the house light 5 min after initiating the Med-PC program. In each session, rats received 16 presentations each of a 10-s white noise and a 10-s clicker (5 Hz) stimulus. One stimulus was designated as the CS+ and was paired with 0.2 ml of fluid delivered into the fluid port across 6 s, starting 4 s after CS+ onset. The second stimulus, the CS−, was not explicitly paired with fluid delivery. Each trial consisted of a 10-s pre-CS interval, a 10-s CS interval, and a 10-s post-CS interval. The intertrial interval (ITI) was 45, 60, or 90 s. The ITI did not include the pre-CS and post-CS intervals and was presented pseudorandomly with a mean ITI of 67.5 s. A total of 3.2 ml of fluid was delivered in each Pavlovian discrimination training session, and ports were checked to ensure that all the fluid was consumed.

For each rat, Pavlovian discrimination training occurred in a specific context, referred to hereafter as "context A." Designation

of the white noise or clicker as the CS+, as well as the context configuration that served as context A was based on creating matched groups according to ethanol intake averaged across the last 2 days of sessions of home cage ethanol exposure or sucrose intake averaged across both sessions.

Extinction and Renewal

Two days after the last Pavlovian discrimination training session, extinction was conducted across 12 daily 1-h sessions. The same Med-PC program used during Pavlovian discrimination training was used for extinction. The CS+ and CS− were presented as during Pavlovian discrimination training and syringe pumps were activated but did not contain syringes.

The day after the last extinction session, responding to the CS+ and CS− in the absence of fluid delivery was tested in either the renewal context or a comparison context. Test 1 was followed by five Pavlovian retraining sessions, nine re-extinction sessions, and a second test. Both tests were counterbalanced and followed a within-subjects design.

ABA renewal

Pavlovian discrimination training was conducted in context A, extinction in context B, and renewal in contexts A and B (ABA vs. ABB). The test in context B provided a within-subjects comparison context against which to assess renewal in context A (Crombag and Shaham, 2002).

AAB renewal

Pavlovian discrimination training and extinction both occurred in context A, followed by renewal tests in context B or context A (AAB vs. AAA). The AAB design evaluated the hypothesis that removal from the extinction context is sufficient to precipitate renewal (Bouton et al., 2012). The test in context A provided a within-subjects comparison context against which to assess renewal in context B (Fonteyne and Baeyens, 2011). Context B is sometimes referred to as a "novel" context in this design; however, rats were familiarized to this context in a single session before the start of Pavlovian discrimination training. Previous studies have observed AAB renewal in animals that were familiarized to context B (Bouton et al., 2011; Campese and Delamater, 2013; Todd, 2013).

ABC renewal

Pavlovian discrimination training was conducted in context A, extinction in context B, and renewal in context A and context C (ABA vs. ABC). As with AAB renewal, the ABC design evaluated if removal from the extinction context was sufficient for renewal. Rats were familiarized to context C in a single session before the start of Pavlovian discrimination training. In the ABC design, context B can serve as a comparison context against which to assess renewal in context C. However, we sought to compare renewal in context A and context C and used responding at the end of extinction in context B as a baseline against which to assess ABC renewal. ABA has been used as a comparison against ABC in both renewal and false memory tasks (Fonteyne and Baeyens, 2011; Bae et al., 2015).

Using these procedures, we examined ABA, AAB, and ABC renewal in separate experiments that were run concurrently. We conducted two sequential studies that included all three renewal experiments. In study 1, the CS+ was paired with 15% ethanol ("alcohol"), whereas in study 2, the CS+ was paired with 10% sucrose ("sucrose"). With the exception of home cage fluid exposure, both studies used identical behavioral training procedures and were conducted by the same researcher in the same set of conditioning chambers.

Data Analysis

We recorded port entries during 10-s pre-CS, CS, and post-CS intervals, as well as during the variable ITI. Our primary variable of interest for assessing renewal was a difference score (Δ CS port entries), which was calculated by subtracting pre-CS port entries from port entries made during the corresponding CS. This variable accounted for individual differences in port entry behavior across rats and has been used previously in appetitive Pavlovian conditioning experiments (Campese and Delamater, 2013; Sciascia et al., 2014; Panayi and Killcross, 2018; Khoo et al., 2019).

All analyses were conducted using SPSS 24 (IBM, New York, NY, United States). Data were analyzed using *t*-tests and analysis of variance (ANOVA), as specified in Section "Results." Greenhouse–Geisser corrections were applied to degrees of freedom following a significant Mauchly test of sphericity (all $\varepsilon < 0.75$). Results where $p < 0.05$ were considered statistically significant.

Discrimination between Δ CS+ and CS− port entries at the end of Pavlovian discrimination training was assessed using a paired *t*-test. ABA and AAB renewal were assessed using an ANOVA with cue (CS+ and CS−) and context (renewal context and comparison context) as within-subjects repeated measures. All *post hoc* comparisons were Bonferroni adjusted. For ABC renewal, we included an extinction baseline in the analysis to determine if responding at test in context C or context A was significantly different from extinction responding in context B. This extinction baseline was obtained by averaging data from the last session of extinction and re-extinction. Thus, the ANOVA for this experiment included cue (CS+ and CS−) and context (extinction in context B, test in context A, and test in context C) as within-subjects repeated measures. We also examined renewal with *t*-tests on data collapsed across both experiments. Finally, we compared extinction conducted in the Pavlovian discrimination training context (context A) with extinction conducted in a different context (context B; collapsed across ABA and ABC experiments) using a mixed-design ANOVA with cue and session as within-subjects repeated measures and context (context A and context B) as a between-subjects factor.

Data Availability

The datasets generated and analyzed for this article can be found in figshare (Khoo et al., 2020).

RESULTS

Pavlovian Conditioning for Alcohol and Sucrose

All rats acquired an appetitive Pavlovian response to a CS that predicted either alcohol or sucrose. There were no statistically significant differences across ABA, AAB, or ABC experiments in CS+ port entries at the end of Pavlovian discrimination training for alcohol- or sucrose-trained rats. For alcohol-trained rats, mean ± SEM Δ CS+ port entries in the final session of training were 28.75 ± 6.45, 22.33 ± 3.92, and 22.58 ± 7.93 for the ABA, AAB, and ABC experiments, respectively, and one-way ANOVA showed no significant difference between experiments [$F(2,33) = 0.33$, $p = 0.72$]. For sucrose-trained rats, mean ± SEM Δ CS+ port entries in the final session of training were 24.27 ± 5.61, 14.36 ± 2.57, and 17.36 ± 2.08, for the ABA, AAB, and ABC experiments, respectively. One-way ANOVA did not reveal any significant differences between experiments [$F(2,30) = 1.826$, $p = 0.18$].

Renewal of Responding to an Alcohol-Predictive Cue

ABA Renewal (*n* = *12*)

By the end of Pavlovian discrimination training in context A, rats trained with alcohol significantly discriminated between the CS+ and CS− (**Figure 1A**). Port entries averaged across session 19 of Pavlovian discrimination training and session 5 of retraining were significantly higher during the CS+ than during the CS− [$t(11) = 4.98$, $p < 0.001$].

After extinction in context B, renewal was tested in the extinction context (ABB) and in the Pavlovian discrimination training context (ABA) (**Figure 1B**). Overall, port entries at test were higher in context A [context, $F(1,11) = 10.13$, $p = 0.009$] and during the CS+ [cue, $F(1,11) = 19.19$, $p = 0.001$]. However, there was a selective ABA renewal of CS+ port entries at test in context A [Cue × Context, $F(1,11) = 9.19$, $p = 0.011$]. Bonferroni-corrected *post hoc* comparisons showed that CS+ port entries were higher in context A compared to context B ($p = 0.009$), with no difference in CS− port entries as function of context ($p = 0.075$).

AAB Renewal (*n* = 12)

By the end of Pavlovian discrimination training in context A, rats significantly discriminated between the CS+ and CS− (**Figure 1C**). Port entries averaged across the final sessions of Pavlovian discrimination training and retraining were significantly higher during the CS+ than during the CS− [$t(11) = 3.97$, $p = 0.002$].

After extinction in context A, renewal was tested in the training/extinction context (AAA) and in a different context (AAB) (**Figure 1D**). Overall, port entries were higher during the CS+ than during the CS− [cue, $F(1,11) = 16.42$, $p = 0.002$] but were surprisingly lower at test in context B than in context A [context, $F(1,11) = 10.02$, $p = 0.009$]. There was no cue × context interaction [$F(1,11) = 2.08$, $p = 0.18$]. Thus, AAB renewal did not occur, and in fact, we observed AAB suppression, because CS responding was significantly lower in context B than in context A.

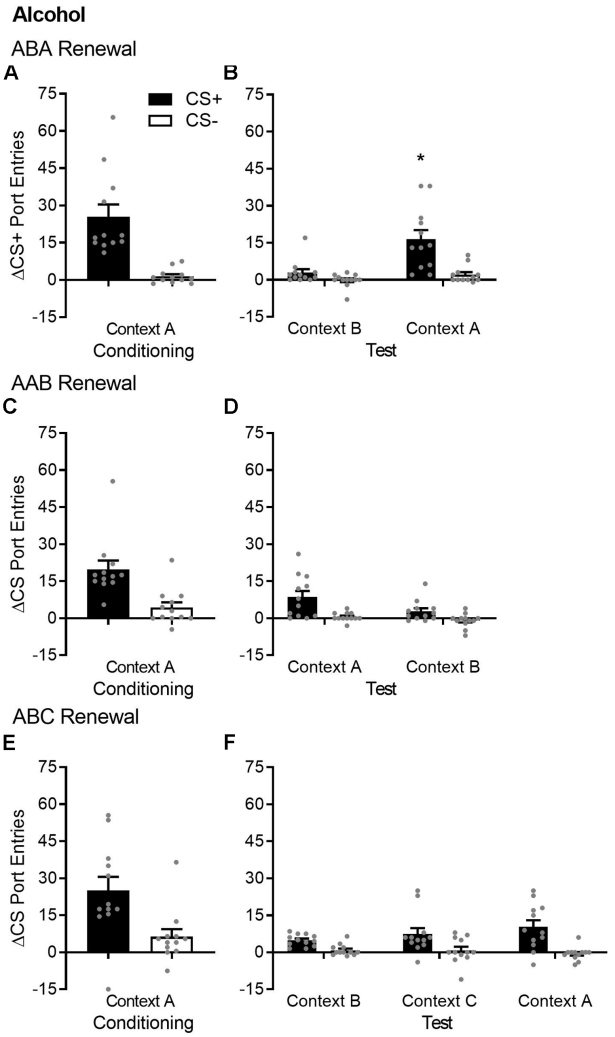

FIGURE 1 | ABA but not AAB or ABC renewal of Pavlovian conditioned responding in alcohol-trained rats. **(A,C,E)** Average port entries during the CS+ (black bars) and CS– (white bars) across the final session of Pavlovian discrimination training and retraining in context A in the ABA, AAB, and ABC renewal experiments. In all cases, CS+ port entries were significantly higher than CS– port entries. **(B)** In the ABA renewal experiment, CS+ port entries at test in context A were significantly elevated compared to the control test in context B. **(D)** In the AAB renewal experiment, overall CS port entries were significantly lower at test in context B, relative to the control test in context A. **(F)** In the ABC renewal experiment, there was no significant difference in responding during either CS across an extinction baseline in context B (averaged across the final session of extinction and re-extinction) and renewal tests in context C or A. All data are mean ± SEM Δ CS port entries (CS minus pre-CS). $n = 12$ per experiment. Data from individual rats are overlaid as symbols on the bar graphs. *$p < 0.05$, Bonferroni-corrected *post hoc* comparison of CS+ port entries in context A versus context B.

ABC Renewal (*n* = 12)

By the end of Pavlovian discrimination training in context A, rats significantly discriminated between the CS+ and CS−

(**Figure 1E**). Port entries averaged across the final sessions of Pavlovian discrimination training and retraining were significantly higher during the CS+ than during the CS− [$t(11) = 2.69$, $p = 0.021$].

After extinction in context B, renewal was tested in a different context (ABC) and in the Pavlovian discrimination training context (ABA) (**Figure 1F**). To evaluate ABC and ABA renewal relative to baseline responding during extinction in context B, the ANOVA included data from both tests and from an extinction baseline. Overall, port entries were elevated during the CS+ compared to the CS− [cue, $F(1,11) = 32.536$, $p < 0.001$]. However, there was no main effect of context [$F(1.34,14.68) = 0.52$, $p = 0.53$, $\varepsilon = 0.67$] or cue × context interaction [$F(1.19,13.04) = 2.45$, $p = 0.14$]. Thus, according to this analysis, ABC renewal did not occur.

Renewal of Responding to a Sucrose-Predictive Cue

ABA Renewal (n = 11)

By the end of Pavlovian discrimination training in context A, rats trained with sucrose significantly discriminated between the CS+ and CS− (**Figure 2A**). Port entries averaged across the last sessions of Pavlovian discrimination training and retraining were significantly higher during the CS+ than during the CS− [$t(10) = 9.64$, $p < 0.001$].

After extinction in context B, renewal was tested in the extinction context (ABB) and in the Pavlovian discrimination training context (ABA) (**Figure 2B**). Overall, rats responded more to the CS+ than to the CS− [cue, $F(1,10) = 33.76$, $p < 0.001$], with no significant main effect of context [$F(1,10) = 2.27$, $p = 0.16$]. However, ABA renewal was revealed by a significant cue × context interaction [$F(1,10) = 22.13$, $p = 0.001$]. Bonferroni-corrected *post hoc* tests showed that compared to those in context B, CS+ port entries were significantly higher in context A ($p = 0.011$), whereas CS− port entries were significantly lower ($p = 0.004$).

AAB Renewal (n = 11)

By the end of Pavlovian discrimination training in context A, rats significantly discriminated between the CS+ and CS− (**Figure 2C**). Port entries averaged across the final sessions of Pavlovian discrimination training and retraining were significantly higher during the CS+ than during the CS− [$t(10) = 2.96$, $p = 0.014$].

After extinction in the same context (context A), renewal was tested in the training/extinction context (AAA) and in a different context (AAB) (**Figure 2D**). ANOVA results indicated the absence of AAB renewal [context, $F(1,10) = 0.0004$, $p = 0.984$; cue, $F(1,10) = 3.45$, $p = 0.093$; cue × context, $F(1,10) = 0.028$, $p = 0.87$].

ABC Renewal (n = 11)

By the end of Pavlovian discrimination training in context A, rats significantly discriminated between the CS+ and CS− (**Figure 2E**). Port entries averaged across the last sessions of Pavlovian discrimination training and retraining were significantly higher during the CS+ than during the CS− [$t(10) = 2.61$, $p = 0.026$].

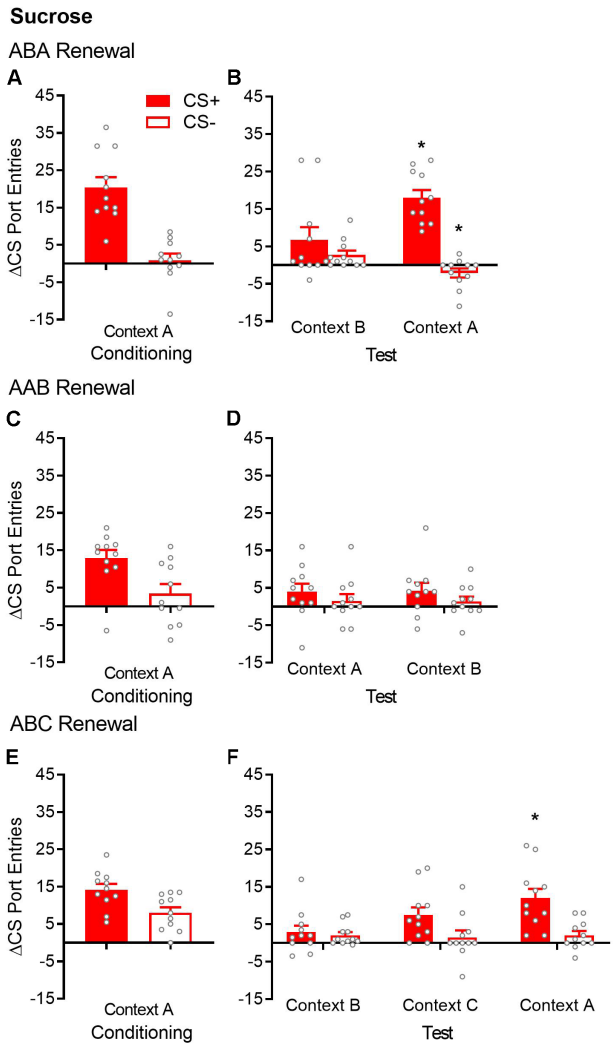

FIGURE 2 | ABA but not AAB or ABC renewal of Pavlovian conditioned responding in sucrose-trained rats. **(A,C,E)** Average port entries during the CS+ (red bars) and CS– (white bars) across the final session of Pavlovian discrimination training and retraining in context A in the ABA, AAB, and ABC renewal experiments. In all cases, CS+ port entries were significantly higher than CS– port entries. **(B)** In the ABA renewal experiment, CS+ port entries at test in context A were significantly elevated compared to the control test in context B. **(D)** There was no evidence of AAB renewal. **(F)** In the ABC renewal experiment, CS+ port entries were significantly higher at test in context A relative to extinction in context B, indicative of ABA renewal. CS+ port entries at test in context C did not differ significantly from either extinction in context B or test in context A. All data are mean ± SEM Δ CS port entries (CS minus pre-CS). n = 11 per experiment. Data from individual rats are overlaid as symbols on the bar graphs. *$p < 0.05$ for a Bonferroni-corrected *post hoc* comparison between context A and context B.

After extinction in context B, renewal was tested in a different context (ABC) and in the Pavlovian discrimination training context (ABA) (**Figure 2F**). Overall, rats continued to discriminate between the CS+ and CS− across extinction in context B and tests in contexts C and A [cue, $F(1,10) = 9.37$, $p = 0.012$]. Responding did not differ significantly across the

three contexts, although the main effect showed a trend toward statistical significance [context, $F(2,20) = 3.44$, $p = 0.052$]. Interestingly, ANOVA revealed a significant cue × context interaction [$F(2,20) = 5.37$, $p = 0.014$]. Bonferroni-corrected *post hoc* tests supported ABA renewal, as CS+ port entries were significantly higher at test in context A, compared to extinction in context B ($p = 0.034$). However, CS+ port entries at test in context C did not differ from the extinction in context B ($p = 0.20$) or from that in context A ($p = 0.23$). These results support visual inspection of the data, which suggest that ABC renewal occurred in a subset of rats but was overall less consistent than ABA renewal.

Renewal Effects on Data Collapsed Across Experiments

The results presented above suggested that ABA renewal was the most reliable effect, AAB renewal was negligible, and ABC renewal was at best a modest effect. To explore these conclusions further, we calculated difference scores for each renewal experiment. For the ABA and AAB experiments, we subtracted Δ CS+ port entries at test in the comparison context from Δ CS+ port entries at test in the renewal context. For the ABC experiment, we subtracted Δ CS+ port entries during extinction in context B from Δ CS+ port entries at test in context C. Based on these calculations, a positive number indicated greater responding at test in the renewal context than in the comparison/extinction context. **Figure 3** depicts these data collapsed across both experiments. To provide a measure of effect size, 95% confidence intervals of the difference are provided.

In the ABA renewal experiment (**Figure 3A**), 19 rats had positive difference scores, whereas four rats had negative difference scores. A *t*-test on the combined data from both the alcohol and sucrose experiments indicated that relative to the test in context B, responding was significantly higher in context A, confirming robust ABA renewal [$t(22) = 4.51$, $p < 0.001$, 95% CI = 6.74, 18.22].

In the AAB renewal experiment (**Figure 3B**), 6 rats had positive difference scores, 1 rat had a difference score of 0, and 16 rats had negative difference scores. A *t*-test on the combined data from both studies found no significant difference between the test in context A and the test in context B [$t(22) = -1.46$, $p = 0.16$, 95% CI = −6.41, 1.89].

Finally, in the ABC renewal experiment (**Figure 3C**), 15 rats had positive difference scores, while seven rats had negative difference scores and one rat had no difference. A *t*-test on the combined data indicated that the difference approached statistical significance [$t(22) = 1.91$, $p = 0.069$, 95% CI = −0.3, 7.3].

Comparing Extinction in the Pavlovian Discrimination Training Context and in a Distinct Context

Because ABA, AAB, and ABC renewal experiments were run concurrently, we compared extinction in the Pavlovian discrimination training context (context A) and in context B (**Figures 4A–D**). For both the alcohol and sucrose studies, data from the extinction phase in context B were collapsed across ABA and ABC experiments and compared to the extinction phase in context A from the AAB experiment. This analysis revealed that in both alcohol- and sucrose-trained rats, CS+ port entries were transiently but significantly elevated in context A than in context B at the start of the first extinction phase (**Figures 4A,B**), as well as the second extinction phase (**Figures 4C,D**) that occurred between tests. These statements are supported by statistical analyses that revealed a significant three-way interaction of cue × session × context for each condition (see **Table 3** for all main effects and interactions).

Figure 4A shows Δ CS port entries for the extinction phase that preceded the first renewal test in alcohol-trained rats. CS+ port entries were significantly elevated in context A relative to context B in extinction sessions 1 and 9. Similar results were obtained in sucrose-trained rats (**Figure 4B**), where CS+ port entries were significantly elevated in context A relative to context B in sessions 1–2 and 9–11.

In the nine sessions of re-extinction that occurred between tests, CS+ port entries in alcohol-trained rats (**Figure 4C**) were higher in context A than in context B in sessions 1–3 and in session 5. However, there was also a significant difference between contexts in CS− port entries in session 9. In sucrose-trained rats (**Figure 4D**), CS+ port entries were significantly higher in context A than in context B in sessions 1–3 and in session 6.

DISCUSSION

In the present experiments, we observed reliable ABA renewal of conditioned responding to a discrete, auditory CS that predicted either alcohol or sucrose. This effect was selective for the CS+ and did not occur for a CS− that was not explicitly paired with fluid delivery. In contrast, removal from a context associated with conditioning and extinction (AAB renewal) did not produce renewal in sucrose-trained rats, and in alcohol-trained rats it resulted in a surprising overall reduction in CS port entries. In both alcohol- and sucrose-trained rats, ABC renewal was not statistically significant, although for sucrose-trained rats, CS+ port entries at test in context C did not differ from CS+ port entries at test in either context A or context B. Finally, in a comparison of extinction, CS+ port entries were significantly higher in context A than in context B at the start of the extinction phase. The theoretical and clinical implications of these results, along with methodological considerations, are presented below.

ABA Renewal

As predicted, we found evidence of ABA renewal in alcohol- and sucrose-trained rats. Across both experiments, the majority of rats responded more to the CS+ at test in context A, compared to context B (19 out of 23). These results are consistent with our prior research (Chaudhri et al., 2008b, 2013; Sciascia et al., 2014; Lacroix et al., 2017) and extend it to show that ABA renewal occurs using a within-subjects design. As before, ABA renewal was selective for the CS+, and a return to the conditioning context following extinction in a different context had no impact on CS− port entries. Overall, these findings

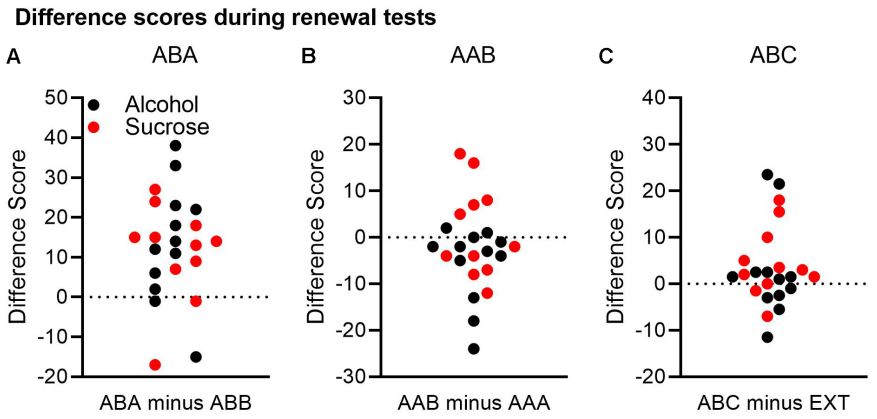

FIGURE 3 | A comparison of renewal across both the alcohol and sucrose studies. A difference score was calculated for each renewal experiment by subtracting Δ CS port entries in the control context from Δ CS port entries in the renewal context. In this analysis, a positive difference score would support a renewal effect. **(A)** In the ABA renewal experiment, 19 of 23 rats had positive difference scores. **(B)** In the AAB experiment, 6 of 23 rats had positive difference scores, and 16 rats had negative difference scores. **(C)** In the ABC renewal experiment, 15 of 23 rats had positive difference scores, and 7 had negative differences scores.

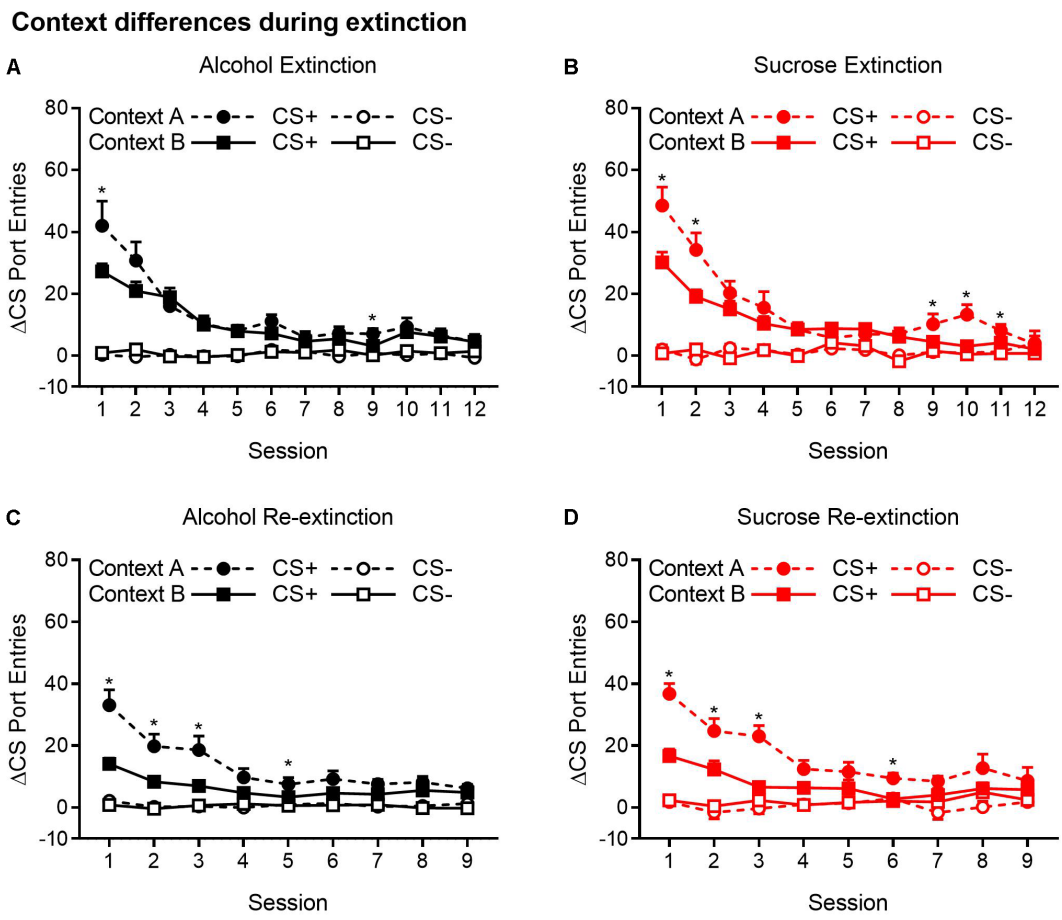

FIGURE 4 | CS+ port entries were transiently elevated during extinction in context A compared to context B. Port entries across 12 extinction sessions prior to test 1 that occurred in **(A)** alcohol-trained rats (n = 12) and **(B)** sucrose-trained rats (n = 11). After the first renewal test, rats were retrained for 5 days and subjected to 9 days of re-extinction. Port entries are shown for **(C)** alcohol-trained and **(D)** sucrose-trained rats. All data are means ± SEM. *$p < 0.05$, Bonferroni-corrected *post hoc* comparisons for CS+ responding between the two extinction contexts.

TABLE 3 | Statistical results of analysis of context-based differences in extinction.

	Alcohol extinction (Figure 4A)	Sucrose extinction (Figure 4B)	Alcohol re-extinction (Figure 4C)	Sucrose re-extinction (Figure 4D)
Cue	$F(1,34) = 113.79$	$F(1,31) = 122.38$	$F(1,34) = 136.12$	$F(1,31) = 96.58$
	$p < 0.001$*	$p < 0.001$*	$p < 0.001$*	$p < 0.001$*
Context	$F(1,34) = 1.29$	$F(1,31) = 4.95$	$F(1,34) = 19.54$	$F(1,31) = 6.21$
	$p = 0.264$	$p = 0.034$*	$p < 0.001$*	$p = 0.018$*
Session	$F(4.97,169.02) = 31.15$	$F(6.29,194.89) = 33.19$	$F(4.74, 161.06) = 21.46$	$F(4.90,152.01) = 11.45$
	$p < 0.001$*	$p < 0.001$*	$p < 0.001$*	$p < 0.001$*
	$\varepsilon = 0.45$	$\varepsilon = 0.57$	$\varepsilon = 0.59$	$\varepsilon = 0.61$
Cue × Context	$F(1,34) = 2.76$	$F(1,31) = 5.29$	$F(1,34) = 18.03$	$F(1,31) = 23.60$
	$p = 0.11$	$p = 0.028$*	$p < 0.001$*	$p < 0.001$*
Cue × Session	$F(4.73,160.69) = 32.38$	$F(5.56,172.30) = 30.32$	$F(4.83,164.13) = 20.12$	$F(8,248) = 13.44$
	$p < 0.001$*	$p < 0.001$*	$p < 0.001$*	$p < 0.001$
	$\varepsilon = 0.43$	$\varepsilon = 0.50$	$\varepsilon = 0.60$	
Session × Context	$F(4.97,169.02) = 1.92$	$F(6.29,194.89) = 3.37$	$F(4.74,161.06) = 4.99$	$F(4.90,152.01) = 2.34$
	$p = 0.094$	$p = 0.003$*	$p < 0.001$*	$p = 0.046$*
Cue × Session × Context	$F(4.73,160.69) = 2.45$	$F(5.56,172.30) = 3.18$	$F(4.83,164.13) = 4.66$	$F(8,248) = 2.58$
	$p = 0.039$*	$p = 0.007$*	$p = 0.001$*	$p = 0.01$*

*$p < 0.05$.

concur with numerous published observations of ABA renewal in aversive and appetitive learning paradigms (Bouton and Bolles, 1979; Bouton and King, 1983; Bouton and Swartzentruber, 1991; Crombag et al., 2000; Tsiang and Janak, 2006; Zironi et al., 2006; Chaudhri et al., 2008b; Bouton et al., 2011; Valyear et al., 2017). They establish that our task parameters were sufficient to generate renewal and provide a basis for comparison with AAB and ABC renewal.

AAB Renewal

Although AAB renewal has been reported in aversive Pavlovian conditioning studies (Bouton and Bolles, 1979; Tamai and Nakajima, 2000), we found no evidence of AAB renewal in either alcohol- or sucrose-trained rats. These results are consistent with two studies with drug reinforcers that failed to detect AAB renewal of operant conditioning (Crombag and Shaham, 2002; Fuchs et al., 2005). With a food pellet reinforcer, AAB renewal of operant conditioning was reported by one laboratory (Bouton et al., 2011; Todd et al., 2014), but not by another (Nakajima et al., 2000). A procedural difference between these studies is that the group that reported AAB renewal conducted magazine training in context B before the start of operant conditioning (Bouton et al., 2011; Todd et al., 2014). A context–reinforcer association formed during magazine training in context B may have influenced subsequent operant responding at test in context B.

In the present experiments, alcohol-trained rats in the AAB renewal experiment showed an overall reduction in CS port entries at test in context B, relative to context A, which is the opposite of a renewal effect. While this finding may be due to chance, we observed a similar effect in an unpublished AAB renewal experiment conducted using the shock-probe defensive burying task (Brown and Chaudhri, unpublished). This decrement was specific to CS port entries because there was no significant difference in ITI port entries at test for alcohol-trained rats [$t(11) = -0.093$, $p = 0.928$]. One explanation for this surprising result is a decrement in the generalization of conditioning (CS–US memory) across contexts. To mitigate such a decrement, we had familiarized rats to all three contexts before conditioning. However, the effects of this familiarization may have worn off by the time rats in the AAB renewal experiment were tested in context B. Thus, a switch to context B after training and extinction in context A might have had a non-associative effect on behavior which resulted in a reduction in CS port entries at test in context B.

Sucrose-trained rats did not show AAB renewal, but neither did they show a suppression of CS responding in context B relative to context A as was observed with alcohol-trained rats. Future studies are needed to replicate this difference across drug and non-drug reinforcers. Altogether, the present findings are consistent with studies that failed to detect AAB renewal of appetitive behavior (see **Table 1**).

ABC Renewal

While in the AAB renewal design, extinction is conducted in the same context as conditioning, in the ABC renewal design conditioning, extinction and test all occur in different contexts. In both cases, renewal is tested in a context that differs from that of extinction; however, the ABC renewal design allows for the opportunity to learn that extinction occurs after a context switch.

We did not observe statistically significant ABC renewal in either the alcohol or sucrose experiment. In sucrose-trained rats, although the ANOVA revealed a statistically significant cue × context interaction, follow-up tests were inconclusive. Δ CS+ port entries in the ABC test ($M = 7.5$) were not

significantly different from extinction in context B (M = 2.9) or the renewal test in context A (which was different from extinction with M = 12).

We considered the possibility that the effect size of ABC renewal may be smaller than ABA renewal and that by comparing ABA and ABC renewal, we might have reduced the statistical power that would have been available if we had compared an ABC test to an ABB test. To address this limitation, we collapsed data across both experiments and examined renewal using a difference score measure that subtracted Δ CS+ port entries in the comparison context from ΔCS+ port entries in the test context. For the ABC renewal experiment, we used the extinction baseline obtained in context B as the comparison context. The analysis for this experiment approached statistical significance, suggesting that there may be an ABC renewal effect that was smaller than we were able to statistically detect. Confidence intervals of effect size indicated that ABC renewal was approximately three to four times smaller than ABA renewal. These results therefore suggest that ABC renewal occurs in appetitive Pavlovian conditioning but that this effect is modest and may require greater statistical power to observe.

Extinction Comparisons

When a switch from conditioning to extinction is accompanied by a change in context, there can be a decrement in conditioned responding that, as opposed to rapid extinction, reflects a lack of transfer of the original learning to the second context (Bouton, 2004). This "generalization decrement" is mitigated by exposure to both contexts prior to conditioning. In the present study, rats were familiarized to all three contexts before conditioning in separate, 20-min sessions. Magazine training was not conducted during these sessions, meaning that these contexts had no opportunity to become associated with sucrose or alcohol before Pavlovian discrimination training. Comparing across **Figures 1A,C**, **2A,C**, **4A,C**, there was no decrement in CS+ port entries triggered by a switch to context B, following conditioning in context A.

In both experiments, CS+ port entries in extinction were significantly higher in context A (the conditioning context) than in context B, and this effect occurred during the initial and second extinction phases. These results replicate prior operant conditioning research (Wing and Shoaib, 2008; Todd, 2013) and concur with our published data using a different Pavlovian conditioning procedure that equated exposure to contexts A and B before test (Remedios et al., 2014; Sparks et al., 2014; Millan et al., 2015; Sciascia et al., 2015; Valyear et al., 2018; Khoo et al., 2019).

The elevation in CS+ port entries during early extinction in context A versus context B may be related to context A gaining associative strength during Pavlovian discrimination training. This excitatory property of the context could summate with a CS–US association to energize CS+ port entries during extinction. The possibility that context A gained associative strength may have implications for AAB and ABC renewal. If rats in the AAB experiment experienced extinction of both context A and the CS+, then the resulting inhibitory memory may have been strong enough to prevent generalization of the CS–US memory to context B at test. It may also have contributed to the surprising and previously unreported AAB suppression effect observed in alcohol-trained rats. In contrast, if rats in the ABC renewal experiment only experienced extinction of the CS+, but not of the context–US association, then the inhibitory memory formed during extinction may not have been able to counter the generalization of the CS–US memory to context C at test, resulting in a weak renewal effect.

The similarity between the present data and prior operant conditioning results (Wing and Shoaib, 2008; Todd, 2013) showing a difference in extinction responding across contexts may also suggest that the magazine approach relies (at least in part) on instrumental contingencies. An instrumental contingency might arise from adventitious or superstitious conditioning of port entries that occur immediately before sucrose/alcohol delivery. Unlike port entries or operant responses like lever presses, other conditioned responses acquired during appetitive Pavlovian conditioning (e.g., head jerking) do not show context-based differences during extinction (Bouton and Peck, 1989), which also supports the idea that port entry responding might have an instrumental contingency. However, other studies provide compelling evidence that port entry behavior in appetitive Pavlovian paradigms is predominantly a Pavlovian conditioned response (Harris et al., 2013), and in at least one prior study, context-dependent differences in magazine approach were not observed (Carranza-Jasso et al., 2014). Thus, additional research is needed to delineate the contribution of Pavlovian and instrumental contingencies to port entry responding in appetitive Pavlovian paradigms.

Theoretical, Methodological, and Clinical Considerations

There are a few psychological explanations for renewal that at first pass appear to be mutually exclusive but may ultimately occur in parallel with differential contributions to behavior based on task parameters. One explanation is that the extinction context may function as a negative occasion setter, such that release from this context will generate renewal (Bouton and Swartzentruber, 1986). This idea is supported by findings that extinguishing the conditioning context before test does not abolish renewal (Bouton et al., 2011; Todd et al., 2014) and that renewal is observed when the reinforcement histories of the conditioning and extinction contexts are equated (Todd, 2013). The extinction context may also function as a conditioned inhibitor, in which case removal from the extinction context would restore responding (Harris et al., 2000). However, context A may also acquire associative strength during conditioning, which could summate with residual associative strength of the CS following extinction to produce renewal. This account has been raised to explain why AAB and ABC renewal have often numerically weaker effects than ABA renewal (Polack et al., 2013).

In the present research, ABA renewal was more reliable than ABC renewal, and AAB renewal was not observed. The lack of AAB renewal might be attributed to differential levels of conditioning in this experiment, relative to the ABA and ABC renewal experiments. However, there were no

statistically significant differences across experiment in CS+ port entries at the end of Pavlovian discrimination training, which suggests that the observed differences cannot be attributed to preexisting differences between experimental cohorts in baseline Pavlovian conditioning.

It is possible that using more than three elements in the configuration of contexts might have helped to make the contexts more discernibly distinct, increasing the possibility of detecting AAB renewal. In addition to having visual, olfactory, and tactile elements, we could have varied the shape of the conditioning box and the time of day at which conditioning/test and extinction were conducted. Conducting repeated conditioning, extinction, and test phases might also have increased discernibility across contexts, increasing the chances of detecting renewal.

A final methodological consideration of these experiments is that renewal was only tested in male rats. Other laboratories have reported ABA renewal of operant alcohol-seeking behavior in female rats (Bianchi et al., 2018) or inconsistent renewal of responding to a food-predictive CS in female rats (Anderson and Petrovich, 2015). However, it is notable that much of the historical literature on renewal has been conducted in female rats (Bouton and Peck, 1989; Bouton and Ricker, 1994; Goddard, 1999; Todd et al., 2012; Anderson and Petrovich, 2018a). The present results are consistent with this literature but cannot account for the possible impact of sex differences in appetitive learning that could differentially affect ABA, AAB, and ABC renewal.

Our data support the idea that if exposure-based treatment for substance use disorders occurs in a setting that is distinct from real-world, drug-use environments, then relapse facilitated by the renewal of conditioned responding to drug-predictive cues remains a possibility. Interestingly, the lack of AAB renewal in the present research supports the hypothesis that conducting exposure-based therapy in real-world, drug-use environments might prevent renewal-induced facilitation of relapse. One mechanism for this long-lasting effect may be through the extinction of both context–US and CS–US associations, which could produce a stronger inhibitory memory that does not allow the original CS–US memory to traverse contexts.

Finally, there is a burgeoning literature on the neural basis of ABA renewal in operant conditioning paradigms (Bossert et al., 2004; Fuchs et al., 2005; Chaudhri et al., 2008a; Crombag et al., 2008; Marchant et al., 2009; Marinelli et al., 2010). Fewer studies have examined the neural basis of renewal in appetitive Pavlovian learning paradigms (Chaudhri et al., 2013; Anderson and Petrovich, 2017; Anderson and Petrovich, 2018b; Villaruel et al., 2018), and only a handful of studies have directly compared neural processes underlying ABA, AAB, and ABC renewal. Trask et al. (2017) showed that pharmacological inactivation of the prelimbic cortex attenuated ABA renewal of operant responding for sucrose pellets; however, the same manipulation had no effect on ABC renewal in the same rats. In another study, Campese and Delamater (2013) found that pharmacological inactivation or lesions of the dorsal hippocampus had no effect on ABA or ABC renewal of Pavlovian responding to a cue that predicted food pellets (Campese and Delamater, 2013). Additional research is needed to directly investigate the neural basis of ABA renewal in conjunction with both AAB and ABC renewal designs.

CONCLUSION

In addition to yielding theoretical insights about extinction, understanding the degree to which CS–US and CS–no US associations generalize across contexts may inform how to improve exposure-based treatment for substance use disorders. We observed ABA renewal of appetitive Pavlovian conditioned responding in alcohol- and sucrose-trained rats, which supports the reliability of this effect and suggests that a return to the conditioning context following extinction in a different context is a robust trigger for renewal. ABC renewal occurred in a subset of rats, but the analysis required a larger sample size to approach statistical significance. Combined with the lack of AAB renewal, our data suggest that removal from a context in which extinction was conducted is not a reliable trigger for renewal. Conducting extinction in the same context as conditioning, which produced transiently heightened CS+ port entries in the present research, might result in a stronger inhibitory memory that prevents AAB renewal and, for alcohol-trained rats, caused a surprising and previously unreported AAB suppression effect. These findings support a deep literature on the importance of context in behavioral responding and provide insight into how different approaches to extinction can influence later context-induced changes in behavior.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research Ethics Committee, Concordia University.

AUTHOR CONTRIBUTIONS

NC and JS designed the experiments. JS conducted the experiments. SK analyzed and graphed the data. SK, AB, and NC wrote the manuscript. NC obtained funding and resources for the research and supervised JS, AB, and SK. All authors contributed to editing and finalizing the manuscript for publication.

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MINI REVIEW
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Retrieval-Extinction and Relapse Prevention: Rewriting Maladaptive Drug Memories?

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Addicted individuals are highly susceptible to relapse when exposed to drug-associated conditioned stimuli (CSs; "drug cues") even after extensive periods of abstinence. Until recently, these maladaptive emotional drug memories were believed to be permanent and resistant to change. The rediscovery of the phenomenon of memory reconsolidation—by which retrieval of the memory can, under certain conditions, destabilize the previously stable memory before it restabilizes in its new, updated form—has led to the hypothesis that it may be possible to disrupt the strong maladaptive drug-memories that trigger a relapse. Furthermore, recent work has suggested that extinction training "within the reconsolidation window" may lead to a long-term reduction in relapse without the requirement for pharmacological amnestic agents. However, this so-called "retrieval-extinction" effect has been inconsistently observed in the literature, leading some to speculate that rather than reflecting memory updating, it may be the product of facilitation of extinction. In this mini review article, we will focus on factors that might be responsible for the retrieval-extinction effects on preventing drug-seeking relapse and how inter-individual differences may influence this therapeutically promising effect. A better understanding of the psychological and neurobiological mechanisms underpinning the "retrieval-extinction" paradigm, and individual differences in boundary conditions, should provide insights with the potential to optimize the translation of "retrieval-extinction" to clinical populations.

Keywords: memory reconsolidation, extinction, retrieval-extinction, addiction, rat

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INTRODUCTION

Addiction is a chronic, relapsing disorder characterized by loss of control over drug use, high motivation for drug, and persistence in drug use despite adverse consequences (American Psychiatric Association, 2013). Those who become addicted show a high propensity to relapse following periods of abstinence. Re-exposure to previously drug-associated cues is one major precipitant of relapse: people, places, and paraphernalia repeatedly paired with drugs become conditioned to the drug high in a pavlovian manner, and these pavlovian conditioned stimuli (CSs) subsequently induce relapse (de Wit and Stewart, 1981).

Drug-associated CSs influence relapse through at least three psychologically and neurobiologically dissociable processes (Milton and Everitt, 2010). Until recently, these maladaptive CS-drug memories were believed to be permanent and resistant to change. However, following the

rediscovery of memory reconsolidation (Nader et al., 2000) interest grew in exploiting this process to develop new forms of treatment for mental health disorders including addiction. One such strategy would be pharmacological disruption of drug memory reconsolidation with the administration of amnestic agents (for review, see Milton and Everitt, 2010). Here, we focus on an alternative strategy aiming to capitalize on the hypothesized updating function of reconsolidation; reactivating a memory and introducing "CS-no US" information through the procedure known as "extinction within the reconsolidation window" or "retrieval-extinction." Due to the relative paucity of drug memory retrieval-extinction studies in the literature, we will extrapolate general principles from retrieval-extinction studies of both fear and drug memories, focusing on the influence of individual differences.

RETRIEVAL-EXTINCTION AS A NON-PHARMACOLOGICAL MEMORY INTERFERENCE METHOD

A potential limitation of pharmacological approaches to target memory reconsolidation is the requirement for amnestic agents. Although drugs such as propranolol, the β-adrenergic receptor antagonist used in many reconsolidation studies, are safe to use in humans, many amnestic agents (e.g., protein synthesis inhibitors) are less well-tolerated. Consequently, there has been great interest in capitalizing on the hypothesized role of reconsolidation in memory updating (Lee, 2009) with the use of "retrieval-extinction" procedures.

"Retrieval-extinction" was first described for pavlovian fear memories, and involves reactivating the memory in a brief re-exposure session, followed by a separate prolonged re-exposure/extinction session after a short delay (typically 10–60 min, but theoretically within 3–4 h of the opening of the "reconsolidation window"). The retrieval-extinction procedure persistently attenuates recovery of fear memories in both rats (Monfils et al., 2009) and humans (Schiller et al., 2010), although this has not been universally replicated (e.g., see Luyten and Beckers, 2017).

Shortly after the discovery of retrieval-extinction, a seminal article (Xue et al., 2012) showed that retrieval-extinction could reduce drug-seeking in rodents trained on cocaine- or opiate-conditioned place preference (CPP) or intravenous cocaine self-administration. Furthermore, retrieval-extinction was shown in the same study to reduce craving elicited by heroin CSs in human outpatient heroin abusers. This has a potentially profound impact on addiction treatment, as a relatively minor adjustment to prolonged exposure therapy greatly improved treatment outcomes. Consequently, there has been intense research interest in retrieval-extinction from both preclinical and clinical addiction researchers.

Reductions in CPP following the retrieval-extinction procedure have been replicated with cocaine (Sartor and Aston-Jones, 2014) and morphine (Ma et al., 2012). Retrieval-extinction also reduces alcohol-seeking in rats (Millan et al., 2013; Willcocks and McNally, 2014; Cofresí et al., 2017) and nicotine-seeking in human smokers (Germeroth et al., 2017). However, despite its efficacy in reducing drug-seeking, there remains a lack of definitive evidence that retrieval-extinction for drug memories depends critically upon memory-updating and reconsolidation mechanisms, and not the facilitation of extinction. In several studies where retrieval-extinction effectively reduced one measure of drug-seeking, it was ineffective at reducing other measures: it did not prevent spontaneous recovery of morphine CPP 4 weeks post-intervention (Ma et al., 2012) and it did not retard the reacquisition of alcohol-seeking, as would be expected if the original cue-alcohol memory had been erased (Willcocks and McNally, 2014). Furthermore, the finding that extinction training *prior* to memory reactivation reduces subsequent alcohol-seeking contradicts the hypothesis that memory destabilization is critical for the retrieval-extinction effect (Millan et al., 2013). This is consistent with our previous report that drugs that block fear memory destabilization do not prevent the reduction in fear produced by the retrieval-extinction procedure (Cahill et al., 2019).

However, it may be premature to conclude that retrieval-extinction simply represents the facilitation of extinction that does not engage in memory reconsolidation mechanisms. Some molecular evidence suggests that retrieval-extinction recruits immediate early genes associated with memory reconsolidation, at least for fear memories (Tedesco et al., 2014) and that antagonism of L-type voltage-gated calcium channels, which are necessary for memory destabilization (Suzuki et al., 2008) prevents the reduction in subsequent responding normally observed following retrieval-extinction for a food-associated CS (Flavell et al., 2011). These apparently conflicting findings are difficult to reconcile, but we propose that individual differences may determine whether reconsolidation or extinction mechanisms are engaged under a given set of experimental conditions. In turn, this may account for the inconsistent reports of retrieval-extinction in the literature.

THE INFLUENCE OF INDIVIDUAL DIFFERENCES ON THE EFFICACY OF RETRIEVAL-EXTINCTION

Individual differences pose a potential challenge to the translation of retrieval-extinction to the clinical situation. A relatively understudied phenomenon in retrieval-extinction, individual differences in the acquisition of extinction influence the efficacy of retrieval-extinction for preventing the recovery of fear memories (Shumake et al., 2018) and in turn, the capacity for fear extinction learning correlates with CO_2 reactivity and orexin expression in the lateral hypothalamus (Monfils et al., 2019). To date, there have been no studies examining the impact of these mechanisms on the retrieval-extinction of appetitive memories, but drawing on findings from the fear literature, we consider three factors that are likely to influence retrieval-extinction for drug memories: individual differences in reconsolidation boundary conditions, the attribution of incentive value to appetitive cues, and the influence of stress on mnemonic processes.

Individual Differences in Boundary Conditions

Not all instances of memory retrieval lead to memory reconsolidation; instead, there are hypothesized "boundary conditions" that determine whether a retrieved memory destabilizes and reconsolidates. There is extensive evidence that memory destabilization depends upon a "mismatch" between what is expected and what actually occurs, formalized as "prediction error" (Pedreira and Maldonado, 2003; Pedreira et al., 2004; Sevenster et al., 2012, 2013, 2014; though see Yang et al., 2019, for a discussion of whether uncertainty may also induce memory destabilization). The relationship between prediction error and memory lability is not monotonic, however, as extensive prediction error—for example, during extended periods of reinforcer omission—leads not to reconsolidation of the original memory, but rather the consolidation of new extinction memory, and thus extinction learning. The relationship between reconsolidation and extinction has been extensively investigated for fear memories, with converging evidence showing that the two mnemonic processes are separated by a "limbo" period in which the original memory becomes again insensitive to disruption (Flavell and Lee, 2013; Merlo et al., 2014, 2018; Sevenster et al., 2014; Cassini et al., 2017). To date, this has been studied at the population level with strong conditioning parameters, which may mask individual variability. For drug memories, where individual drug use histories show greater variability, it may be hypothesized that the extent of prediction error required to engage reconsolidation, limbo and extinction mechanisms may differ between individuals. Thus, considering the widely accepted boundary conditions of memory strength and age (Suzuki et al., 2004; Kwak et al., 2012), the extent of re-exposure required for reactivating a cue-drug memory may individually vary.

Individual Differences in Attribution of Incentive Value to Cues

An increasingly large body of research has characterized how individual differences in the attribution of incentive value to drug-associated CSs influence subsequent drug self-administration and relapse (see Robinson et al., 2018, for review). There is variation in the degree to which individuals are attracted to discrete CSs associated with reward ("sign-tracking") as compared to the location of the reward itself ("goal-tracking"), usually measured by a pavlovian conditioned approach using an autoshaping procedure (Meyer et al., 2012). These behaviors are hypothesized to reflect endophenotypes correlated with differences in dopaminergic signaling within the motivational circuitry (Flagel et al., 2011) and differential reliance on model-based (goal-directed) and model-free (habitual) motivational systems (Lesaint et al., 2015). There is also evidence that goal-trackers condition more readily than sign-trackers to contextual cues predictive of reinforcement (Morrow et al., 2011; Saunders et al., 2014), although this has not been universally replicated (Vousden et al., in press).

Sign-trackers and goal-trackers appear to learn differentially about discrete and contextual cues. This may influence whether they perceive the retrieval-extinction procedure to be the same as the previous learning experience (favoring reconsolidation updating) or as a different learning experience (favoring the formation of a new extinction memory). We speculate that sign-trackers and goal-trackers may attribute the retrieval-extinction experience to different "latent causes" (Dunsmoor et al., 2015). Considering that sign-trackers also appear to be resistant to pavlovian extinction (Ahrens et al., 2016), the relative paucity of studies of the influence of these endophenotypes on retrieval-extinction is surprising. Those that have been conducted used a slightly different procedure, classifying rats as "orienters" and "non-orienters" to pavlovian CSs, which are broadly similar to sign-tracking and goal-tracking. Both groups showed reduced spontaneous recovery of fear memory (Olshavsky et al., 2013), but when the appetitive CS-reward memory was targeted for retrieval-extinction, only the orienters/sign-trackers showed reduced appetitive responses (Olshavsky et al., 2014). This may suggest a shift in the boundaries between reconsolidation, limbo and extinction, such that the same re-exposure session may have induced reconsolidation-based updating in the sign-trackers, but limbo or extinction in the goal-trackers, reflecting the increased sensitivity of goal-trackers to contextual cues (including interoceptive, temporal cues) that distinguish the retrieval session from previous learning.

Individual Differences in the Effects of Stress on Extinction

The discrepancies within and between studies of "retrieval-extinction" could potentially be explained by different individual stress levels during either the reconsolidation or the extinction session(s), whether stress is induced through re-exposure to an aversive CS or by frustration by the omission of an appetitive drug reward (e.g., Ginsburg and Lamb, 2018). The effect of stress is usually to impair reconsolidation, as has been reviewed previously (Akirav and Maroun, 2013), so here we focus on the effects of stress on extinction.

The relationship between stress and extinction is complicated, depending critically upon the degree and timing of stress relative to extinction learning and retrieval. Mimicking stress through the administration of low doses of exogenous glucocorticoids enhances, whilst high doses impair, consolidation (Roozendaal, 2003). This depends upon the activation of glucocorticoid receptors in the amygdala, which modulates both the acquisition and consolidation of fear extinction (Yang et al., 2006) in an NMDA receptor-dependent manner (Yang et al., 2007). These dose effects of glucocorticoids depend critically on the receptors activated, with glucocorticoid receptors and mineralocorticoid receptors having differential roles in contextual fear extinction (Ninomiya et al., 2010; Blundell et al., 2011).

Timing of stress relative to extinction learning or retrieval determines whether stress enhances or impairs the behavioral expression of the extinction memory, as articulated in the Stress Timing affects Relapse (STaR) model (Meir Drexler et al., 2019). This model proposes that stress or glucocorticoid administration prior to extinction learning increases consolidation of the extinction memory such that it is less context-specific

(de Quervain et al., 2011), and that post-extinction stress or glucocorticoid administration also enhances its consolidation, but in a context-dependent manner. By contrast, stress or glucocorticoid administration immediately before an extinction retrieval test impairs extinction retrieval, leading to increased fear. However, though the STaR model (Meir Drexler et al., 2019) is well supported by evidence from human studies of contextual fear, the evidence from discrete fear learning (summarized in **Table 1**) is not always consistent with stress enhancing extinction consolidation. The studies presented here show generally that stress, either behaviorally induced or by corticosterone administration, has a neutral or even detrimental effect on the distinct phases of extinction and retrieval. However, in the acquisition or consolidation of extinction in contextual fear, a few studies show enhancing potential. Importantly, for the extinction of maladaptive appetitive drug associations, no studies indicate enhancing the therapeutic potential of stress. The contrast of stress effects between different types of memory likely reflect the different effects of stress hormones in the hippocampus, which is required for contextual fear learning, and the amygdala, required for both contextual and discrete fear learning (McEwen et al., 2016).

To our best knowledge, the effects of stress have not been systemically investigated in the context of retrieval-extinction. Based on the STaR model (Meir Drexler et al., 2019) it may be possible to optimize retrieval-extinction using well-timed glucocorticoid administration. However, based on **Table 1**, we would only expect this to work for contextual fear extinction, and to have a limited or even detrimental effect for appetitive memories, regardless of whether retrieval-extinction is mediated by an extinction or reconsolidation mechanism. Importantly, differences in stress state would be predicted to affect the acquisition, consolidation, and retrieval of extinction, thus potentially explaining the large variation between retrieval-extinction studies.

OPTIMIZING RETRIEVAL-EXTINCTION FOR THE DISRUPTION OF DRUG MEMORIES

Considering the influence of these individual differences on retrieval-extinction, how might the procedure be individually optimized?

Optimizing Memory Reactivation

Reconsolidation deficits are highly selective to the reactivated memory (Dębiec et al., 2006; Doyère et al., 2007), which could limit the efficacy of reactivation based on the presentation of CSs. Furthermore, individual differences exist in attention and engagement with CSs (Meyer et al., 2012), which could account for differences in the efficacy of retrieval-extinction, such as those seen with appetitive memories (Olshavsky et al., 2014).

US presentation can also be used to reactivate memories. It was first shown in studies of fear memory that unsignalled re-exposure to footshock could destabilize the fear memory and make it susceptible to disruption with protein synthesis inhibition (Dębiec et al., 2010). Similarly, re-exposure to the US induced susceptibility to retrieval-extinction, and led to reductions in fear to all CSs associated with the US, rather than individual CS-US associations (Liu et al., 2014). US-based reactivation has also been shown to extensively reduce reactivation-induced CREB expression, compared to CS-based reactivation (Huang et al., 2017).

A similar US-based reactivation approach has been used in studies of drug memory reconsolidation. In rats extensively trained to self-administer cocaine, reactivation of the drug memory through experimenter-administered injections of cocaine, followed by drug memory extinction, reduced reinstatement, spontaneous recovery and renewal (Luo et al., 2015). Importantly, the retrieval-extinction effect was also observed when instead of cocaine, the stimulant methylphenidate was administered. As noted by the authors (Luo et al., 2015), this overcomes the difficult ethical issue of administering an illegal drug to a patient who is trying to maintain abstinence. However, these findings do raise questions regarding the mechanism by which US-based reactivation occurs. It may reactivate a "US engram" in the brain, propagating destabilization along the network of associated CSs. Alternatively, US exposure could lead to experiencing interoceptive cues that reactivate the drug memory which may account for the increased efficacy of US-based reactivation procedures. A specific test of the latter hypothesis would be to determine whether drug isoforms that do not cross the blood-brain-barrier—and so could only produce central effects through the detection of peripheral interoceptive cues—would be as effective in reactivating the memory as drugs that do cross the blood-brain-barrier. To our knowledge, this remains to be investigated.

Optimizing Extinction

The fact that there are no standardized procedures to destabilize memory makes the interpretation of studies failing to replicate retrieval-extinction difficult. Although memory destabilization—at least for pavlovian memories—is thought to depend on inducing a "violation of expectations" or "prediction error" (Pedreira et al., 2004; Sevenster et al., 2013, 2014), it is widely accepted that the relationship between prediction error and memory destabilization is complex. As noted above, re-exposure to a single previously fear-associated CS will induce memory reconsolidation, but greater re-exposure (with more prediction error) leaves the original memory intact and instead promotes the consolidation of an extinction memory after a "limbo" period (Lee et al., 2006; Merlo et al., 2014, 2018). Therefore, the relationship between prediction error and memory destabilization is not linear, leading some to hypothesize that destabilization may instead be driven by the attribution of an unexpected experience to the same underlying "latent cause" as has been experienced in the original consolidation of the memory (Dunsmoor et al., 2015; Gershman et al., 2017). The difficulty in empirically determining whether an experience is attributed to the same or different latent cause—which could also differ between individuals—leads us to hypothesize that the failures to replicate the retrieval-extinction effect may be due to engaging the facilitation of extinction, rather than destabilization of the original memory.

TABLE 1 | Modulation of different phases of extinction by behavioral stress, glucocorticoid administration, and secondary interventions.

Type of memory	Mnemonic phase	Effect	Method of stress induction	References	Secondary intervention	Total effect	References
CS-US fear conditioning and extinction	Acquisition or consolidation of extinction	Impaired	Behavioral	Izquierdo et al. (2006), Akirav and Maroun (2007), Yamamoto et al. (2008)[2], Akirav et al. (2009)[1], Farrell et al. (2010), Knox et al. (2012b), Maroun et al. (2013), Keller et al. (2015) and Sawamura et al. (2016)	Dexamethasone Metyrapone Infralimbic lesion Diazepam D-cycloserine D-cycloserine	Rescued Exacerbated Impaired Rescued No effect Rescued	Sawamura et al. (2016) Keller et al. (2015) Farrell et al. (2010) Akirav and Maroun (2007) Akirav et al. (2009)[1] Yamamoto et al. (2008)[2]
		No effect	Behavioral	Miracle et al. (2006); Garcia et al. (2008); Wilber et al. (2011) and Knox et al. (2012a)			
			CORT	Wang et al. (2014)			
	Retrieval of extinction	Impaired	Behavioral	Miracle et al. (2006), Garcia et al. (2008), Farrell et al. (2010) Wilber et al. (2011), Knox et al. (2012a), Deschaux et al. (2013), Maroun et al. (2013) and Xing et al. (2014)	Fluoxetine Infralimbic lesion	Rescued Rescued	Deschaux et al. (2013)[3] Farrell et al. (2010)
		No effect	CORT	Wang et al. (2014)			
Context-fear conditioning and extinction	Acquisition or consolidation of extinction	Impaired	Behavioral	Akirav and Maroun (2007), Yamamoto et al. (2008)[2] and Akirav et al. (2009)	D-cycloserine Diazepam	Rescued Rescued	Yamamoto et al. (2008)[2] and Akirav et al. (2009) Akirav and Maroun (2007)
			CORT	Gourley et al. (2009)	Mifepristone	Mimicked	Gourley et al. (2009)
		No effect	Behavioral	Knox et al. (2012a)			
		Enhanced	Behavioral	Kirby et al. (2013)			
			CORT	Cai et al. (2006), Abrari et al. (2008) and Blundell et al. (2011)			

(Continued)

TABLE 1 | Continued

Type of memory	Mnemonic phase	Effect	Method of stress induction	References	Secondary intervention	Total effect	References
Instrumental conditioning for drug reward and cued extinction	Acquisition or consolidation of extinction	No effect	Behavioral	Eagle et al. (2015) and Manvich et al. (2016)			
	Retrieval of extinction	No effect	Behavioral	Eagle et al. (2015)[4]			
		Enhanced reinstatement	Behavioral	Erb et al. (1998)[5], Graf et al. (2013)[6] and Manvich et al. (2016)[4]	ADX ADX + CORT	Rescued Reinstated	Erb et al. (1998)[5] and Graf et al. (2013)[6] Erb et al. (1998)[5]
			CORT	Graf et al. (2013)[6]	Mifepristone	No effect	Graf et al. (2013)

Abbreviations: ADX, adrenalectomized; CORT, corticosterone. Note that this table includes only rodent studies. Other exclusions consist of: studies that applied the stress before conditioning when this had a significant effect on conditioning, e.g., studies using early life stress, or when they did not provide any conditioning data as this renders it impossible to conclude on the effects on extinction alone. For the effects on retrieval of extinction as determined by performance during reinstatement, only studies were included which targeted the stress specifically to the extinction session, and not to the reinstatement session. Also, articles that did not provide controls for stress/CORT induction were excluded. No articles on retrieval of extinction within contextual fear, nor articles which used CORT to induce stress in instrumental conditioning were found after these exclusions. Specific excluded articles, as it is beyond the scope of this table: effect of strain in mice (Brinks et al., 2009); diurnal changes in corticosterone (Woodruff et al., 2015); gender (Baran et al., 2009); exposure to novel context (Liu et al., 2015); conditioning using conditioned place preference (Leão et al., 2009; Taubenfeld et al., 2009; Karimi et al., 2014; Meng et al., 2014; Ebrahimian et al., 2016; Taslimi et al., 2018). The severity of behavioral stress induction, nor CORT dose showed no clear effect and is thus for clarity not included.

[1]This study was the only one in the CS-US category where conditioned taste aversion was used to establish the CS-US association. All others used classical cue-fear conditioning, where a fear-related US, typically an electrical shock, is paired to a CS, typically a tone.

[2]This study used classical cue-fear conditioning but used only the context for extinction.

[3]The fluoxetine was given for 21 days after extinction. The behavioral stress consisted of elevated platform prior to retrieval. The decrease in freezing could also be interpreted as an enhancing effect of fluoxetine on the consolidation of extinction, rather than retrieval, or could be ascribed to the general anxiolytic effects of fluoxetine.

[4]Reinstatement was not cocaine-primed.

[5]Reinstatement was cocaine-primed.

[6]This effect was only observed when animals received a priming dose of cocaine vs. saline prior to the reinstatement test.

One major challenge in distinguishing between these two accounts of retrieval-extinction is the reliance on a single behavioral readout. We have previously argued (Cahill and Milton, 2019) that corroborating molecular evidence would be useful in this respect.

Certainly, our own data are more consistent with a "facilitation of extinction" account of retrieval-extinction. We observed (Cahill et al., 2019) the retrieval-extinction effect for fear memories despite behavioral manipulations of prediction error and selective pharmacological blockade of the D_1-subtype of dopamine receptor, which is required for memory destabilization (Merlo et al., 2015). Furthermore, considering studies showing facilitation of extinction following exposure to a novel environment (de Carvalho Myskiw et al., 2013; Liu et al., 2015), at least some of the published putative retrieval-extinction effects could be due to the facilitation of learning by a proximal behavioral experience. This phenomenon, in which novelty exposure facilitates subsequent learning, is known as "behavioral tagging" (Moncada and Viola, 2007; Moncada et al., 2011). One test of the "facilitation of extinction" account of retrieval-extinction would be to expose animals to a novel context prior to extinction training, rather than a memory reactivation session; if the "retrieval-extinction" effect persists despite a lack of memory reactivation, this would cast doubt on the reconsolidation-based account of the phenomenon.

Determining whether retrieval-extinction depends upon reconsolidation or extinction mechanisms is of great potential importance in optimizing this therapeutic strategy. For example, if dependent primarily on extinction mechanisms, then it may be possible to facilitate retrieval-extinction further with the administration of drugs such as the glutamate receptor partial agonist D-cycloserine (Das and Kamboj, 2012). However, the use of drugs to enhance retrieval–extinction may reduce the non-pharmacological appeal of the intervention. Alternatively, if individual differences determine whether reconsolidation-update or extinction mechanisms are engaged by the retrieval-extinction procedure, then identification of these differences—for example, by classifying individuals as sign-trackers or goal-trackers, or determining stress reactivity—could be used to optimize the retrieval-extinction procedure by targeting the dominant mnemonic process in each individual.

CONCLUSIONS

Although the mechanisms underlying retrieval-extinction remain unclear, and retrieval-extinction has not been universally replicated, this process has great potential for the treatment of drug addiction. Understanding the contribution of individual differences to the boundary conditions underlying reconsolidation, limbo, and extinction, and how these interact with factors such as the attribution of incentive value to appetitive stimuli and stress, may provide insight into the apparent inconsistencies in the literature, and guide future optimization of retrieval-extinction for clinical use.

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Default Mode Network Efficiency Is Correlated With Deficits in Inhibition in Adolescents With Inhalant Use Disorder

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It is well established that alterations in cognitive function and damage to brain structures are often found in adolescents who have substance use disorder (SUD). However, deficits in executive cognitive functioning in adolescents related to the vulnerability and consumption of such substances are not well known. In this study, we use graph theoretic analysis to compare the network efficiency in the resting state for three networks—default mode network (DMN), salience network (SN) and fronto-parietal network (FPN)—between inhalant-consuming adolescents and a control group (12 to 17 years old). We analyzed whether the efficiency of these functional networks was related to working memory, mental flexibility, inhibition of response, and sequential planning. We found that, when compared to the control group, inhalant-consuming adolescents presented with important deficits in communication among brain regions that comprise the DMN, SN, and FPN networks. DMN is the most affected network by inhalant abuse during adolescence. The mediation analyses suggested that the relationship between inhalant abuse and inhibitory control and sequential planning was partly mediated by DMN efficiency.

Keywords: executive-function, resting-state functional magnetic resonance imaging, substance use disorder, functional connectivity, adolescents

INTRODUCTION

The consumption of illicit drugs by young people has increased in recent years, becoming a public health issue in several countries (1). Numerous clinical and epidemiologic studies [*e.g.*, (2–4)] have indicated that cognitive and emotional effects are more severe and persistent if their use begins during childhood or adolescence rather than if it happens in adulthood. Thus, research on addiction in the context of a developing brain is currently of utmost importance. Prevention and early intervention programs are needed to face the growing indexes of addictive behavior among the young population.

A recent study showed that many individuals with addiction present neuropsychological deficits across a range of functions: inhibition, compulsivity, action selection, expectancy, and reward learning and valuation. These functions could be linked with vulnerability to and the treatment and diagnosis of substance use disorders (SUDs) (5).

Several clinical investigations in adults, including those in subjects with chronic job-related industrial solvent exposure, have shown that chronic use of inhalants is associated with significant brain abnormalities. Among the most commonly reported effects are leukoencephalopathy (6, 7) and atrophy of both the cerebellum and corpus callosum (8). It is worth mentioning that inhalants are commonly one of the earliest drugs used by adolescents, and a few authors have pointed out that such encephalopathies can be observed in young consumers (9, 10).

Neuropsychological research has contributed to relating the brain damage associated with inhalant consumption with alterations in the cognitive function of adolescents. Thus, it is interesting to note that Takagi and collaborators [see (11, 12)] have reported that inhalant users show, when compared with consumers of other drugs and controls, the worst performance in tasks that measure attention, memory, cognitive control, and processing speed. In line with this, (13, 14) have also described more significant deficits in working memory, processing speed, concept formation, and mental flexibility in adolescents that have used several drugs, including inhalants, compared to adolescents with no inhalants in their consumption history.

Complex cognitive processes, as previously mentioned, involve the integration of information through functional communication between anatomically distributed brain regions (15). Recently, a number of neurocognitive disorders have been studied using functional magnetic resonance imaging (fMRI) from the perspective of complex disorders of functional connectivity. Those disorders include autism (16, 17), attention-deficit disorder (18, 19), and dementia (20, 21). In addition, functional connectivity alterations have also been revealed in regard to some aspects of addictive behavior (22–25). We know that inhalants affect anatomical connectivity. It is yet unknown, however, what functional connectivity networks are affected, although we know that functional connectivity is constrained by anatomical structural connections (26). Structural connections are affected in inhalant users (10), particularly during adolescence, when a large-scale refinement of the functional networks takes place (27).

In this case–control study, we explored the performance of complex cognitive functions in inhalant-consuming adolescents and whether intranetwork communication efficiency is acting as a mediator of the effects of consumption on executive cognitive functions. We decided to examine executive functions to identify deficits with SUD as in previous studies. Based on the results of previous inhalant abuse studies, in which damage to structural connections and cognitive deficits were linked to inhalant use, we hypothesized that inhalant abuse during adolescence which is a maturational period of executive function (EF) and the brain, affects functional brain networks' topologies that would lead to cognitive deficits. We focused on three functional networks that have received special attention in the investigation of the neural correlates of executive function development. Recently, studies have shown that modular segregation of the fronto-parietal network (FPN) and global efficiency mediated improvements in executive functions with age in structural networks and further that reciprocal activation of the FPN and deactivation of the default mode network (DMN) as well as the flexible transition between these networks are correlated to executive function across adolescence (28–30). Recent evidence suggests the switch between the DMN and FPN is modulated by the anterior insula which is an important region in the salience network (SN) (31). Graph theoretic analysis was applied to the analysis of functional connectivity. This method is based on the conception of the brain as a complex and efficient "small world" network, which can be qualitatively described through a variety of measurements (32–34).

METHODS

Participants

A total of sixty subjects participated in this study: 30 inhalant-consuming adolescents (IC) and 30 healthy controls (HC), with ages ranging from 12 to 17 years. Three HC were excluded from the study after the fMRI due to image processing difficulties related to excessive movement of the subject during image acquisition. Age (IC: 15.1 ± 1.3 years, HC: 15.0 ± 1.4 years (= 0.71, Student's t-test) and sex (IC: 24 men/6 women, HC: 16 men/11 women; p = 0.15, chi-square test) showed no significant differences between the two groups.

Inhalant use was characterized by structured interviews. The adolescent consumers had a diagnosis of mild or moderate SUD according to the Diagnostic and Statistical Manual for Mental Disorders (DSM)-V. All of the IC had been systematic inhalant users (more than three times a week) for at least a year. Most of them were polydrug consumers, with a preferential use of inhalants (see **Table 1**). Eighty percent of the consumers reported the use of solvents (paint thinners, acetone, and

TABLE 1 | Basic demographic and substance use histories for the inhalant and control groups.

		Inhalant group (IC) n = 30	Control group (HC) n = 27
Age	Mean (SD)	15.1 (1.3)	15.0 (1.4)
Years of education	Mean (SD)	8.1 (1.1)	9.9 (1.4)
Sex	% male	80	59
Age of first self-reported inhalant use (in years)	Mean (SD)	13.03 (1.6)	N/A
Duration of regular inhalant use (in months)	Mean (minimum–maximum)	24 (12–60)	N/A
Duration of regular use (months):	Mean		N/A
Tobacco	(minimum–	20 (1–72)	
Alcohol	maximum)	18 (4–60)	
Cannabis		16 (6–48)	
Cocaine		4 (1–36)	

gasoline), and 20% also used aerosols and adhesives (paints, deodorants, and glues). The subjects were in the first week of remission under pharmacological treatment to minimize the effect of these compounds on cognitive performance and neurovascular coupling in the brain.

None of the subjects had a history of premature birth, birth weight lower than 2.5 kg, or had suffered a neurologic disease, according to a neurodevelopment interview conducted with the parents. Additional exclusion criteria were the following: a) I.Q. lower than 70 (intellectual disability); b) lack of a family environment to avoid family abandonment and homeless situations; c) sensorial and motor disabilities; and d) any condition that prevented the use of a magnetic resonator, such as metallic implants, pregnancy or claustrophobia.

Procedure

The adolescents comprising the IC were engaged in the study through the Centers for Youth Integration, while the HC came from high and senior high schools, which were all in Mexico City. All IC were recruited by professionals with experience on addictive disorders through a complete assessment based on the DSM-V, taking into account the study's objectives and eligibility criteria. All subjects underwent a consent process in the presence of a family member, where they received detailed information regarding confidentiality, risks and benefits, psychometric tests, length of the study, *etc*. For the IC, relevant information regarding the use of addictive substances was also acquired in the same session by a structured interview. On this first encounter, either the Wechsler Intelligence Scale for Children (35) or the Wechsler Adult Intelligence Scale (36) was applied depending on the age of the subject. Family members were also interviewed in the same session to gain insight regarding their neurodevelopmental characteristics.

Both neuropsychological tests and resting-state fMRI acquisition were applied during a second session to all volunteers who fulfilled the eligibility criteria. All the procedures of the protocol were approved by both the Scientific Research Committee of the Centers for Youth Integration and the Ethics Committee of the Children's Hospital of Mexico "Federico Gomez".

Cognitive Functioning Assessment

Four complex cognitive functions, known as executive functions due to their role in the generation, regulation, execution, and readjustment of behaviors, were studied to obtain information on the subject's short-, medium- and long-term goals (37, 38), as these measures can be potentially affected in inhalant users (13, 39). We used the Neuropsychological Battery of Executive Functions and Frontal Lobes (BANFE), which consists of a number of worldwide-validated cognitive tests. The Mexican standardization sample was 500 children and adolescents who were divided by age into five groups; internal consistency reliability had a mean of.85 for test (40), and since age and education have been shown to have an important influence on executive performance, individual scores were transformed to standardized scores following norms for the Mexican population that take both characteristics into account.

- **Verbal working memory:** This function, which involves the ability to maintain and manipulate verbal information, was assessed by using the Working Memory Index from the Wechsler intelligence scales.
- **Mental flexibility:** This function was assessed with the Wisconsin Card Sorting Test (WCST), which allows an assessment of the mental flexibility of an individual through his/her capacity to change a classification criterion when it is inadequate. In the test, the subject must take one card from a deck—the card may contain figures with different colors, geometrical shapes and numbers—and to choose were to place it according to a changing classification criterion. For this study, the total number of correct answers was used (41).
- **Cognitive control:** The capacity to inhibit an automatic response was assessed by the application of the Stroop test. The test measures the inhibition abilities and the resistance to interference related to external stimuli through the capacity of the individual to inhibit the highly automated habit of reading every time a word is presented to him/her (42). Volunteers are presented with printed words, ordered in columns and are instructed to describe the color of the word and not read the word when they were underlined. The number of errors according to the given instructions was registered and used as the performance of the subject in this test.
- **Action planning and sequencing:** This cognitive function was assessed with the Tower of Hanoi test (TOH), which requires sequential planning of upcoming intermediate steps to achieve a final goal. The test consists of moving discs from an initial state to a finished state in the smallest number of steps as possible while following a specific set of rules: a) only one disc can be moved at the time; b) the discs can only be moved to a different plug and c) a disc cannot be placed on top of another disc that is smaller than itself (43). There are two levels of complexity, with three discs at the first level and four discs at the second level. The total number of movements in the task with the maximum level of complexity was considered the performance measure.

Resting-State fMRI Data Acquisition and Preprocessing

The fMRI data were acquired while the subjects remained silent and stared at a cross mark on a Siemens Trio 3.0 Tesla MRI scanner at the National Institute of Neurology and Neurobiology "Manuel Velasco Suárez" by a physician specialized in radiology and brain imaging. All the subjects underwent a 6-minute echo-planar sequence in the resting state, using a multiband sequence with repetition time (TR)/echo time (TE)/precession angle = 720 ms/29 ms/44°; 48 slices; 500 volumes; acceleration factor 8; 82 × 82 matrixes; 268 mm field of view (FOV); and a 3 × 3 × 3 mm3 voxel size. In addition, an anatomical reference image was acquired with contrast T1 and a 3DMPRAGE sequence with TR/TE = 2200 ms/2.45 ms and a voxel size of 1 × 1 × 1. Three scans were excluded due to high in-scanner motion (defined as mean framewise displacement (FD) > 0.3 mm or maximum FD > 1.3 mm).

The anatomical data preprocessing of the T1-weighted (T1w) images was corrected for intensity nonuniformity (INU) with

'N4BiasFieldCorrection' distributed with ANTs 2.2.0. The T1w reference was then skull-stripped with a "Nipype" implementation of the 'antsBrainExtraction.sh' workflow (from ANTs) using OASIS30ANTs as the target template. Brain tissue segmentation into cerebrospinal fluid (CSF), white matter (WM), and gray matter (GM) was performed on the brain-extracted T1w image using 'fast' [FSL 5.0.9]. Volume-based spatial normalization to one standard space (MNI152NLin2009cAsym) was performed through nonlinear registration with 'antsRegistration' (ANTs 2.2.0) using brain versions of both the T1w reference and the T1w template. The following template was selected for spatial normalization: "ICBM 152 Nonlinear Asymmetrical template version 2009c" [MNI152NLin2009cAsym]. The blood oxygen level-dependent (BOLD) reference was then coregistered to the T1w reference using 'flirt' [FSL 5.0.9] with the boundary-based registration [@bbr] cost-function. Coregistration was configured with nine degrees of freedom to account for distortions remaining in the BOLD reference. Head-motion parameters with respect to the BOLD reference (transformation matrixes and six corresponding rotation and translation parameters) were estimated before any spatiotemporal filtering using 'mcflirt' [FSL 5.0.9]. The BOLD time series (including slice-timing correction when applied) were resampled onto their original, native space by applying the transforms to correct for head motion. These resampled BOLD time series are referred to as "preprocessed BOLD in original space" or just "preprocessed BOLD". The BOLD time series were resampled into standard space, generating a "preprocessed BOLD run in ['MNI152NLin2009cAsym'] space". First, a reference volume and its skull-stripped version were generated using the custom methodology "fMRIPrep". Several confounding time series were calculated based on the "preprocessed BOLD": framewise displacement (FD), spatial deviation of successive difference images (DVARS) and three region-wise global signals. FD and DVARS were calculated for each functional run, both using their implementations in "Nipype". The three global signals were extracted within the CSF, the WM, and the whole-brain masks. Additionally, a set of physiological regressors were extracted to allow for component-based noise correction ["CompCor"]. Principal components were estimated after high-pass filtering the "preprocessed BOLD" time series (using a discrete cosine filter with 128 s cut-off) for the two "CompCor" variants: temporal (tCompCor) and anatomical (aCompCor). tCompCor components were then calculated from the top 5% variable voxels within a mask covering the subcortical regions. This subcortical mask was obtained by heavily eroding the brain mask, which ensures that it did not include cortical GM regions. For aCompCor, the components were calculated within the intersection of the aforementioned mask and the union of the CSF and WM masks calculated in T1w space, after their projection to the native space (using the inverse BOLD-to-T1w transformation). The components were also separately calculated within the WM and CSF masks. For each CompCor decomposition, the "k" components with the largest singular values were retained, such that the retained components' time series were sufficient to explain 50% of the variance across the nuisance mask (CSF, WM, combined, or temporal). The remaining components were dropped from consideration. Despiking was performed with AFNI's 3DDESPIKE utility and the 36 parameters from the global confound regression.

Atlas Selection

Each subject's cortex was parcellated using the Power Functional Atlas and comprised 264 functional regions Regional BOLD time series were estimated by averaging time series over all voxels in each parcel Power (2011).

Time Series Extraction and Graph Calculation

Time series of the fMRI data were decomposed on six levels using the maximum overlap discrete wavelet transform (MODWT), which allows signal decomposition at different resolution levels using the Daubechies least asymmetric wavelet filter (8). The decomposition was carried out with the R package *brainwaver* 1.6. Since the wavelet decomposition depends on the repetition time (720 ms), the maximum frequency obtained was 0.69 Hz. The relevant information was on decomposition level 4, corresponding to a 0.043–0.087 Hz frequency range (44).

From the signals obtained at decomposition level 4, the correlations (Pearson) between the time series of pairs of ROIs were calculated, and a correlation matrix per subject was built. The passing from a correlation matrix to an adjacent or binary matrix—to study connectivity patterns using the network theory—was achieved by choosing a threshold that allows establishing whether a particular connection was significant or not. The connection density used for this purpose in the present study was 5% (45). The procedure employed a connectivity conservation criterion by fixating the number of edges and chose a threshold that kept that number and varied for every matrix. Individual network measures were determined using the R *igraph* 1.0.1 package (46). Global efficiency was calculated to assess the functional organization of the networks. This measure quantifies the efficiency of information transmission between any of the nodes by multiple and parallel paths (47, 48). Global efficiency is the average of the efficiencies over all pairs of vertices and is denoted as follows:

$$E = \frac{1}{n}\sum_{i\in N} E_i = \frac{1}{n}\sum_{i\in N}\frac{\sum_{j\in N, j\neq i} d_{ij}^{-1}}{n-1} \quad (1)$$

(49, 50) the distance d_{ij} between any two vertices i and j in a graph is the number of edges in shortest path between i and j. An analog measure of global efficiency was used for the networks of interest (default mode network, salience network, and fronto-parietal network) where instead of N a subset of nodes N_s was used, where these nodes belong only to the network of interest, and n was replaced by n_s, which is the number of nodes in the network of interest. Making these substitutions in equation (1), we define the resulting E, which we call E_s, to be the network efficiency, which only refers to information exchange between these subnetworks and not between these subnetworks and the whole brain.

Data were processed with the *IBM SPSS* 24.0 package for Windows. Descriptive statistics were used for demographically characterizing the sample and for describing the characteristics of the use of addictive substances. Normality and homoscedasticity were verified. The parametric Student's t-test for independent samples was used for comparing the groups regarding the functional connectivity efficiency. The comparative analysis of cognitive functioning was achieved through the nonparametric Kruskal–Wallis test for independent test groups, owing to the noncompliance the assumption of normality for these variables and False Discovery Rate (FDR) correction for multiple comparisons to obtain adjusted *p*-values less than 0.05.

Once the descriptive analysis was performed and the intergroup differences on cognitive measures and on the connectivity of the three networks were determined, the second step was a mediation analysis to estimate the direct effects of the use of inhalants on the cognitive functions, as well as the mediation effects of the three functional networks between use and cognition. We regressed out the effects of nuisance covariates (sex, age, and other substance use) on dependent (Y) and mediating (M) variables. The residuals were then used in our mediation analysis. The significance of the indirect effect was evaluated using bootstrapped confidence intervals within the R package *lavaan*.

RESULTS

Comparison Between Inhalant-Consuming and Nonconsuming Adolescents on Cognitive Performance

Analysis of the difference in means was performed using the Kruskal-Wallis test for independent samples since the condition of normality was not fulfilled. **Table 2** presents the means and standard deviation values for both groups. The cognitive performance of the IC group with respect to the HC group was assessed using these data while considering that due to the normalization of the scores, higher scores mean better executive performance.

The performance of inhalant consumers was significantly lower than that of the controls in all cognitive tests. The IC presented a lower level of success on the WCST; that is, they generated fewer classification criteria. On the Stroop test, the consumers made more mistakes when they were asked to state the color of the word instead of reading it. They also needed more steps in the TOH test to solve the exercise, thus evidencing less efficiency in action planning and sequencing. Finally, the IC showed a lower index of verbal working memory, denoting a lower capacity to mentally maintain and manipulate verbal information.

TABLE 2 | Executive Functioning. Descriptive statistics and differences between groups (Kruskal–Wallis test for independent samples).

Test	HC Mean (SD)	IC Mean (SD)
Working Memory Index	102.4 (13.2)	82.40 (8.2)*
Wisconsin Card	12.2 (2.9)	7.1 (3.1)*
Stroop	8.4 (4.9)	6.9 (4.3)*
Tower of Hanoi	9.8 (4.1)	7.2 (4.4)*

*Significant differences between groups (p < 0.01) are indicated with *.*

Comparison of the Global Efficiency and Network Efficiency: Default Mode Network, Salience Network, and Fronto-Parietal Network

The network efficiency index showed significant levels of difference in means $p < 0.05_{FDR}$ for all three networks between the inhalant and nonconsumer adolescents. The DMN, however, showed the greatest difference between the two groups. The network efficiency scores, as shown in **Figure 1**, were significantly lower in inhalant-consuming adolescents than nonconsuming individuals. The global efficiency in the HC group was significantly greater than for the IC group (HC: 0.44 ± 0.21; IC: 0.26 ± 0.03 $p = 0.0001$).

Mediation of the Relationship Between Inhalant Use and Cognitive Functioning by Network Efficiency

A path analysis was applied to explore whether the lower cognitive efficiency observed in IC subjects compared to noninhalant consumers may be related to differences in their functional brain communication. For that purpose, *lavaan* package was used. The variables of the model and their relationship were initially set in accordance with the working hypothesis (see **Figure 2**). The feasibility of using such a model was confirmed by the degrees of freedom ((Df > 0) = 10) involved in the comparisons, and the parameters were determined using the method of maximum likelihood. The model was adjusted on the basis of the modification indexes by eliminating the connections with nonsignificant coefficients ($p > 0.05$), and an adequate adjustment was achieved (X2 = 26.7, $p = 0.11$; CFI = 0.96; RMSEA = 0.05). The final adjusted model (**Figure 3**) differed from the initial one with the elimination of the assumption of a relationship between inhalant usage and the Stroop and TOH tests, as well as the elimination of the direct connections between the efficiency of the FPN and SN and the cognitive functions.

By considering the sign and magnitude of the estimated standardized parameters, the results showed that the use of inhalants had a negative effect on the efficiency of the functional connectivity in all three networks. Thus, the presence of inhalant use allowed for the prediction of a lower efficiency in communication between the brain regions that comprise these three networks. The strongest negative effect was observed towards the DMN, where the use of inhalants explained 30% of the variance.

Inhalant usage also has a direct negative effect on the working memory index and on the performance in the card sorting test, with coefficients of −0.53 and −0.58, respectively. The use of inhalants allowed the prediction of a lower capacity in the verbal

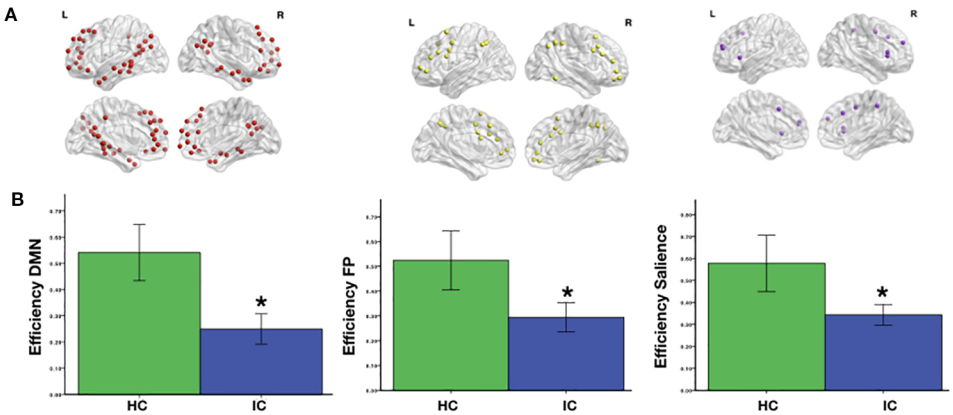

FIGURE 1 | (A) Brain regions comprising the default mode (DMN), salience (SN), and fronto-parietal (FPN) networks according to the spatial coordinates from Power's Functional Atlas (2011). **(B)** Significant differences (Student's t-test) between the two groups ($*_{PFDR} < 0.05$) are indicated.

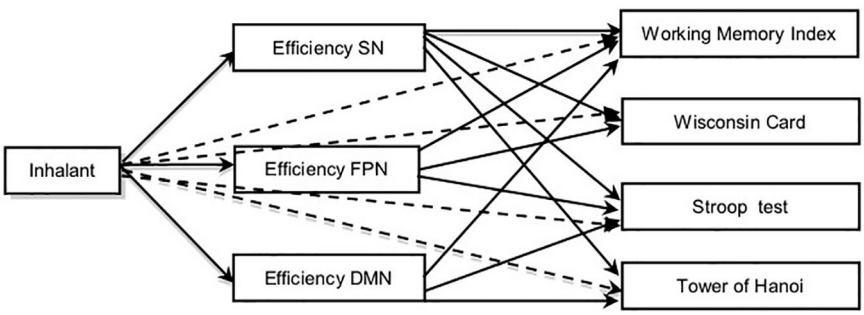

FIGURE 2 | Graphic representation of a model on the effect of inhalants on cognitive functions. Discontinuous lines: direct effects of inhalant use.

working memory task and a lower performance in the card sorting test, which was used to assess the capacity of mental flexibility of an individual. No direct effects were observed between inhalant abuse and the Stroop and TOH tests.

When analyzing the role of connectivity efficiency as a mediator in the effects of inhalant use on cognitive performance, in opposition to the initial hypothesis, the analyzed data showed that the differences in cognitive performance were not mediated by how well connected the SN and FPN were. In contrast, the efficiency of the DMN did show a significant mediating effect, as this indirect path was the only explanation of the differences in performance in the Stroop and TOH tests between inhalant consumers and nonconsumers.

DISCUSSION

The present study compared complex cognitive functions in inhalant-consuming and nonconsuming adolescents. Both groups of individuals were also compared regarding the capacity of information exchange between nodes or regions of three functional networks DMN, SN, and FPN using the network efficiency and global efficiency obtained from the graph theoretical analysis. Finally, we explored whether network communication efficiency acted as a mediator of the effect of inhalant use on cognitive performance. The main findings were (1) a lower degree of working memory, mental flexibility, inhibitory control, and sequential planning in the IC group, (2) a lower functional connectivity efficiency for all three networks in the IC group, and (3) the occurrence of a mediator effect of the DMN between consumption and performance on the neuropsychological tests that explored inhibitory control and sequential planning functions.

For three networks, The IC group showed a lower network efficiency index when compared to the control group, thus evidencing a different functional organization characterized by a diminished capacity of information propagation and integration across the these networks. The executive function regulatory processes that monitor goal-directed cognitive operations are crucial for development to adulthood. The neural mechanisms underlying the normative maturation of

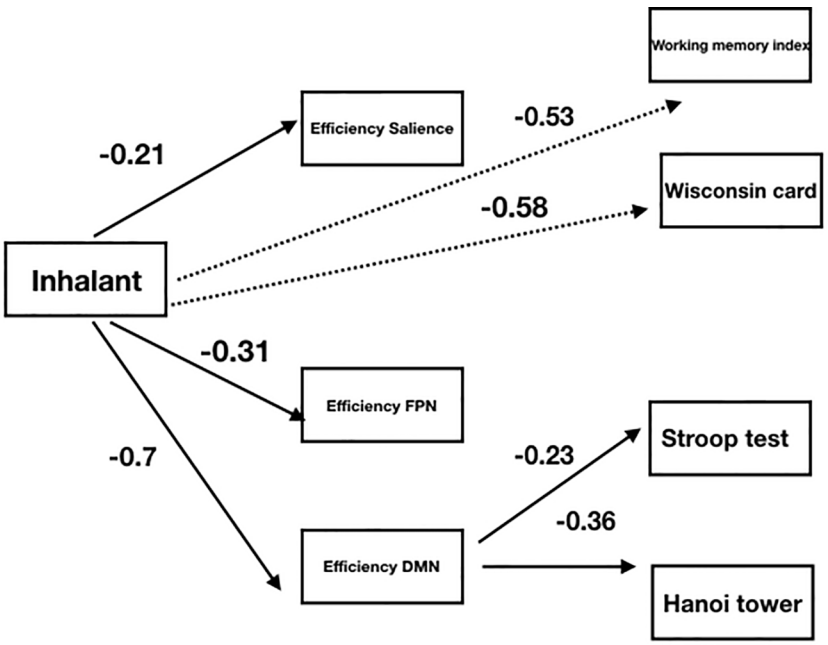

FIGURE 3 | Graphic representation of an adjusted model using standardized coefficients($p < 0.05$) and explained variance (R^2) on the effect of inhalants on cognitive functions. Discontinuous lines: direct effects of inhalant use.

executive functioning are related with segregation of networks modules and increase within-module connectivity mainly on DMN and FPN and further reciprocal activation FPN and DMN deactivation as well the flexible transition between this networks (28–30). Our study focused on four executive functions: verbal working memory, mental flexibility, inhibitory control and sequential planning. We found a significantly lower performance on tasks that measure each of these functions in inhalant-consuming adolescents than the controls. The cross-sectional design of the research is a limitation for analyzing whether the differences in cognitive functioning in the IC predated the use of drugs.

Recent longitudinal investigations (51, 52) have identified that weak working memory and poor impulse control can act as predictors of the progressive use of drugs in adolescents. Lower working memory capacity has been correlated with increased delay discounting and therefore has an effect on reward evaluation (53–55). Future longitudinal studies, which incorporate the assessment of different executive functions, might identify other cognitive predictors of addiction-related behaviors. Our data confirm the presence of alterations in cognitive functions that are fundamental for choosing and maintaining actions in everyday life (56, 57) in adolescents with inhalant use disorder and the need to include rehabilitation or cognitive stimulation among early intervention strategies.

Our results show that the DMN is the most affected network by inhalant abuse during adolescence. The mediation analyses suggested that the relationship between inhalant abuse and inhibitory control and sequential planning was partly mediated by DMN efficiency. Although this network has been mainly related to self-referential functions, numerous studies (58–60) have identified its indirect yet important contribution in the response to external cognitive demands. The high demands of executive functions require the availability of resources, which is achieved by diminishing the activation of the DMN. A deficit in the efficiency of the DMN in adolescents with inhalant use disorder might affect the induced deactivation of this network and hence compromise their executive functions (28). In favor of this hypothesis, there are recent data regarding the contribution of the DMN to the executive function deficit identified in patients with Alzheimer's disease (59) and attention-deficit/hyperactivity disorder (61).

We must point out that the neuropsychological exploration performed here was focused on only four measures of executive functioning based on previous reports of these measures being affected in inhalant users (14, 39). It is possible that the detected decreased efficiency in the DMN, FPN, and SN may be responsible for other cognitive alterations and mental health problems also described among inhalant-abusing adolescents (62), which were not assessed in our research subjects.

According to the specific cognitive domains that have been linked to each network—the default network in self-referential functions, including autobiographical memory, the SN in identifying the most subjectively relevant stimuli, and the FPN in higher-order cognitive and attention control (63)—we expected to find a mediating effect of the FPN between inhalant abuse and poor performance in inhibitory control and sequential planning tasks. The absence of this effect may be related to the partitioning of the FPN into branches and the

specific contribution of each sub-network in cognitive domains. (64) identified two main branches within the FPN: a dorsal spatial/motor network, which connects regions of the superior parietal lobe and the superior frontal lobe and a ventral non-spatial/motor network, which connects the inferior parietal lobe with the inferior and middle frontal gyri. When the FPN is analyzed based on its different components, we think that the behavior in these tests might be mediated by organizational characteristics of each branch instead of parameters of the overall FPN connectivity.

On the other hand, some authors have described the FPN as a flexible cognitive control center, with the ability to adapt its composition by recruiting diverse regions or networks, depending on the demands of the present task (65). We should also consider that each of the applied tests involves, in addition to the regions associated with the executive functions, other areas underlying different cognitive domains: the Hanoi Tower test involves regions associated with motor and visual/spatial abilities, while the Stroop tests involves regions that are associated with perception and verbal abilities. The nature of the executive demand determines the inclusion of the nodes in the FPN; therefore, the topological measurements may vary as a function of the accommodation of this functional structure. Furthermore, deficits associated with the inherent flexibility of the functional network for adapting its constitution as a response to the particular demands of each of the cognitive tests may be responsible for the differences in the executive behavior observed in the inhalant-consuming adolescents.

Finally, the IC group showed a lower global efficiency, similar results have been recently reported for alcohol-dependent patients. Sjoerds et al. (66) reported that resting brain functions were less efficient with longer alcohol dependence duration, while Wang et al. (67) found that brain networks of adult patients with alcohol use disorder showed decreased global efficiency compared to those of controls. Our results are consistent with previous research and suggest a diminishing in the efficiency of functional connectivity in individuals with SUDs. According to our research, this reduced global network efficiency might be present from an early age. Further recently study show that global efficiency mediated the improvement of executive functions with age in structural networks (29), however we do not have a global score of executive functions.

CONCLUSIONS

This study explored novel neurocognitive aspects of addictive substance abuse, specifically inhalant use, during adolescence. Our findings show alterations in the topological organization of three functional brain networks of great importance for cognition: the DMN, the SN, and the FPN. Overall, we detected important variations in information transmission and integration between the brain regions that comprise these networks.

Important alterations in a number of executive functions were detected among the inhalant-consuming group, as well as the association between these alterations and the connectivity of the DMN. Taken together, our results demonstrate the usefulness of the analysis of functional brain networks in the resting state for improving the understanding of the changes in neural functioning underlying inhalant use disorder and suggest the need for its wider application in the field of SUDs. If these findings are validated in much larger samples, they could lead to the development of intervention strategies that consider the functional and structural brain plasticity present in adolescence, thus enabling the restoration of the functional architecture of the affected brain networks.

LIMITATIONS

Limitations of this research include the following:

1. Throughout the paper we focus in the default, fronto-parietal, and salience networks. However, it is known that brain topology of other systems relating to cognitive control and attention, such as the Cingulo-Opercular, Dorsal Attention, and Ventral Attention networks are directly involved in executive functions. It would be desirable to explore these correlations in a future study.
2. Its transversal design prevents establishing causality between inhalant use and the efficiency of functional connectivity, as well as between inhalant use and the state of executive brain functions.
3. The modest size of the sample limits the analysis of the results and the reach of its interpretation.
4. Some variables, such as the socioeconomic status of the subjects, the quality of their educational institutions, the presence of family stress and other variables related to their family history having a potential impact on the brain and cognitive development, were not considered.
5. Substance use history was established from an interview, where the subjects may have minimized their use of inhalants or other drugs.
6. Most of the subjects were on initial remission under pharmacological treatment, and both conditions may have affected cognitive performance.
7. Many inhalant users also consumed other substances, although to a lesser extent. The results must accordingly be interpreted while taking into account the occurrence of polydrug use, which is characteristic of this population.

Finally, our results should be considered within the limits of the adolescence period, which is an important stage for brain development. The study of the difference in the topological organization of brain networks and its impact on cognitive functioning in adult inhalant users may lead to different results and constitute a future line of research.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available as the participants did not give their informed consent for the

public availability of their data. Requests to access the datasets should be directed to NG-G, nagonzalez@himfg.edu.mx.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Children's Hospital of Mexico "Federico Gomez". Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NG-G, DH-A, and CT-R conceived the study and participated in the data collection. NG-G, DH-A, LP, and RV-S analyzed the data and carried out the statistical analysis. DH-A, NG-G, and MP wrote the manuscript. All authors have approved the final manuscript.

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A Critical Period for Prefrontal Network Configurations Underlying Psychiatric Disorders and Addiction

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The medial prefrontal cortex (mPFC) has been classically defined as the brain region responsible for higher cognitive functions, including the decision-making process. Ample information has been gathered during the last 40 years in an attempt to understand how it works. We now know extensively about the connectivity of this region and its relationship with neuromodulatory ascending projection areas, such as the dorsal raphe nucleus (DRN) or the ventral tegmental area (VTA). Both areas are well-known regulators of the reward-based decision-making process and hence likely to be involved in processes like evidence integration, impulsivity or addiction biology, but also in helping us to predict the valence of our future actions: i.e., what is "good" and what is "bad." Here we propose a hypothesis of a critical period, during which the inputs of the mPFC compete for target innervation, establishing specific prefrontal network configurations in the adult brain. We discuss how these different prefrontal configurations are linked to brain diseases such as addiction or neuropsychiatric disorders, and especially how drug abuse and other events during early life stages might lead to the formation of more vulnerable prefrontal network configurations. Finally, we show different promising pharmacological approaches that, when combined with the appropriate stimuli, will be able to re-establish these functional prefrontocortical configurations during adulthood.

Keywords: prefrontal networks, decision-making, critical period, ventral hippocampus, basolateral amygdala

THE mPFC AS A HIGH-ORDER COGNITIVE AREA

A remaining question in the field of neuroscience is how our brain shapes the decision-making process, i.e., the ability to coordinate thought and action to achieve internal goals. Classic studies show the medial prefrontal cortex (mPFC) is an important region involved in the decision-making process, and patients with damage in the frontal lobe show deficits in attention and behavior control (Hagberg, 1987) and inability to acquire and use behavior-guiding rules (Shallice, 1982; Wise et al., 1996). Recent research, not only in humans but also in rodents, has shown that the mPFC is a key region for high executive functions such as

rule learning and other different aspects related to working memory (Kesner and Churchwell, 2011). In this regard, lesion studies with damage in the prelimbic (PrL) and infralimbic (IL) cortices have been shown to produce deficits in both spatial (Horst and Laubach, 2009) and visual working memory (Di Pietro et al., 2004). Another task related to working memory is the temporal order memory; it requires the animal to remember the last visited arm in a maze and has also been shown to be dependent on the PrL and the anterior cingulate (AC) cortices (Chiba et al., 1997; Barker et al., 2007). Interestingly, the PrL cortex has also been suggested to mediate behavioral flexibility, i.e., the ability to change learned rules to obtain a reward, as in rotating rewards and inverting patterns for reward retrieval (Dias and Aggleton, 2000; Rich and Shapiro, 2007).

Regarding the decision-making process, lesions in the AC lead to disruptions in effort-based tasks, such as reduced willingness to climb a barrier to get a large reward (Rudebeck et al., 2006). Supporting these findings, effort-based decisions increased activity not only in the AC but also in the PrL and other prefrontocortical regions such as the lateral orbital cortex (Endepols et al., 2010). Similarly, the willingness to wait for a larger reward is also dependent on regions of the mPFC (Mobini et al., 2002; Churchwell et al., 2009).

TOP-DOWN CONTROL OF mPFC ON NEUROMODULATION

One of the most accepted theories of how the mPFC controls the decision-making process is the top-down control from the mPFC (Miller and Cohen, 2001), which states that the mPFC provides biased signals representing the goals and the means to achieve those goals. These signals would be sent to efferent regions throughout the brain, including sensory or motor executive regions, but also those areas related to memory retrieval and emotions. These biased signals would determine how the flow of neural activity is guided to achieve those goals (Miller and Cohen, 2001). However, the mPFC also connects, both directly and indirectly, to important regions responsible for the modulation of mood, decision making and addiction, such as the ventral tegmental area (VTA) and the dorsal raphe nucleus (DRN; Russo and Nestler, 2013). This neuromodulatory route represents an alternative top-down pathway through which the mPFC controls behavior (Challis and Berton, 2015).

After the establishment of the physiological and computational principles underlying reward prediction coding (Schultz et al., 1997), a vast number of studies have reported that multiple behaviors function according to the reward prediction principles and its associated neuromodulatory circuits (Dayan et al., 2000). These networks are responsible for the learning of expected behavioral outcomes through the enhanced release of dopamine (DA) on specific locations (Schultz, 2016). While previously the focus has been set on the different routes through which DA is secreted: mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular pathways (Luo and Huang, 2016), we want to stress the relevance of a reverse circuit in the regulation of certain behaviors related to addiction and neuropsychiatric disorders: from the mPFC to the striatum and then to the dopaminergic regions. In this line, the main efferent projections from the PrL target the caudate-putamen and the core of nucleus accumbens (NAcc), while the IL targets the medial shell of NAcc (Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007). Interestingly, the NAcc core and shell display different activities during the establishment of rewards: While the core is active before a newly-devalued cue, the shell decreases its activity (West and Carelli, 2016); hence granting both PrL and IL cortices different roles in the reward circuitry.

These basal ganglia have been long suggested to loop with the mPFC, being these loops key for the incentive learning and control of motor behaviors through activity in the substantia nigra (SN) and the VTA (Allen and Tsukahara, 1974; Lanciego et al., 2012; Leisman et al., 2014). These two regions are the source of dopaminergic transmission in the brain and have been suggested to feed back to the basal ganglia (Haber et al., 2000), pointing to the relevance of the mPFC in the DA circuitry. Therefore, the mPFC would exert an integrative role in processing motor and sensory inputs; and together with the basal ganglia, would be controlling the learning of appropriate and incentivized series of motor behaviors that include motor skills and habits (McNab and Klingberg, 2008; Graybiel and Grafton, 2015). Similarly, we suggest that a similar mechanism might exist, by which the integrative role of the mPFC would control higher cognitive behaviors as well.

In this line, the study of inputs to the VTA and the SN indicates strong projections from different areas such as the NAcc as indicated, but also from the dorsal striatum and the lateral hypothalamus (LH), among others (Geisler and Zahm, 2005; Watabe-Uchida et al., 2012). Interestingly, a similar pattern has been described when studying the input projections to the dorsal raphe, the area responsible for serotonergic innervation. Serotonin (5HT) has also been shown to modulate decision making, as well as to interact with the reward prediction error circuitry. Moreover, the altered expression of 5HT is associated with the onset of different mood disorders, including major depressive disorder (Challis and Berton, 2015). Interestingly, the areas projecting to the dorsal raphe include the ventral and dorsal striatum, the LH and the septum (Gabbott et al., 2005; Commons, 2016; Ogawa and Watabe-Uchida, 2018).

It is important to note that different parts of these dopaminergic and serotoninergic regions, as well as their specific inputs, have been suggested to mediate different functions of the prediction reward system (Ogawa and Watabe-Uchida, 2018). However, it is also relevant to support our hypothesis that most of the areas projecting to the dopaminergic and serotoninergic regions are also the main targets of the mPFC (Gabbott et al., 2005; see **Figure 1**).

The DRN is the main source of 5HT in the brain and receives dense monosynaptic input from the mPFC that contacts both serotoninergic and GABAergic neurons. Optogenetic activation of the mPFC onto the DRN produces strong disynaptic inhibition (Zhou et al., 2017). Nevertheless, the net effect

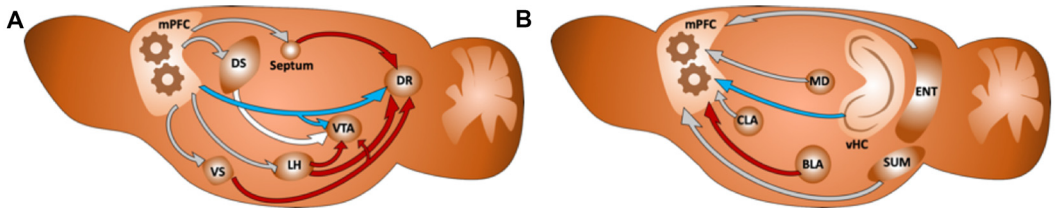

FIGURE 1 | (A) Diagram of a sagittal section of the rodent brain illustrating how the integration of information in the medial prefrontal cortex (mPFC) affects both directly (in blue) and indirectly (in red) monoaminergic nuclei in the brain, as an example for the top-down control of the mPFC. The rest of the connections are colored in gray. **(B)** Diagram illustrating some of the main inputs to the mPFC. We have colored in red the projection from the BLA and in blue the projection from the vHC: two regions we hypothesize to compete for target innervation in the mPFC. The rest of the connections are colored in gray.

of mPFC input on DRN activity, caused by the balance between direct excitation to serotonergic neurons vs. disynaptic inhibition, can be modulated not only by endocannabinoids (Geddes et al., 2016) but also by stressful experiences such as social defeat (Challis et al., 2014). A disrupted balance between excitation and inhibition (E/I) in the DRN after mPFC activation has been hypothesized to be an underlying factor for developing depressive-like symptomatology and that high-frequency deep brain stimulation (DBS) protocols of the mPFC (as performed in humans and rodents) can restore the E/I balance towards a higher excitation onto serotonergic neurons, thus enhancing DRN output and providing a mechanistic explanation for the antidepressant action of DBS of the mPFC (Challis and Berton, 2015).

Altogether, these data suggest that top-down control from the mPFC is exerted through the action of monoamines, such as DA) and 5HT. These two systems have been long suspected to play opposite roles in the reward circuitry (Solomon and Corbit, 1974), with DA reinforcing positive reward prediction error, and 5HT facilitating learning of new adaptive behaviors and inhibiting non-adaptive responses (Boureau and Dayan, 2011; Cools et al., 2011). Recent findings have shown that these processes are not only caused by the different effects of these neurotransmitters—5HT promoting LTD (He et al., 2015) and DA facilitating LTP (Li et al., 2003; Otani et al., 2003)—but are caused by their different release dynamics. In fact, in a conditioning paradigm, the action of DA is faster after reversal learning than that of 5HT. However, in that same experiment, DA was also shown to be withdrawn more rapidly during the negative prediction error, favoring the slow 5HT signals (Matias et al., 2017). Suggesting that DA reinforces a positive reward, but 5HT indicates a mismatch between expectations and reality.

Nevertheless, the mPFC is also interconnected to other regions that mediate the activity of neuromodulatory areas, such as the medial thalamus, or the periaqueductal gray area (Cameron et al., 1995; Vertes et al., 2012). In this line, a recent functional imaging study has shown that the connectivity between the mPFC and these regions is altered in the rumination of patients with chronic pain (Kucyi et al., 2014).

It is also important to note that an increasing amount of evidence is highlighting the relevance of critical periods for the establishment of functional connectivity between the mPFC and its afferent regions, and how such connectivity is disrupted in different psychiatric disorders such as addiction or depression (Crews et al., 2007; Contreras-Rodríguez et al., 2016; Pujol et al., 2019).

INPUTS TO THE mPFC

The data discussed here supports the classical view of the mPFC as a key player in our decision-making process. However, to better understand how it achieves these goals, we focus here not only on its outputs but also on its inputs. It has been long suggested that mPFC has a role in integrating diverse information from many different brain regions (Fuster, 1985, 1995). In this line, several studies have described the connectivity of the mPFC (Gabbott et al., 2005; Hoover and Vertes, 2007) revealing that the mPFC is a very interconnected structure within its different parts and with other regions, forming part of a brain-wide network of interconnected structures involved in mood control (Gilbert et al., 2010; Riga et al., 2014). It receives direct input from the ascending neuromodulatory monoaminergic systems, like the DRN, the VTA or the locus coeruleus. Also, its most dorsal part receives strong projections from the somatosensory and motor cortices, resulting in the motor response, or attention control (Passetti et al., 2002). On the other hand, the ventral parts are usually associated with cognitive spatial and mnemonic processes (Heidbreder and Groenewegen, 2003) receive strong projections from limbic structures, such as the hippocampal formation and the amygdala, as well as other regions such as the claustrum, the entorhinal cortex, the mediodorsal nucleus of the thalamus or the supramammillary nucleus in the hypothalamus (see **Figure 1**; Hoover and Vertes, 2007).

From these regions, the ventral Hippocampus (vHC) and the basolateral amygdala (BLA) are two of the most important regions conveying different types of information to the mPFC due to their role in different functions such as memory formation, spatial navigation or fear processing (Spellman and Gordon, 2015). We hypothesize that the integration that takes place during early life stages in the mPFC between these two different sources of information is a key factor, that determines the behavioral outcome of the animal in a given scenario, i.e., depending on the strength of each input and how it is processed by the mPFC.

A DISTRIBUTED BRAIN NETWORK FOR MOOD CONTROL IN HEALTH AND DISEASE

Focusing on these two regions directly projecting to the mPFC, the vHC has been traditionally thought to be important for emotional memories (Fanselow and Dong, 2010), although recent studies have shown that this area also manages spatial information (Kjelstrup et al., 2008; Wirt and Hyman, 2017). On the other hand, the BLA would convey more emotional-related information to be processed into the mPFC (Garcia et al., 1999; Senn et al., 2014), and is necessary to express fear-related behaviors such as freezing (Helmstetter and Bellgowan, 1994). Alterations of its connectivity in humans has already been suggested to underlie aggressive behaviors (Leutgeb et al., 2016).

Interestingly, when reviewing the literature, opposite alterations in these two structures can be found after different experimental conditions: patients suffering from major depression show reduced activity and volume in the hippocampus (Sheline et al., 1999; Campbell et al., 2004; Videbech and Ravnkilde, 2004; Milne et al., 2012), but an increase in these parameters when measured in the amygdala (Anand et al., 2005; Hamilton et al., 2012). In animal models of depression, such as chronic or prenatal stress, animals show dendritic atrophy in the hippocampus (Sousa et al., 2000; Mychasiuk et al., 2012) and dendritic growth in the amygdala (Vyas et al., 2002). These changes can be reversed by antidepressants such as Fluoxetine (Magariños et al., 1999; McEwen and Chattarji, 2004), by promoting an increase in spine density of pyramidal neurons in the hippocampus (Hajszan et al., 2005). Fluoxetine and other antidepressants also produce an increase in BDNF mRNA in the hippocampus (Nibuya et al., 1995; Larsen et al., 2008). While stress produces a decrease in BDNF in the hippocampus, it leads to an increase in the amygdala (Lakshminarasimhan and Chattarji, 2012), suggesting an important role of BDNF in the structural changes observed.

In humans, a common polymorphism in the BDNF gene is the substitution of Val to Met at codon 66, known as Val66Met (Shimizu et al., 2004). This allele has been associated with several neuropsychiatric disorders (Harrisberger et al., 2015). Interestingly it has been shown that this polymorphism of BDNF is associated with an increased activity of the amygdala and, conversely, a decreased activity in the hippocampus. This has been suggested as the underlying reason for an impaired fear extinction in patients and slightly impaired memory retrieval (Hariri et al., 2003; Soliman et al., 2010; Hajek et al., 2012).

Furthermore, recent evidence measuring functional connectivity after fear acquisition shows that the connectivity between the amygdala and the mPFC is decreased during fear memory consolidation, while that between the hippocampus and the insular cortex, another important region in the decision-making process (Droutman et al., 2015; Von Siebenthal et al., 2017), is enhanced (Feng et al., 2014).

Together, these results point to a prefrontocortical network configuration in major depression dominated by a reduced functional connectivity with the amygdala. Interestingly, an increased functional connectivity between the prefrontal cortex and the amygdala have been reported in animal models of autism (Huang et al., 2016) as well as in human subjects with autism spectrum disorders (Iidaka et al., 2019). Highlighting the relevance of precise mechanisms controlling the functional connectivity balance across this distributed network of brain structures for mood control. Furthermore, prefrontocortical configurations associated with the disease not only span top-down afferents but also hyperconnectivity and hyperplasticity of local microcircuits have been reported in animal models of autism (Rinaldi et al., 2008).

On the other hand, recent evidence has shown that certain pharmacological treatments, such as antidepressants, seem to reverse certain pathological network configuration, through structural plasticity dependent mechanisms, to a balance of these two inputs and, interestingly, when administered to control animals, it leads to a hippocampal dominated network configuration. It has been shown recently that the activation of the projection from the vHC to the mPFC both optogenetically and chemogenetically in the adult brain, can replicate the effects of the fast-acting antidepressant ketamine for a short time (Carreno et al., 2016).

A CRITICAL PERIOD OF OPPORTUNITY

Critical periods are defined as temporal windows of enhanced plasticity in brain development, during which a specific circuit or region is highly sensitive to experience (Hensch, 2005).

It has been shown in several sensory cortices that, during these critical periods, the incoming developing inputs compete in an experience-dependent manner for target innervation: i.e., left and right eye compete for innervation of the visual cortex (Hubel and Wiesel, 1970) or different whiskers compete for space in the barrel cortex (Van der Loos and Woolsey, 1973).

During the critical period of the fear system, at a time point when fear memories can be extinct easily, there is an increase in the connectivity from the vHC to the mPFC (Pattwell et al., 2016), which is in agreement with other studies suggesting that the projection from the ventral hippocampus to the mPFC can disrupt expression of fear memories (Sotres-Bayon et al., 2012). We have shown evidence for the vHC and the BLA competing for target innervation in the mPFC (Guirado et al., 2016). In this line, we hypothesize that the mPFC has a critical period in which its different incoming inputs compete in an experience-dependent fashion. Moreover, the specific results of this competition would determine a specific prefrontal network configuration, which, we believe, is a key element to understand neurodevelopmental trajectories to different psychiatric disorders.

Interestingly, not only the inputs onto the mPFC undergo late development and susceptibility during this critical period. It has been shown that the prefrontal-amygdalar output undergoes late development in mice (Arruda-Carvalho et al., 2017) as late as 45 days of postnatal development. Moreover, the increase in serotonergic tone, produced by the blockage of serotonin

transporter by antidepressants during this period, has been shown to increase the output innervation from the mPFC to the DRN (Soiza-Reilly et al., 2019). It is worth noticing that the alteration in the strength of mPFC projections to the amygdala and the DRN has been shown as a factor associated with increased risk to develop anxiety and depression (Chen et al., 2018; Soiza-Reilly et al., 2019).

In line with the idea of a critical period in the mPFC, both classical and fast-acting antidepressants, as well as optogenetic and chemogenetic stimulations, only revert temporarily the behavior in paradigms of mental diseases (Carreno et al., 2016). Therefore, we propose that to achieve long-lasting effects, a critical period plasticity context is required for the remodeling of prefrontal network configurations. Most animal models of neuropsychiatric disorders are based on manipulations during early life and/or adolescence (Nestler and Hyman, 2010; Andersen, 2015), probably during that critical period in the mPFC.

We hypothesize that after the closure of this critical period, the brain will remain with a specific network configuration according to the strength of its mPFC inputs during such a time window. Moreover, we suggest two specific mPFC configurations: one dominated by the amygdala and one by the ventral hippocampus (see **Figure 2**). These specific configurations can be correlated with specific behaviors through adulthood, as discussed above, building a bridge between behavior and network connectivity that could explain, to a certain extent, the decision-making process.

Epidemiological data have shown that early life trauma, including physical abuse, sexual abuse or neglectful parenting is correlated not only with alcohol abuse (Clark et al., 1997), but also to other substances, including THC (Bensley et al., 1999; Dube et al., 2003). Moreover, vulnerability to stress and depression has been associated with rodents with increased drug abuse (Riga et al., 2018), showing the interconnectivity of these disorders and a possible common origin. In this line, we propose here that aversive experiences during early life lead to a prefrontocortical network dominated by the amygdala, which would be more vulnerable to brain diseases such as neuropsychiatric disorders or addiction. The impact of early life trauma seems to be restricted to certain time windows, supporting the idea of a prefrontal cortex critical period (Rutter et al., 2007).

The good news is that the combination of drugs that reopen critical period plasticity, such as Fluoxetine (Guirado and Castrén, 2018), with the proper experimental conditions, can effectively allow the network configuration to change to a healthy state during adulthood. We have proven this principle both in the visual (Maya Vetencourt et al., 2008) and in the fear systems (Karpova et al., 2011).

Moreover, we have recently found that the combination of behavioral therapy (re-socialization in isolated animals) after chronic Fluoxetine treatment, increases the strength of the projection from the vHC to the mPFC and reduces abnormal aggressive behavior in a long-time manner (Mikics et al., 2018). Interestingly, other substances whose efficiency as antidepressants is undergoing a revision both in the context of clinical treatment as well as basic research, such as MDMA (Nardou et al., 2019), ketamine (Berman et al.,

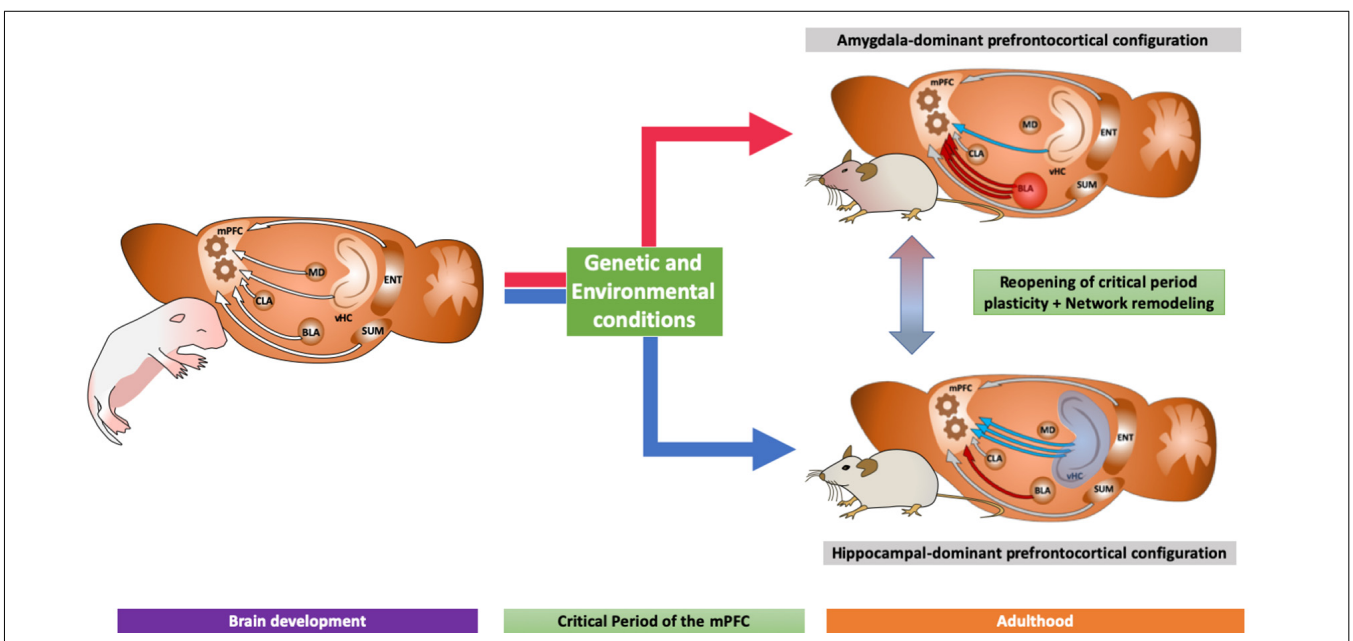

FIGURE 2 | Diagram showing two different neurodevelopmental trajectories according to its prefrontocortical inputs: (1) an amygdaloid-dominant scenario, with strong projections from the basolateral amygdala to the mPFC, with increased volume in the amygdala which, as discussed in the main text, is related to increased fear expression and neuropsychiatric disorders; and (2) a hippocampal-dominated network, associated to antidepressant action and increased neuroplasticity. We also indicate the possibility of new treatments able to revert the network configuration as explained in the main text.

2000; Li et al., 2010) or isoflurane (Antila et al., 2017), have been shown to reopen a critical period for social behavior through different molecular mechanisms of plasticity, including oxytocin, mTOR and TrkB (Li et al., 2010; Antila et al., 2017; Nardou et al., 2019).

Thus, new approaches using drugs aiming at the reopening of critical periods of plasticity (Castrén and Antila, 2017; Guirado and Castrén, 2018), together with environmental conditions or the experimental manipulation of the activity of these prefrontal inputs, will provide a new proof of concept to explore new and efficient treatments for addiction and neuropsychiatric disorders. Furthermore, future research studying how different inputs are integrated and processed in the mPFC will help us understand further the decision making process and the human mind.

AUTHOR CONTRIBUTIONS

RG has written the draft and designed the review. All authors have contributed significantly reviewing the manuscript.

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OPINION
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What Is the "Trigger" of Addiction?

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Addiction is a multidimensional condition (European Monitoring Centre for Drugs Drug Addiction., 2014) which has traditionally been explored from different perspectives, including biological, social, and psychological approaches. Inside of these main levels, the specific contribution of multiple sublevels to the addiction development and treatment have been investigated. This research field draws an amazing addiction puzzle which is built by a diverse set of pieces coming from the genetic, epigenetic, molecular, neurobiological, and psychological levels, but also from live experiences, the environment, and from cognitive traits. The complex combination of these factors determines the addiction process.as well as Drug use affects each factor differently.

Any attempt to study this complexity through one single level is insufficient. In fact, addiction models usually need to include elements from different models in order to provide a satisfactory explanation of the disease.

Most addiction research has classically focused on the neurobiological level, trying to figure out the neuroadaptations that repetitive drug use produces over the brain systems and their behavioral consequences, including effects on the reward system, emotional or cognitive functioning. Currently, one of the most accepted neurobiological theories postulates that the development of drug addiction is a progressive process through a three-phase cycle: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation (Koob and Volkow, 2016).

However, there is no doubt that addictive disorders have a strong subjective component that is not fully fitted with the present models. There is increasing literature showing how some factors related to subjective processes can impact the neurobiology of addiction by increasing the vulnerability such as early childhood experiences (Kim et al., 2017; Lee et al., 2018), social context (Schriber and Guyer, 2016; Burke et al., 2017), environment (Zucker et al., 2018), maturation (Romer et al., 2017) or personality (Jauk and Dieterich, 2019; Ramirez-Castillo et al., 2019) factors. Of special interest is the role of the so called "Big Five" personality traits in the risk for drug addiction (Andreassen et al., 2013).

RE-UNDERSTANDING THE TRIGGER

The current maladjustment between the neurobiology and the subjective human condition can be observed in the concept of "trigger." Understood as "a stimulus that elicits a reaction" (APA dictionary, 2019), the trigger is considered a key element in the craving response showed by addicts. This external stimulus would lead the individual to repeat drug use or relapse after a period of abstinence. Addiction models constructed upon this observation consider the trigger as a stimulus able to activate drug related memories leading to reward anticipation and craving responses. As a consequence, derived therapeutic approaches suggest to avoid the trigger or provide the individuals with cognitive capabilities to control that emotional response provoked by the trigger. Such cognitive-behavioral therapies include operant conditioning, contingency management or coping skills training (Witkiewitz et al., 2019).

In this way, where "trigger" is considered as an "external" stimulus inducing a reaction, its scope is only at the psychological level and does not address the uniqueness of complexity.

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Instead, we offer a re-understanding of the "trigger" as something "internal" that relates all levels of complexity and requires dialogue between different levels mentioned above. Moreover, the stimulus-response association was already questioned by PK Anokhin (Egiazaryan and Sudakov, 2007), who proposed to come out of a causal reading (the trigger provokes a behavior) and assumed a systemic conception in which the behavior is due to a global situation of the whole system (Thelen and Smith, 1994; Smith, 2005; Anderson et al., 2012).

The change from outside to inside is also justified by discovering that due to the high subjectivity of addiction, it makes no sense to "blame" something outside. In this sense, a term to refer to all this subjective complexity is suggested here: "frustration." Frustration cannot be understood without breaking expectations (Amsel, 1992). The interesting point about this term is that it evidences aspects of interiority, but its conceptual basis also allows its use in the different levels of complexity. If we define frustration as widely as possible, we would say that it is the emotional result of the perception of the distance between the expected (needed) and the found. At all levels, even in the most molecular, frustration would indicate a mismatch between one molecular situation and another. Frustration is understood as a global experience that can have many types of "mismatches," whether molecular or in terms of expectations. This mismatch needs to be perceived either by cognitive (if we talk about expectations) or biological processes (if we talk about biological levels). Frustration would thus be a meeting point for all levels of complexity.

Classically, frustration and other emotions were considered as an evaluation of the actual need and estimation of probability of its satisfaction (the "need-informational theory of emotions"), linked to the participation of specific key brain structures (Simonov, 1984, 1997). However, recent meta-analyses found little evidence that discrete emotion categories can be consistently and specifically localized to distinct brain regions (Lindquist et al., 2012). Therefore, a set of interacting brain regions commonly involved in basic psychological operations are active during emotion experience and perception across a range of discrete emotion categories (Kober et al., 2008; Lindquist et al., 2012). Therefore, the current model of emotions is systemic and linked to other psychological functions (Pessoa, 2013), which is in line with the proposal of "frustration" as a personal global condition which (after the whole personal evaluation of a stimulus) "triggers" a response.

What is usually conceptualized as a trigger (cause attributed to an external element) would be a simplification because it denies the role of the inner experience. If we accept the frustration is previous to the external trigger, then the clinical approach should be headed to search and treat the emotional "tangle" underlying frustration and its relationship with the external stimulus. Because if we avoid the external trigger without treating the previous subjective cause, then the probability of relapse is high. The treatment of the subjective emotional state will help to provide a new meaning to that external stimulus, an action that we call to "re-meaning" the trigger.

This therapeutic fact of giving a new meaning to the trigger does not exclude the traditional therapeutic avoiding of the trigger, which is an urgent aim at the beginning of the treatment. Nevertheless, after that initial phase, the inner problem should also be addressed. Actually, both are necessary, one to get initial abstinence and the other to help the addict to resolve the frustration underlying drug addiction.

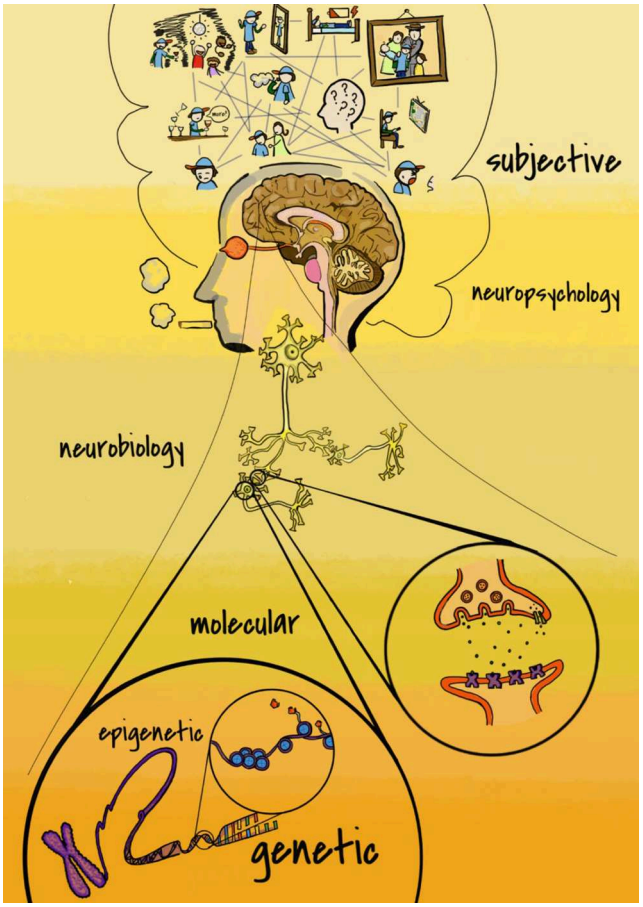

FIGURE 1 | Different levels for the study of addiction range from molecular **(lower)** to subjective **(upper)**.

Our proposal about the individual subjectivity implies understanding that the emotion is lived more as an expression of the complexity of a person's life in specific circumstances, than as a reaction to the outside (Barrett, 2017).

THE SUBJECTIVE CONCEPT AND NEUROBIOLOGY OF STRESS AND ADDICTION

Similarly to addiction, stress disorders are also related to a trigger stimulus evoking a strong subjective experience. A neurobiological overlap between these two conditions would therefore be expected.

The responses to psychosocial stressful stimuli in healthy individuals also involve the participation of hippocampus, amygdala, insula and prefrontal cortices (Shin and Liberzon,

2010). Specifically, limbic circuits underlie the coherent contextualization of different neural inputs (Bird, 2017), as well as the formation of episodic memories and the integration of emotional processing; essential elements in craving and relapse by exposure to the context of drug addicts.

Moreover, drug addicts, psychiatric patients, and subjects that suffered early child abuse show similar brain alterations such as volume reductions of the hippocampus, amygdala and anterior cingulate cortex, or hyperactivity of the amygdala and insula, vs. a decreased response of prefrontal cortex when dealing with stress (Etkin and Wager, 2007; Shin and Liberzon, 2010; McCrory et al., 2012). The cue-reactivity paradigm used in fMRI addiction studies has pointed out limbic and prefrontal cortices as the key systems in response to stimuli (Chase et al., 2011). However, a more recent meta-analysis concludes the absence of a consensus in relation to the brain response to conditioned drug stimuli (Zilberman et al., 2019). The loss of consensus can possibly be partially explained by the role of frustration (subjective personal factors) triggering the negative perception of the reality (inner trigger), an element shared in both, stress and addiction disorders.

Our suggestion is to promote resilience as a therapeutic tool to treat frustration. It is known that the subjective perception of the event is a determining point to understand the experience lived (Burr, 1982). Therefore, the best way to work on resilience is through the re-meaning of the so-called stressor or trigger (Lazarus and Launier, 1978; Boss, 2002), but working on the meaning attributed to the stressor instead of the stressor itself. A creative act is necessary because resilience is not a mere adaptation to new circumstances, but implies a global personal growth (Walsh, 2002; Cicchetti, 2010).

From a therapeutic view, the capability of psychotherapeutic treatments (alone) has been demonstrated to restoring the biological normality of brain structure and function (Barsaglini et al., 2014). This is of especial interest when only limited effects have been documented by pharmacological treatments, for example in the drug addiction (Dakwar and Nunes, 2016). Psychological symptoms, including depression, anxiety, hostility, psychological pain, embarrassment, blame, panic and obsession, are complex and difficult to characterize but treating them is crucial and essential for rehabilitation (Dakwar and Nunes, 2016).

TURNING BACK TO THE COMPLEXITY

Usually, the emphasis for relapse prevention is focused on avoiding trigger stimuli by means of healthy habits, but, once again, subjective elements play a central role, and are related to the complexity of personal relationships and self-assessment (Marlatt and Gordon, 1985). Therefore, relapse is seen as the effect of not having coping strategies. Moreover, it has been shown that high percentages of drug addicted patients allege intrapersonal determinants related to frustration as the main cause of relapse (Ramirez-Castillo et al., 2019).

It is clear that resilience to stress or addiction must be studied at all levels from the most biological to the most subjective (Cicchetti, 2010) in order to attend the globality and uniqueness of the person since the absence of risk factors or the presence of protective elements alone are not enough to explain whether an individual using drugs will become addicted or whether an addict will be rehabilitated (Luthar et al., 2000).

This brief journey opens the possibility of accepting the term "frustration" as a global subjective element, leading the therapeutic intervention toward the inner patient condition, for example, through work on the resilience, more than the avoiding of external stimuli.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Sex Differences and the Role of Estradiol in Mesolimbic Reward Circuits and Vulnerability to Cocaine and Opiate Addiction

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Although both men and women become addicted to drugs of abuse, women transition to addiction faster, experience greater difficulties remaining abstinent, and relapse more often than men. In both humans and rodents, hormonal cycles are associated with females' faster progression to addiction. Higher concentrations and fluctuating levels of ovarian hormones in females modulate the mesolimbic reward system and influence reward-directed behavior. For example, in female rodents, estradiol (E2) influences dopamine activity within the mesolimbic reward system such that drug-directed behaviors that are normally rewarding and reinforcing become enhanced when circulating levels of E2 are high. Therefore, neuroendocrine interactions, in part, explain sex differences in behaviors motivated by drug reward. Here, we review sex differences in the physiology and function of the mesolimbic reward system in order to explore the notion that sex differences in response to drugs of abuse, specifically cocaine and opiates, are the result of molecular neuroadaptations that differentially develop depending upon the hormonal state of the animal. We also reconsider the notion that ovarian hormones, specifically estrogen/estradiol, sensitize target neurons thereby increasing responsivity when under the influence of either cocaine or opiates or in response to exposure to drug-associated cues. These adaptations may ultimately serve to guide the motivational behaviors that underlie the factors that cause women to be more vulnerable to cocaine and opiate addiction than men.

Keywords: estrogen, dopamine, female, withdrawal, mesolimbic reward system

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INTRODUCTION

Drug addiction or substance use disorder is a chronic, relapsing, neuropsychiatric illness characterized by a loss of control over drug seeking and intake, persistent drug craving, and high motivation to take the drug (Reid et al., 2012). Here we provide an updated review, of the literature in which we discuss sex differences in the development of cocaine and opiate addiction, with an emphasis on the influence of estradiol (E2) on signaling within the mesolimbic reward circuit. We provide an overview of the mechanisms of the mesolimbic reward circuit and have described in detail how E2 influences it at the circuit level and also at the level of dopaminergic signaling. Furthermore, we have included discussions of molecular mechanisms underlying the sex differences in the behavioral responses to cocaine and opiates and have demonstrated the

role of E2 in the modulation of these mechanisms. Taken together, this review demonstrates the link between sex differences in cocaine and opiate addiction and the role of E2 as a major driver of these sex differences. Many excellent reviews have been published on sex differences in addiction and drug abuse. Some of the most recent of these have detailed sex differences in the balance between dorsal and ventral striatal circuits (Becker, 2016); quantitative, population, and mechanistic sex differences in addiction (Becker and Koob, 2016); basic biological differences between males and females that influence addictive behaviors (Becker et al., 2017); sex differences in biology, epidemiology, and treatment of substance use disorder (McHugh et al., 2018), and, most recently, a comprehensive review which highlights sex differences in the neural mechanisms and developmental events influencing addiction vulnerability (Becker and Chartoff, 2019).

Sex Differences in Addiction to Cocaine

Cocaine use disorder is a serious public health concern. In the United States, approximately 2.2 million people report regular use of cocaine and 1 million individuals met criteria for cocaine use disorder in the past year (Substance Abuse and Mental Health Services Administration, 2018). Although, overall, more men use and are addicted to cocaine than women, women exhibit a more rapid progression from initial cocaine use to dependence than men (Kosten et al., 1985; Brady and Randall, 1999). Women also report experiencing enhanced positive subjective effects (feelings of euphoria) of cocaine than men. The enhancement of positive subjective effects is speculated to be the reason women more rapidly progress through the stages of addiction than men (reviewed in Becker and Hu, 2008). During periods of abstinence, women report experiencing higher levels of cocaine craving and relapse rates than men. Lastly, during bouts of relapse women take larger amounts of cocaine than men (Kosten et al., 1985; Brady and Randall, 1999; Robbins et al., 1999; Chen and Kandel, 2002; Gallop et al., 2007; Ignjatova and Raleva, 2009). Collectively, these data indicate that women may be more severely affected by cocaine use than men.

Sex Differences in Addiction to Opiates

Opioid use disorder (OUD) has reached epidemic proportions, claiming ∼115 lives daily to opioid overdoses in the US alone (Substance Abuse and Mental Health Services Administration, 2015; The, 2017; Volkow et al., 2019). Opioids acutely attenuate perceptions of pain and induce euphoria and relaxation in users. Despite the acute benefits, long-term opioid use can lead to the development of OUD (The, 2017; Volkow et al., 2019). Individual differences in addiction liability exist, and behavioral traits like impulsivity and risk-taking behavior often heighten the risk for users to transition from recreational drug use to drug dependence (Kreek et al., 2005; Belin et al., 2008). The influence of sex/gender in the physiological/psychological risks for developing OUD has also been reported (Fillingim et al., 2009; Green et al., 2009; Back et al., 2010). Overall, more men than women report lifetime and past-year use of all opioid drugs. However, women are more likely to report the non-medical use of prescription opioids as their primary drug of abuse (Center for Behavioral Health Statistics and Quality, 2015). This is likely because women are more likely to suffer from chronic pain conditions (Darnall et al., 2012), be prescribed prescription pain relievers, and use them for longer time periods than men (Berkley, 1997; Gureje et al., 1998; Fillingim et al., 2009; Mogil, 2012; Centers for Disease Control and Prevention, 2013; Substance Abuse and Mental Health Services Administration, 2014; Serdarevic et al., 2017). In addition, compared to men, women (*a*) are more likely to use opioids for non-medical conditions (Green et al., 2009; Hemsing et al., 2016; Marsh et al., 2018); (*b*) transition more rapidly from casual to non-medical use of prescription opioids to opioid dependence; (*c*) experience higher levels of craving and relapse during abstinence; (*d*) consume larger amounts of drug during relapse; and (*e*) are less likely to seek treatment for their opioid addiction (Kosten et al., 1985; Brady and Randall, 1999; Ignjatova and Raleva, 2009; Bobzean et al., 2014a). In addition, during periods of abstinence, women report higher numbers of distressing physical/affective withdrawal symptoms compared to men (Soldin et al., 2011; Fox et al., 2013; Fernandez-Montalvo et al., 2017; Dunn et al., 2018). While, men are more likely to die from opioid overdose than women, overdoses related to opioids have greatly increased in women compared to men: from 1999 to 2017 opioid overdose-related deaths increased in women by 471% as compared to 218% among men (Paulozzi et al., 2011; Serdarevic et al., 2017; VanHouten et al., 2019). Therefore, it is not surprising that several studies identify women as one of the most vulnerable subpopulations for non-medical prescription opioid use and abuse (Fillingim et al., 2009; Green et al., 2009; Back et al., 2010).

THE ROLE OF THE MESOLIMBIC REWARD SYSTEM IN ADDICTION

The mesolimbic reward system is necessary for organisms to engage in reinforcing behaviors and to motivate actions that produce rewarding feelings of pleasure (Gardner, 2011). Midbrain dopamine (DA) neurons that arise in the ventral tegmental area (VTA) and substantia nigra project to forebrain regions (striatum, prefrontal cortex, hippocampus, and amygdala) that regulate the motivational and cognitive processes necessary for organisms to engage in behaviors that produce rewarding feelings of pleasure (Wise, 2004) (**Figure 1**). These pathways modulate information flow through the limbic system to regulate and/or promote behaviors related to survival and perpetuation of the species (i.e., feeding, drinking, mating, maternal and paternal behaviors, and social interaction) (White and Milner, 1992; Gardner, 2011). Chronic drug use produces a persistent enhanced activation of these systems which results in long-term structural and functional changes (Nestler, 2016). Drug-induced enhanced activation of these systems, in turn, alters the motivational and cognitive processes arising from these systems in such a way that the drive to obtain and use drugs supersedes other drives (Anselme, 2009; Goldstein and Volkow, 2011; Buchta and Riegel, 2015; Zorrilla and Koob, 2019).

VTA in Drug Addiction

The VTA contains two major classes of DA projection systems: the mesolimbic system which regulates rewarding motivational processes including feeding, drinking, sex, drug

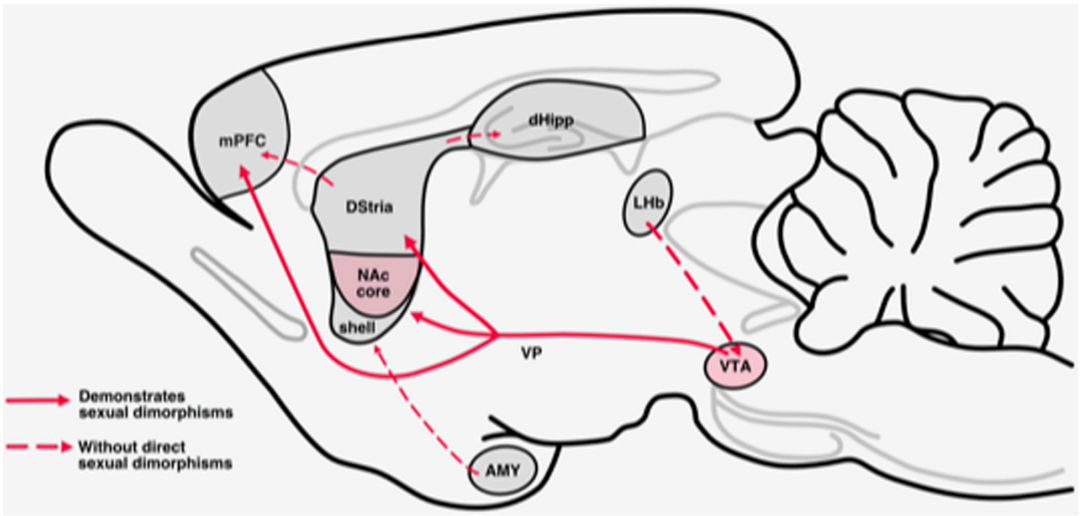

FIGURE 1 | Mesolimbic reward circuits. The mesolimbic pathway originates with dopaminergic cell bodies in the VTA. Dopamine cell bodies in the VTA are tonically inhibited by GABAergic neurons in the VTA. This pathway projects primarily to the NAc, dorsal striatum (Dstria), and medial prefrontal cortex (mPFC). Sexual dimorphisms that are established for projections within these pathways and are delineated with a solid line.

consumption and continued drug use, and the mesocortical DA system which regulates cognitive/executive functions such as working memory, drug-cue associations, cue-induced reinstatement of drug use (**Figure 1**) (Le Moal and Simon, 1991; Bjorklund and Dunnett, 2007).

Striatal Complex in Drug Addiction

The striatal complex, generally speaking, gathers inputs from the neocortex, and then sends projections to other nuclei of the basal ganglia which ultimately reach cortical areas implicated in motor planning and execution. The striatum is a structure that is highly conserved across species and critically important for broad range of cognitive, sensorimotor, and limbic-related functions. The striatal complex is anatomically divided into the ventral striatum (nucleus accumbens) and dorsal striatum (caudate putamen) (Voorn et al., 2004; Yin et al., 2004).

Dorsal Striatum

The dorsal striatum (DS) can be subdivided into dorsomedial striatum (DMS), which is vital for goal-directed learning, and the dorsolateral striatum (DLS) implicated in stimulus-response learning (Yin et al., 2005, 2006). Compulsive responding to drug-associated cues is established when the DS is engaged by the intrastriatal loops between the NAc and DS. Thereafter, exposure to drug-associated cues induces activation of the DS without the presence of the drug. Such activation of DS neurons may induce craving for the drug and has been proposed to be the initiator of drug seeking and relapse (Koob and Volkow, 2016).

Nucleus Accumbens (a.k.a. Ventral Striatum) in Addiction

The NAc is a heterogeneous structure and is subdivided into shell and core components which are chemoarchitecturally and functionally distinct (Zahm and Brog, 1992; Groenewegen et al., 1999; Di Chiara, 2002). The central area of the NAc, called the NAc core, is distinct and is surrounded medially, ventrally, and laterally by the NAc shell. The differences between the NAc core and NAc shell are defined by a number of histochemical, electrophysiological, connectional, and cellular criteria (Zahm and Brog, 1992; Groenewegen et al., 1999; Di Chiara, 2002). Anatomically, the NAc core/shell subdivisions can be differentiated in terms of the input they receive from prefrontal cortical regions: The NAc core receives the majority of its prefrontal input from the prelimbic region of the cortex and lateral orbitofrontal cortex; the NAc shell receives its cortical input from infralimbic cortex and medial lateral orbitofrontal cortex (Berendse et al., 1992; Wright and Groenewegen, 1996). These accumbal subregions are also dissociable in terms of their pallidal/nigral projection outputs: The core projects predominantly to the substantia nigra while the shell targets the pallidum and the VTA (Heimer et al., 1991; Zahm and Brog, 1992; Zahm and Heimer, 1993; Ikemoto, 2007). There is some evidence to suggest communication between MSNs within the ventral striatum. Within the NAc, the core and shell share important intra-structural connections which may be important for the combination of diverse limbic inputs to be later integrated for output to other structures (van Dongen et al., 2005, 2008).

The NAc core is critically involved in the development and expression of addiction-related behaviors (Koob and Volkow, 2010) and is recruited in Pavlovian conditioning (Koos and Tepper, 1999; Lof et al., 2007; Sunsay and Rebec, 2014). For example, typically, NAc core interacts with brain regions associated with motor circuitry, thus coordinating behavioral output, while the shell interacts with limbic and autonomic brain regions, indicating significant regulation of reward, emotional, and visceral responses to stimuli (Heimer and Alheid, 1991; Zahm and Brog, 1992; Everitt et al., 1999). As

such, the shell is suspected to mediate the reinforcing properties of novelty, feeding behavior, rewarding substances and stimuli which induce drug relapse, while the core seems to play a role in spatial learning, conditioned responses, responses to motivational stimuli, and impulsive choices. Together, the NAc core and shell control the enactment and reinforcement of conditioned behaviors through interaction with reward circuitry (Meredith et al., 2008). In this way, it is generally accepted that the NAc shell is more involved in shorter-term aspects of addiction, for instance reward; whereas, the NAc core plays a role in longer lasting reward-directed behaviors (Ito et al., 2004; Meredith et al., 2008).

Prefrontal Cortex in Drug Addiction

The VTA sends dopaminergic (DA) projections to the medial prefrontal cortex (mPFC). These DA projections activate the glutamatergic systems of the prefrontal cortex. Glutamatergic projections from the prefrontal cortex (PFC) directly activate mesocortical DA neurons in the VTA and exert excitatory control over DA cell firing and release in the PFC (Geisler and Wise, 2008). The PFC also sends glutamatergic projections to the dorsal and ventral striatum which modulates the control of pallidal/nigral pathways. In this way the PFC is in a good position to regulate salience and conditioned behavior in response to salient stimuli (Koob and Volkow, 2016). Protracted abstinence from drugs of abuse leads to over activation of the glutamatergic systems that induce strong craving-like responses via glutamatergic activation of the NAc (McFarland and Kalivas, 2001; De Witte et al., 2005), thus indicative of this circuit's importance in cravings associated with drugs of abuse. Drug-induced reinstatement also involves glutamatergic projections to the NAc that modulate DA release within the NAc (Koob and Volkow, 2016).

Hippocampus in Addiction

The hippocampus has been implicated in the formation of drug-context memories, drug-cue associations, and reconsolidation of drug memories. In addition, the hippocampus has been implicated in reinstatement of drug-taking behavior leading to relapse via cue and contextual triggers (Kutlu and Gould, 2016).

Amygdala in Addiction

The basolateral amygdala (BLA) plays a critical role in the response to natural reward and drug-associated cues. Anatomically, the BLA receives DA inputs from the VTA and provides outputs to neurons in the NAc (Floresco et al., 1998, 2001; Bissière et al., 2003; Ford et al., 2006). Because the BLA serves as an interface between VTA DAergic inputs and outputs to the PFC and NAc, it is well positioned to subserve associative memory functions. Through the convergence of DAergic inputs with sensory-associative information, BLA neurons encode emotionally salient memories (Grace and Rosenkranz, 2002; Rosenkranz and Grace, 2002). The BLA is implicated in the associative properties of opiate-related learning (Fuchs and See, 2002). Increases in activity in adjacent central nucleus of the amygdala (CeA) are associated with the anxiety-like effects of acute withdrawal and the increased drug intake associated with dependence (Koob and Le Moal, 2008).

SEX DIFFERENCES ON MESOLIMBIC REWARD SYSTEM FUNCTION

In this section, we discuss sex differences that have been identified and described within the mesolimbic reward system. Sexual dimorphisms have been established in both the underlying organization of the midbrain DA circuitry, as well as the influence of estradiol on DA activity (**Figure 1**).

Sex Differences in the VTA

Sex and levels of ovarian hormones influence DA cells in the VTA (Morissette et al., 2008; Gillies and McArthur, 2010; Johnson et al., 2010). Female rodents have a significantly greater proportion of DA neurons in the VTA compared to their male counterparts (Kritzer and Creutz, 2008). In addition, sex differences in the shape and volume of the VTA as well as in the distribution and size of DA cell populations have been identified (McArthur et al., 2007).

Although baseline firing activity of DA cells in the VTA of male and female rodents is reported to be equivalent (Locklear et al., 2017; Rincon-Cortes and Grace, 2017), the activity of VTA-DA neurons appears to be sensitive to circulating levels of estradiol (E2). For example, basal firing rates of DA neurons of the VTA vary during the different phases of the rodent estrous cycle: DA firing rates are highest in estrus, lowest in proestrus, and intermediate in diestrus (Zhang et al., 2008). Moreover, E2 replacement to ovariectomized (OVX) rats influences firing rate, spontaneous activity, DA release, DA transporter activity, and overall responsiveness of striatal neurons to DA (Zhang et al., 2008; Calipari et al., 2017). In addition, DA receptor auto-inhibition is also E2 sensitive, demonstrating greater inhibition with increasing levels of E2 over the estrous cycle and increased inhibition in OVX-E2 treated mice (Vandegrift et al., 2017). Overall, the ability of E2 to influence activity of VTA-DA neurons strongly suggests the involvement of locally expressed estrogen receptors (Shughrue et al., 1997; Creutz and Kritzer, 2002; Milner et al., 2010).

Sex Differences in the Striatal Complex

The GABAergic medium spiny neuron (MSN) is the predominant striatal neuron type (∼95%) (Gerfen and Surmeier, 2011). GABAergic MSNs of the DS are capable of influencing both motor and cognitive behaviors via their projections to other brain regions (Yager et al., 2015). GABAergic MSNs work intricately to integrate inputs from a number of different brain areas to determine the final output of the striatum. For example, GABAergic MSNs of the NAc make inhibitory connections with cells in the ventral pallidum and VTA, and receive excitatory input from the prefrontal cortex, ventral subiculum of the hippocampus and basolateral nucleus of the amygdala (Sesack et al., 2003; Kauer and Malenka, 2007). MSNs also receive other inputs (predominately excitatory) from multiple brain regions

implicated in mediating striatal function (Russo and Nestler, 2013; Scofield et al., 2016).

Robust sex differences and hormone sensitivity in the NAc core are well documented (Becker, 1999; Becker and Hu, 2008; Yoest et al., 2014). Reports of sex differences/hormone sensitivity in NAc shell are less robust and/or more variable compared to core and likely depend upon interactions with other environmental influences (Forlano and Woolley, 2010; Brancato et al., 2017). The sex differences in the NAc core appear to be mediated primarily via influences on excitatory synaptic and electrophysiological properties of NAc neurons and striatal terminals (Mermelstein et al., 1996; Wissman et al., 2011; Dorris et al., 2015). In addition, sexual dimorphisms in synaptic properties and dendritic spine density of GABAergic MSNs in the NAc core have also been identified. In females, GABAergic MSNs of NAc core have anatomically larger spines and higher dendritic spine density than in males (Forlano and Woolley, 2010) and the frequency of mEPSCs in the core is higher in females than males (Wissman et al., 2011). In both the core and shell there is no evidence for sex differences in the number of DA neurons (Forlano and Woolley, 2010; Wissman et al., 2012). Other neuroanatomical attributes such as MSN soma size, cellular density and gross region volume have not been found to be sexually dimorphic (Meitzen et al., 2011; Wong et al., 2016). Electrophysiological properties of GABAergic MSNs in the core change across the estrous cycle (Proano et al., 2018). For example, during diestrus, the excitatory synaptic input onto these MSNs decreases in magnitude, while intrinsic excitability increases. In other words, mESPC frequency and amplitude are decreased during diestrus compared to other estrous cycle phases, while properties such as action potential rheobase, threshold, input resistance, and resting membrane potential change to increase cellular excitability (Proano et al., 2018). During proestrus and estrus excitatory synaptic input increases and intrinsic excitability decreases. Frequency and amplitude of mEPSCs are also increased compared to diestrus phase (Proano et al., 2018). These findings demonstrate the likelihood that higher and lower levels of E2 differentially regulate the electrophysiological properties of GABAergic MSNs of the NAc core. Based on these data, it can be inferred that lower levels of E2 during diestrus may permit tonic activation of GABAergic MSNs while higher levels of E2 during proestrus and estrus induces GABA release.

The major target of the VTA-DA projections is striatal GABAergic MSNs (**Figure 1**). Within the NAc, subpopulations of MSNs are distinguished based on expression of DA receptor subtype and connectivity to other structures (Gerfen et al., 1990). These include, D1 receptors which have an excitatory influence on movement and reward project directly to the substantia nigra reticulata and D2 receptors which most often have inhibitory effects and project to the external segment of the globus pallidus (Deng et al., 2006; Garcia-Carmona et al., 2015; Volkow and Morales, 2015). These neurons can be further defined by their transcriptional profiles: D1substance P and dynorphin and D2 enkephlin (Gerfen et al., 1990; Lobo and Kennedy, 2006; Heiman et al., 2008). It has been suggested that 5–15% of dorsal striatum MSNs can express both D1 and D2 receptors (Lester et al., 1993; Bertran-Gonzalez et al., 2008; Perreault et al., 2011). D1-MSNs are equally distributed throughout the core and shell (Gangarossa et al., 2013). D2-MSNS are homogeneously distributed in the core but in the shell, they are more expressed in the medial and ventral shell (Gangarossa et al., 2013).

The striatum of male rats contains about 10% more D1 DA receptors than that of intact female or OVX rats. Generally speaking, there are no sex differences in the number or binding characteristics of striatal D2 DA receptors (Hruska and Silbergeld, 1980; Levesque and Di Paolo, 1988), however, one experiment reported female rats had fewer D2 receptors than males (Miller, 1983). Interestingly, E2 rapidly downregulates D2 DA receptor binding in the striatum of females (Bazzett and Becker, 1994).

Sex differences and E2 sensitivity of striatal DA kinetics have been well documented (Yoest et al., 2018). For example, administration of E2 to OVX rats increases DA release, turnover, and DA uptake (Becker and Ramirez, 1981a; Becker and Beer, 1986; Di Paolo, 1994). In addition, E2 acutely increases DA receptor density and DA binding (Di Paolo et al., 1985; Levesque and Di Paolo, 1989; Di Paolo, 1994; Shieh and Yang, 2008). Evidence from studies using intact rodents add to the above studies and clearly demonstrate sex differences in baseline DA activity and stimulated DA activity in the striatum (Becker, 1999; Becker and Hu, 2008; Becker et al., 2012). More specifically, female rats exhibit greater basal concentrations of DA and stimulated DA concentrations in the striatum compared to those of males (Castner et al., 1993; Walker et al., 2000). The ratio of levels of striatal DOPAC/DA (a measure of neurotransmitter turnover) are highest during the proestrus stage of the estrous cycle as compared to the other stages of the cycle, suggesting a greater magnitude of DA turnover when circulating levels of E2 are high (Xiao and Becker, 1994).

SEX DIFFERENCES IN THE BEHAVIORAL RESPONSE TO DRUGS OF ABUSE

Like humans, female rodents escalate administration of cocaine and opioids more rapidly than males (Becker and Chartoff, 2019). Females rodents also demonstrate higher motivation to consume cocaine and opioids than their male counterparts (Becker and Chartoff, 2019). In addition, females consume larger quantities of drug (under some but not all conditions) and experience increased rewarding effects of cocaine and morphine compared to their male counterparts (reviewed in Becker and Koob, 2016). Post abstinence, female rodents consume greater amounts of the drug and demonstrate greater dysregulation of drug intake when given free access to drugs than males (Lynch and Taylor, 2004, 2005; Fuchs et al., 2005; Kosten and Zhang, 2008). Females also demonstrate increased responsivity toward drug taking under stress than males indicating a higher intensity of negative physical and psychological withdrawal symptoms and therefore, higher relapse probability (Fox and Sinha, 2009). Lastly, female rodents more readily reinstate drug use after a

period of abstinence in the absence of any reinforcing cues when compared to males (Anker and Carroll, 2010; Buffalari et al., 2012). Taken all together, these data demonstrate sex differences in preclinical rodent models of drug administration/reward and recapitulate and extend findings from the clinical literature. Specifically, female rodents are decisively more vulnerable to developing addiction-like behaviors after exposure to drugs of abuse than males. Although animal models of drug addiction do not emulate exactly what happens in humans, the assessment of the behaviors that occur after ingestion of drugs of abuse have demonstrated good face validity in that they allow for the objective understanding of specific signs and symptoms of the addiction process. They also allow quantification of psychological constructs like drug reward and affective states. Ultimately, much of the extant scientific literature involved in the understanding of underlying neurobiology and pathophysiology of drug addiction comes from the use of animal models of drug addiction. With regard to this review, almost all evidence-based inferences to demonstrate the interaction between estradiol and mesolimbic reward circuitry toward the vulnerability of women to drug addiction have been derived from the preclinical rodent literature. This further elaborates the validity of the use of these rodent models toward understanding the basis of sex differences in drug addiction.

The Menstrual and Estrous Cycle Influence Addictive Behaviors

In females, the phase of the menstrual/estrous cycle and the release of reproductive hormones associated with each phase, influences synaptic transmission, sex-specific motivated behaviors, as well as motivation related to drug-taking and addiction-related behaviors (**Figure 2**). Therefore, it is essential that the hormonal conditions of women be taken into consideration when discussing sex differences in addiction.

The human menstrual cycle has a 28-day duration and consists of follicular, periovulatory, and luteal phases. During the follicular phase (10–12 days), estradiol (E2) is secreted from the ovary as the follicle develops and circulating concentrations of E2 increase daily. The next phase is the peri-ovulatory phase (2–4 days) during which a surge of E2 prompts the release of luteinizing hormone from the pituitary that induces ovulation. The next phase is the luteal phase (10–12 days) which is characterized by the release of relatively high concentrations of both E2 and progesterone from the remains of the ruptured follicle (corpus luteum). Menstruation occurs at the end of the luteal phase after the fall in progesterone and E2 secretion once the corpus luteum has regressed (unless pregnancy occurs). Hormone levels are at their lowest points during menstruation indicating the beginning of the next follicular phase (Becker et al., 2005). Rats and mice have a 4–5 days estrous cycle comprised of phases that function similarly to the phases in the human menstrual cycle. The rat/mouse follicular phase (2–3 days) is called diestrus. The periovulatory phase of the rat and mouse is called proestrus and occurs during the day of E2 and progesterone surges. The estrus phase of the rat/mouse cycle occurs on the day following the E2 and progesterone surges; this is when the female rodent ovulates and is sexually behaviorally receptive (Becker et al., 2005).

Cocaine

It is well established that compared to males female rats demonstrate more robust operant behavior during acquisition of cocaine self-administration, escalation of drug intake, and reinstatement of extinguished drug-taking behavior (Lynch and Carroll, 1999, 2000; Roth and Carroll, 2004). Moreover, female rats acquire cocaine self-administration more quickly and at lower doses than males (Lynch and Carroll, 1999; Davis et al., 2008).

Estrous cycle phase has been shown to influence an animal's motivation to self-administer cocaine (Roberts et al., 1989); cocaine-self administration is highest during proestrus and estrus and lowest during diestrus (Feltenstein et al., 2011). In other words, female rats consume greater amounts of cocaine when circulating levels of E2 are high and cocaine consumption is reduced at times when E2 levels are lower. The notion that different levels of circulating ovarian hormones are important for differences in the reinforcing properties of drugs is further supported by self-administration paradigms using OVX-hormone-treated rodents. Such experiments have consistently demonstrated a role for E2 in enhancing the responsivity to cocaine. More specifically, ovariectomy alone decreases the rate of acquisition of cocaine self-administration and reinstatement of previously extinguished cocaine-seeking behavior. E2 administration to OVX animals restores acquisition of cocaine self-administration to levels comparable with those of intact female rodents (Lynch et al., 2001; Hu et al., 2004; Larson et al., 2005; Frye, 2007). Furthermore, this effect was specific for female rodents since there was no effect of E2 replacement on cocaine self-administration behaviors of male rodents (Jackson et al., 2006).

Continued drug use, drug-seeking and relapse to former patterns of drug use during abstinence are heavily dependent on learning associations between the drug and environmental cues and/or contexts as well as the individual's physical and emotional reactivity to these stimuli (O'Brien et al., 1990; Enmark et al., 1997). Susceptibility of females to cocaine-associated cues and contexts is an important underlying factor in the sex-differences seen in cocaine addiction (Robbins et al., 1999). The conditioned place preference paradigm (CPP) is used to determine the conditioned rewarding effects of drugs in rodents because the contextual (environmental) cues used within the paradigm acquire secondary appetitive properties when paired with a rewarding stimulus (i.e., drug of abuse) (Bardo and Bevins, 2000; Tzschentke, 2007). Additionally, the cues/context itself acquires rewarding properties that are directly associated with the subjective rewarding effects of the drug. Sex differences in the rewarding properties of cocaine have been well illustrated using this paradigm by our group and others. Specifically, female rats demonstrate acquisition of conditioned place preference to lower doses of cocaine compared to males (Russo et al., 2003b; Zakharova et al., 2009). This indicates that at lower doses females experience an increase in the magnitude of the rewarding effects of cocaine when compared to males (Russo et al., 2003b;

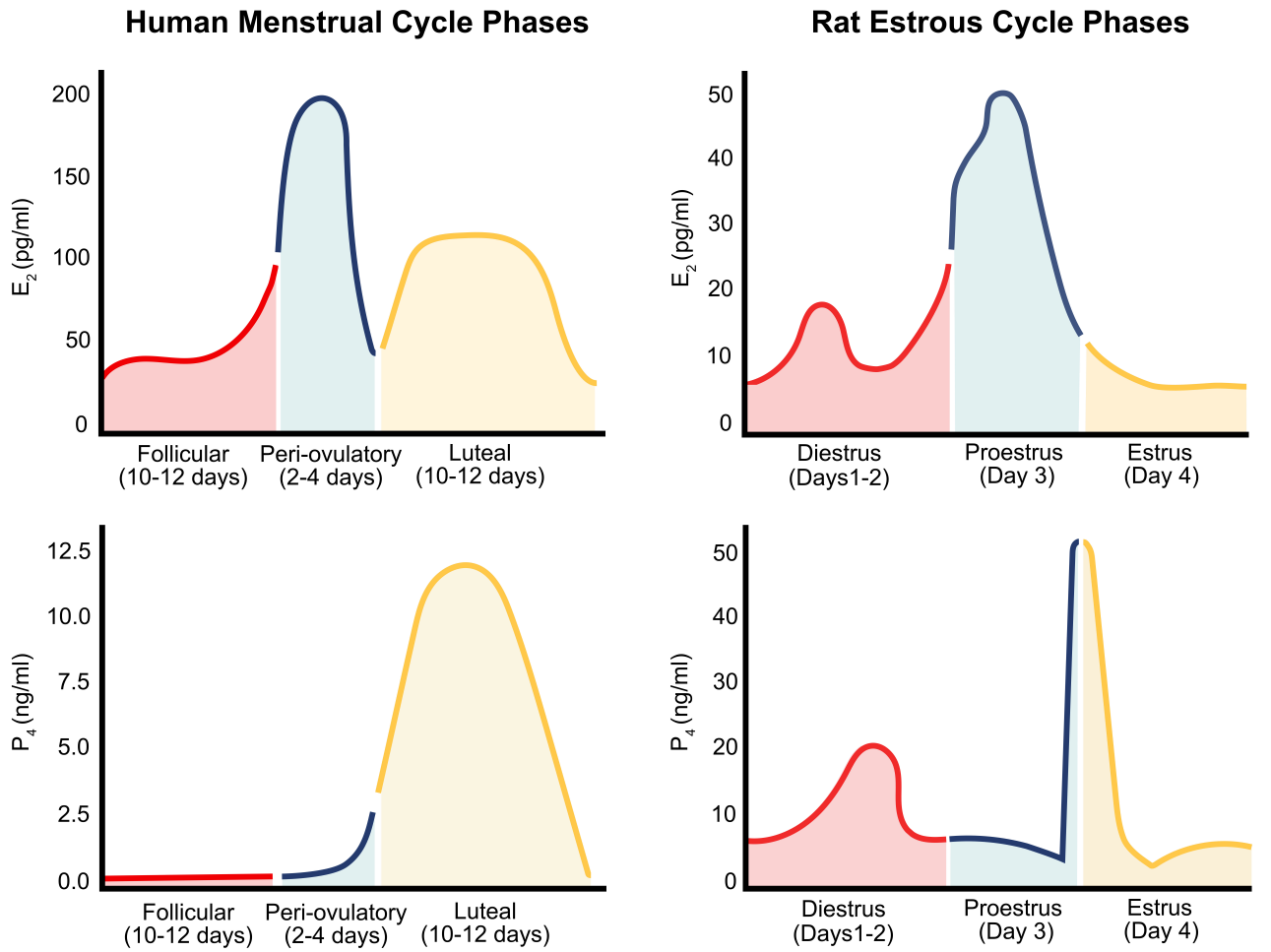

FIGURE 2 | The human menstrual cycle and rat estrous cycle. The human menstrual cycle **(left)** occurs over 28 days and is comprised of fluctuating levels of E2 (E2); (top) and progesterone (P4; bottom). Levels of E2 rise to a peak during the peri-ovulatory phase. E2 levels then drop, rise, and plateau between during the mid-luteal phase (approximately Days 14–28). Progesterone (P4) levels begin to at the end of the ovulation (Days 0–14) and reach their peak during the mid-luteal phase of the cycle (approximately Day 7). The rat estrous cycle **(right)** is similar to the human menstrual cycle but occurs over a 4/5-day period. Four phases; metestrus, diestrus, proestrus, and estrus comprise the rat estrous cycle. Estrogen levels (top) peak toward the end of diestrus and beginning of proestrus. Progesterone levels (bottom) reach a peak during mid-proestrus.

Zakharova et al., 2009). However, at higher doses, cocaine place preference is similar between males and females. This suggests that female sensitivity to cocaine's effects is dose dependent and maybe acutely enhanced at lower doses. Circulating levels of E2 influence the magnitude of CPP, however, these effects vary according to the dose and length and time course of hormone treatment. We and others have reported that OVX female rats demonstrate lower CPP scores compared to intact females (Russo et al., 2003a; Kokane et al., 2019). In a series of experiments in which chronic continual administration of E2 was given throughout the duration of the CPP paradigm, E2 treatment alone did not influence CPP when compared to untreated animals (Russo et al., 2003a). Recent studies from our laboratory, demonstrate that low dose E2 administration to OVX female rats during the conditioning phase of CPP enhances expression of cocaine place preference (Kokane et al., 2019). Conversely, a single injection of E2 prior to the test phase of cocaine CPP, blunted the expression of cocaine place preference in OVX rats (Bobzean et al., 2014a). This finding indicates that elevations in E2 after drug-associations have taken place, my actually serve to inhibit the conditioned response or decrease the salience of the stimulus. Taken together these data elaborate the influence of E2 on the rewarding and reinforcing properties of cocaine. It is also evident from the above studies that the timing of the elevations in E2 levels is important toward these effects.

Morphine

Overall, female rodents acquire both oral and IV self-administration of opioids faster than males, at lower doses, and under a wider variety of environmental housing factors (Alexander et al., 1978; Lynch and Carroll, 1999; Cicero et al., 2003). Moreover, females consume greater amounts of opioids and will work harder for a dose of an opioid drug than males (Cicero et al., 2003). In tests of CPP, female mice

demonstrate preferences for environments associated with lower doses of morphine compared to males (Karami and Zarrindast, 2008). Taken together, these experiments have identified sex differences in opiate reward and reinforcement. In addition, there are limited data suggesting that E2 enhances the salience of cues paired with opioid reward (CPP) (Roth et al., 2002; Mirbaha et al., 2009). Therefore, these findings suggest that E2 augments the rewarding/reinforcing properties of opioids and that fluctuating levels of E2 in females may increase (or decrease) their vulnerability to opioid taking behaviors.

SEX DIFFERENCES IN THE NEUROBIOLOGY OF ADDICTION

Sex differences in behavioral effects of cocaine are largely the result of underlying neurobiological changes brought about by the interactions between cocaine and ovarian hormones within reward-related circuitry. Although specific neurobiological mechanisms remain to be fully elucidated, there is much evidence implicating the combined effect of cocaine and E2 on VTA DAergic neurotransmission and on striatal GABAergic, DAergic, and glutamatergic neurotransmission. Additionally, it has been demonstrated that drug use, whether it be cocaine or opiates, produces persistent enhanced activation of the mesolimbic reward circuit resulting in long-term structural and functional changes. These neuroadaptive changes include enhancement in neurotransmitter release, increased synaptic plasticity and dendritic arborization within areas of the mesolimbic reward pathway. The increased neurotransmission (GABAergic, dopaminergic, and glutamatergic) activates downstream molecular mechanisms within these areas. Estradiol has been shown to modulate the activity of these molecular mechanisms. In this section, we discuss the influence of E2 on the molecular mechanisms which underlie the persistent effects of cocaine and morphine on the structural and functional changes in the mesolimbic reward system.

Cocaine

Cocaine exerts its psychomotor stimulant effects by increasing extracellular DA levels by binding to the dopamine transporter (DAT) in the striatum; a membrane protein located on nerve terminals responsible for the reuptake of DA from the synaptic cleft (Mortensen and Amara, 2003; Zhu and Reith, 2008). Through this inhibition of DA reuptake, cocaine increases synaptic DA levels thereby potentiating activation at postsynaptic DA receptors. Cocaine produces a buildup of DA wherever the brain has DA transporters. However, the psychoactive and addictive effects of cocaine are generated by the drug's ability to produce a buildup of DA in mesolimbic reward structures (Koob et al., 1998; Hyman and Malenka, 2001; Nestler, 2001; Kalivas and McFarland, 2003).

Activation of G-protein-coupled D1 receptors initiates a cellular signaling cascade that enhances phosphorylation of the transcription factor cAMP response element binding protein (CREB) and expression of immediate-early genes (Nestler, 2002; Walters et al., 2003). Generally speaking, the positive rewarding effects of cocaine are mediated via D1-MSNs (Hikida et al., 2010; Lobo et al., 2010; Lobo and Nestler, 2011; Bock et al., 2013; Chandra et al., 2013; Lenz and Lobo, 2013) and many cocaine-induced molecular adaptations occur within these D1 MSNs (Lobo and Nestler, 2011; Grueter et al., 2013; Lobo et al., 2013). The increases in cAMP in response to D1 receptor stimulation initiate activation of downstream protein kinases, including protein kinase A (PKA) and extracellular signal-regulated kinase (ERK). These kinases, along with many other downstream effects, can phosphorylate the transcription factor CREB (Kano et al., 1995). CREB activation in the NAc is essential as a regulator of cocaine reward and for cocaine-dependent alterations in gene expression (McClung and Nestler, 2003; Lemberger et al., 2008; Renthal et al., 2009). Post synaptic D2 receptors either inhibit or have no effect on adenylyl cyclase activity; presynaptic D2 receptors function as auto-receptors (Clark and White, 1987; Meador-Woodruff et al., 1991; Surmeier et al., 2007). Generally speaking, D1 receptors play a role in the primary rewarding properties of cocaine, while D2 receptors play a role in drug-seeking mechanisms (Self et al., 1996; Lobo and Nestler, 2011). The repeated administration of cocaine causes adaptations in such DA receptor stimulated signaling pathways which manifest into the behaviors characteristic of cocaine addiction (Anderson and Pierce, 2005; McGinty et al., 2008; Thomas et al., 2008).

Overview of Signaling Mechanisms of Cocaine Addiction

Molecular mechanisms associated with cocaine reward, reinstatement, craving and dependence involve extracellular signal regulated kinase (ERK), calcium/calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase G (PKG), phosphatidylinositol 3-kinase (PI3K) and its downstream target mammalian target of Rapamycin (mTOR), cyclin-dependent kinase 5 (Cdk5), transcription factors (cAMP response element binding protein – CREB, nuclear factor-kappa B – NF-κB, delta Fos B – ΔFosB) and brain derived neurotrophic factor (BDNF).

CREB, NF-κB, and ΔFosB are transcription factors regulating the expression of several genes involved in synaptic plasticity, neuroadaptations dendritic arborization and have been implicated in the development of cocaine addiction-related behaviors. CREB gets phosphorylated by PKA, CaMKII and ERK signaling pathway. Increased phosphorylation of CREB in the NAc, blunts the rewarding effects of cocaine thereby driving cocaine self-administration (Carlezon et al., 1998; Barrot et al., 2002; Larson et al., 2011). Through empirical evidence, it has been proposed that increased activity of CREB leads to increased excitability of NAc MSNs via increased expression and synaptic transmission of NMDARs. This may in turn induce a negative feedback loop that blunts rewarding effects of cocaine and eventually drives escalation (Dong et al., 2006). Phosphorylation of CREB also drives formation of new dendritic spines by increasing the expression of NMDARs but not AMPARs leading to the formation of "silent synapses" (Murphy and Segal, 1997; Segal and Murphy, 1998). Studies have indicated formation of "silent synapses" to be critical to the enhancement of cocaine

seeking (Huang et al., 2015). NF-κB is upregulated in NAc post chronic cocaine administration (Ang et al., 2001). It is involved in expression of cocaine reward and the induction of dendritic spines in the MSNs of NAc (Russo et al., 2009). Genes transcribed by NF-κB are essentially required in the regulation of synaptic plasticity of neurons within the mesolimbic reward regions (Engelmann and Haenold, 2016). ΔFosB is induced in the D1-type MSNs by chronic exposure to cocaine and has been proposed to be the molecular switch for addiction (Nestler, 2008; Perrotti et al., 2008). Its expression is regulated by cocaine and CREB within NAc MSNs and increases cocaine reward and self-administration (Nye et al., 1995; Vialou et al., 2012; Robison et al., 2013). It is also necessary and sufficient for cocaine-induced dendritic spine formation through increase in the expression of "silent synapses" in the D1-type MSNs of NAc. Conversely, it decreases their expression in D2-type MSNs of the NAc thereby enhancing rewarding effects of cocaine (Grueter et al., 2013). It has been shown to be critical in mediating the effects of cocaine on NAc's ability to integrate glutamatergic inputs from the hippocampus, mPFC and amygdala (Eagle et al., 2019).

Sex Differences in Signaling Mechanisms of Cocaine Addiction

Sex differences in baseline and cocaine-induced activation of cAMP and PKA pathways in the NAc have been documented. Overall, female rats exhibit higher levels of total PKA protein and phosphorylated DARPP-32 in the NAc compared to males, regardless of drug-treatment condition (drug naïve, saline-treatment, or cocaine-treatment) (Lynch et al., 2007; Nazarian et al., 2009). Moreover, males and females have different basal and cocaine-induced levels of pERK, ΔFosB, and pCREB in the NAc (Nygard et al., 2013). It is possible that this may be due to the differences in DA receptor expression and binding (Yoest et al., 2018). Studies have also shown E2 regulation of cAMP and PKA pathways. For example, the estrous cycle seems to affect the activity of a variety of intracellular signaling cascades in the NAc of female rats regardless of cocaine-treatment (Weiner et al., 2009). Ovariectomized females have been used in attempts to identify a role of E2 on these intracellular signaling cascades. The results of one study show that OVX E2 treated females demonstrate E2-induced initiation of PKA cascades and CREB protein phosphorylation via activation of G-protein dependent cell signaling cascades (Hammes and Levin, 2007). The downstream effects of these results likely contribute to the structural sexual dimorphisms seen in dendritic morphology and spine density (Forlano and Woolley, 2010; Wissman et al., 2011, 2012).

Estrogen Receptors Influence Cocaine-Induced Intracellular Signaling

It is well established that estrogens produce their effects by genomic and non-genomic actions. The so-called "genomic or classical estrogen receptors" are ligand-activated transcription factors which reside in the cytosol and translocate to the nucleus upon ligand binding and dimerization (Nilsson et al., 2001). As with other steroid hormone receptors, ERs can either modulate gene expression directly, by binding to consensus target DNA sequence, or indirectly, by interacting with other transcription factors to activate or repress gene activation. Estrogen also has acute, rapid (non-genomic) effects, which are initiated via binding at plasma associated membrane estrogen receptors (mER) (Boulware et al., 2005; Mermelstein and Micevych, 2008; Micevych and Mermelstein, 2008). Signaling at mERs activates G-protein dependent cell signaling cascades (Hammes and Levin, 2007), including PKA and MAPK (Dhandapani and Brann, 2002; Bjornstrom and Sjoberg, 2005; Ronnekleiv et al., 2007; Vasudevan and Pfaff, 2007). These signaling cascades are similar to those initiated by DA at D1 receptors. In fact, evidence for the role for mERs in mediating the rapid effects of E2 stems from its effects on CREB phosyphorylation (pCREB). ERα and ERβ antagonists mimic the effects of E2 while the mER antagonist ICI 182,780 blocks the rapid effects of E2 on pCREB. In this way, E2 activates both mER and D1 receptor G-protein-dependent cell signaling cascades including activation of the MAPK pathway, and phosphorylation of CREB (Hammes and Levin, 2007).

Adult female rats predominantly express membrane-bound ERs (GPER1, membrane associated ERα and ERβ) in MSNs of the DS and NAc, but express few or no nuclear ERs (Mermelstein et al., 1996; Kuppers et al., 2008; Schultz et al., 2009; Grove-Strawser et al., 2010; Almey et al., 2012, 2015). Membrane ERs are expressed on axon terminals, somas, and dendritic spines (Almey et al., 2012, 2015, 2016). Functionally, the activation of MSN membrane ERs have been shown to increase sensitivity to drugs of abuse in females (Tonn Eisinger et al., 2018) and change dendritic spine morphology and density in the NAc (Peterson et al., 2015). Previous work has established that application of E2 rapidly increases DA (Becker, 1990; Pasqualini et al., 1996) and decreases GABA production (Hu et al., 2006) in the NAc and DS which suggests that E2 may indirectly influence DA signaling by first releasing inhibition of GABAergic signaling, and perhaps also directly upon DA-producing regions (such as the VTA). In striatal MSNs, E2, acting through membrane-associated ER alpha and beta receptors coupled to mGluRs modulates phosphorylation of the transcription factor CREB (Mermelstein et al., 1996; Grove-Strawser et al., 2010).

Similar to the DS, E2 also rapidly modulates glutamate signaling in NAc core and these effects are sex-specific and bidirectional (Krentzel et al., 2019). The mechanism whereby E2 enhances drug-induced plasticity is via interactions with mGluRs. Specifically, E2 activates mGluR5 signaling in the NAc core, which in turn, leads to alterations in dendritic structure (Grove-Strawser et al., 2010; Martinez et al., 2014; Peterson et al., 2015). These alterations induce neuroadaptations which are long-lasting and cause long-term enhanced activation of the mesolimbic reward pathway. As a result, activation of the reward pathway and DA release in response to "naturally" rewarding stimuli is insufficient. Hence, normal rewarding stimuli become less rewarding and full activation of the reward pathway requires drug consumption.

Estradiol and DA systems interact to modulate striatal function and resultant behavior (Di Paolo et al., 1985; Becker and Beer, 1986; Becker, 1990; Bitar et al., 1991). For example, E2 increases striatal DA release and turnover (Becker and Ramirez, 1981b; Becker et al., 1984; Di Paolo et al., 1985; Becker and

Beer, 1986) and density of striatal DA uptake sites (Morissette et al., 1990). Post-synaptically, E2 increases striatal D1 receptors, while decreasing high-affinity D2, and increasing low-affinity D2 binding (Di Paolo et al., 1985; Levesque and Di Paolo, 1988, 1989; Morissette et al., 1990; Shieh and Yang, 2008). Presynaptically, E2 potentiates amphetamine-induced DA release and turnover in the NAc (Becker et al., 1984; Di Paolo et al., 1985; Thompson and Moss, 1994). Additionally, E2 promotes the sensitivity of VTA-DA neurons to cocaine (Zhang et al., 2008) which, in turn, enhances cocaine-stimulated release of DA in the striatum (Peris et al., 1991; Febo et al., 2003). Therefore, the potentiating effects of E2 on striatal DA activity are, in part, a cause of the sex and hormone-related differences in subjective and physiological responses to cocaine (Becker and Ramirez, 1981b; Becker and Rudick, 1999; Walker et al., 2001; Quinones-Jenab, 2006). The effect of E2 in the female striatum is also mediated by E2 receptors on GABAergic MSNs that enhance DA release via disinhibition of local DAeric terminals (Mermelstein et al., 1996; Mermelstein, 2009; Schultz et al., 2009; Grove-Strawser et al., 2010). Thus effects of E2 on cocaine self- administration and CPP may be due to the ability of E2 to act on mesolimbic DA system to regulate reward and motivation, through the ability of E2 to increase cocaine-stimulated DA release in NAc (Tobiansky et al., 2016) and alter signaling pathways and gene expression in striatum (Le Saux et al., 2006; Grove-Strawser et al., 2010; Peterson et al., 2015, 2016).

Interactions between E2 and cocaine have been demonstrated in that E2 enhances the sensitivity of the DAergic neurons of the VTA to cocaine and increases cocaine-induced DA release in the striatum (Peris et al., 1991; Zhang et al., 2008). A recent study by Calipari et al. (2017) demonstrated that female mice in estrus showed increased cocaine-induced DA release compared to female mice in diestrus or male mice. Indeed, these neurobiological effects resulted from greater firing rate of VTA-DAergic projections and DA release in the NAc of female mice in estrus (Zhang et al., 2008; Calipari et al., 2017). Moreover, these estrous females also displayed increased phosphorylation of dopamine transporter (DAT) protein. Taken together, it can be hypothesized that higher levels of circulating E2 increase DA levels in the NAc which is due to increased inhibition of DAT activity under the influence of cocaine (Calipari et al., 2017).

E2 stimulation of mERs rapidly stimulates MAPK-dependent pCREB, and peripheral administration of E2 initiates MAP kinase and PKA signaling pathways (Mhyre and Dorsa, 2006; Dewing et al., 2007, 2008; Kelly and Ronnekleiv, 2008) and decreases L-type calcium channel-mediated CREB activity (Gu et al., 1996; Zhou et al., 1996; Boulware and Mermelstein, 2005; Boulware et al., 2005, 2007). Inhibition of either MEK or PKC significantly inhibits E2-mediated DA efflux, while inhibiting PI3 kinase or PKA does not affect E2-mediated DA efflux (Alyea and Watson, 2009). E2 induction of TH involves membrane-initiated E2 signaling, rapid activation of dual PKA/MEK signaling pathways, leading to pCREB activity (Maharjan et al., 2010), while the mER antagonist ICI 182,780 blocks the rapid effects of E2 on pCREB. Thus, mER mediated activation of these intracellular signaling cascades influences the activity of a variety of transcription factors which likely contribute to gene transcription independently of nuclear ER (Mhyre and Dorsa, 2006).

Although extensive research in male animals has attributed several different intracellular signaling cascades to different processes associated with cocaine-addiction, studies of these same intracellular signaling mechanisms in females is almost non-existent. However, based on the above discussion, estradiol and cocaine separately or together, affect all of the aforementioned signaling molecules/transcription factors. Further research demonstrating the influence of estrogen/estradiol on the function/activity of these signaling molecules, transcription factors and BDNF is essentially required.

Morphine

Morphine and other opioids exert their rewarding effects through stimulation of mu opiate receptors (MORs) localized at the GABAergic terminals in the VTA (Johnson and North, 1992a; Bonci and Williams, 1997). Opioid activation of MORs disinhibits VTA-GABA neurons, which in turn, increases the release of DA to the NAc (Pontieri et al., 1995; Lecca et al., 2007). The increased release of DA in the NAc induces feelings of euphoria and promotes the development of drug dependence (Wise and Rompre, 1989; Johnson and North, 1992b; Bodnar, 2014).

Overview of Morphine Addiction Mechanisms

The MOR is a conventional G-protein coupled receptor in that it is a cell surface protein with seven transmembrane domains consisting of α, β, and γ subunits, and an effector protein. Upon activation by an agonist, the Gα and Gβγ subunits dissociate from one another and subsequently regulate a variety of intracellular effector pathways. In case of the VTA-GABAergic interneurons, morphine activation of MORs, suppresses the release of GABA within the VTA thereby promoting DA release from the DAergic projections arising from the VTA. Acute morphine treatment followed by prolonged abstinence produces burst firing of VTA-DA neurons which is believed to play a role in encoding reward value (Schultz, 2002; Jalabert et al., 2011; Fields and Margolis, 2015). Chronic exposure to morphine results in compensatory changes in the cell that oppose the initial alterations observed after acute exposure. Chronic morphine treatment has been shown to reduce the size of VTA-DA neurons (Russo et al., 2007) and increase basal firing rate (Koo et al., 2012) in male rats thus upregulating levels of DA in VTA-NAc circuits (Beitner-Johnson et al., 1991). Therefore, the rewarding effects of opioids are linked to the drug's ability to activate the mesolimbic DA pathway (Di Chiara and Imperato, 1988; Pierce and Kumaresan, 2006).

The VTA receives inputs from the tail of the VTA (tVTA)(Perrotti et al., 2005)/rostromedial tegmental nucleus (RMTg) (Jhou et al., 2009a) and the NAc (**Figure 3**). This input is believed to modulate the activity of VTA-DA neurons (Koo et al., 2012; Tan et al., 2012; van Zessen et al., 2012; Taylor et al., 2016). Studies using male rodents show that opioid drugs induce rewarding effects through inhibition of GABAergic interneurons, leading to excitation of VTA-DA neurons (Gysling and Wang, 1983; Johnson and North, 1992b). This VTA-DA excitation is

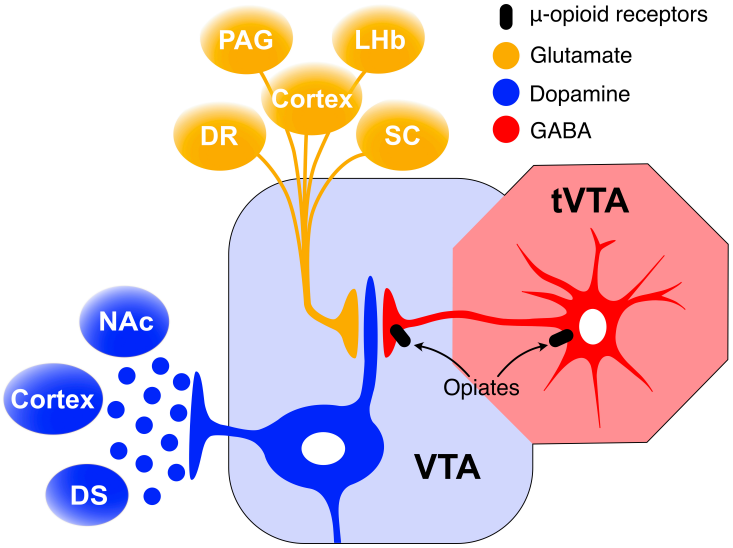

FIGURE 3 | GABA inhibition from the tVTA/RMTg mediates VTA-DA excitation. The main input of the tVTA is a glutamatergic projection from the lateral habenula (LHb) while its main output is a GABAergic projection to the VTA. tVTA-GABAergic neurons are densely packed with mu opioid receptors the activation of which decreases their firing rate. As a result, DA neurons in the VTA are disinhibited and freely transmit DA to projection sites in the NAc, DS, and cortex.

mediated via GABAergic projections from the tVTA/RMTg. The tVTA/RMTg, caudal to the VTA, is a GABAergic area innervating VTA-DA cells (Bourdy et al., 2014). MORs are densely packed on these tVTA-GABAergic neurons (Jhou et al., 2009b, 2012; Jalabert et al., 2011; Kaufling and Aston-Jones, 2015), and MOR binding decreases their firing rate (Jalabert et al., 2011; Kaufling and Aston-Jones, 2015). Consequently, DA neurons in VTA are disinhibited and freely transmit DA to projection sites in the NAc and other limbic regions which leads to overstimulation of the circuitry mediating addiction-related behaviors. Acute morphine treatment activates VTA-DA neurons via inhibition of GABAergic projections from the tVTA/RMTg (Lecca et al., 2012; de Guglielmo et al., 2015; Kaufling and Aston-Jones, 2015). Naloxone precipitated withdrawal from chronic continual morphine treatment has been shown to increase release of GABA in the tVTA of male rats (Kaufling and Aston-Jones, 2015). Although there is no single brain region responsible, a growing body of evidence supports morphine withdrawal-induced increases in tVTA GABA release in triggering the intracellular events that manifest aspects opiate withdrawal syndrome (Madhavan et al., 2010; Kaufling and Aston-Jones, 2015).

The molecular mechanisms underlying opioid reward and dependence are under the direct control of G-protein activation including adenylyl cyclases, ion channels, and components of the mitogen activated protein kinase (MAPK) cascade. For example, agonist stimulation of opioid receptors leads to an inhibition of adenylate cyclase activity and a reduction of cyclic adenosine monophosphate (cAMP) levels in the cell (Sharma et al., 1977; Chakrabarti et al., 2016) as well as a suppression of the activity of protein kinase A (Fleming et al., 1992; Zhang and Pan, 2010). In addition, the Gα subunit directly interacts with G-protein inward rectifying potassium channels leading to increased hyperpolarization of the cell, and reduced cell excitability (North et al., 1987; Law et al., 2000). The dissociated Gβγ subunit is responsible for the direct blockade of calcium channels therefore reducing intracellular calcium concentrations (Moises et al., 1994), which leads to suppression neurotransmitter release.

Following removal of the opiate, the functional activity of the cAMP pathway increases continuously. These changes in the functional state of the cAMP pathway are regulated through stimulation of adenylyl cyclases and protein kinase A as a consequence of chronic administration of opiates. The phosphorylated cAMP response element (CRE)-binding protein (pCREB), and related proteins, are indicators of protein kinase A activity. Studies in male rodents indicate that CREB is essential for morphine-induced changes in gene expression precipitating reward- and withdrawal-associated behavior: opioid withdrawal upregulates cAMP by increasing adenylyl cyclase activity, leading to increased pCREB levels in reward-related brain regions.

Sex Differences in the Neurobiological Response to Opiate Reward

Sex-based differences in the cellular and neurobiological mechanisms underlying sex differences in the rewarding properties of opioids are poorly understood (Dahan et al., 2008; Lee and Ho, 2013; Huhn et al., 2018). Converging evidence from the reproductive and pain literatures suggests interactions among opioids, gonadal hormones, opioid receptors, and estrogen receptors may underlie sex differences in addictive responsiveness to opioid drugs (Lee and Ho, 2013).

There is evidence demonstrating sex differences in the development of tolerance and dependence to morphine (Craft et al., 1999, 2004; Dahan et al., 2008). Sex differences in

the potency of morphine seem to account for differences in tolerance when animals are exposed to the same dose of morphine (Barrett et al., 2001). However, most of what is currently known about opioid dependence and withdrawal comes from studies conducted exclusively in males. Results of the few sex differences studies that have been conducted show that male rodents express a greater magnitude of withdrawal symptoms than females during spontaneous withdrawal from chronic morphine administration (Cicero et al., 2002), but not naloxone-precipitated withdrawal (Ali et al., 1995; Cicero et al., 2002). However, caution should be taken when evaluating the methods and results of these experiments and others; at this point in time, most withdrawal scales have been developed using only male animals. As such, a detailed withdrawal syndrome for the female phenotype is currently unknown.

Recently, work from our lab has begun to establish sex differences in the behavioral response and molecular signaling within the tVTA resulting from morphine withdrawal (Bobzean et al., 2019). We observed sex differences in the expression and duration of spontaneous somatic morphine withdrawal. Morphine dependent male rats displayed a more severe opiate withdrawal syndrome sooner after cessation of morphine, while females displayed a more protracted withdrawal syndrome which lasting at least 72h after termination of morphine treatment (Bobzean et al., 2019). In addition, we demonstrated a correlation between activation of CREB in the tVTA and severity of morphine withdrawal symptoms in females (Bobzean et al., 2019). Overall, these findings indicate that ovarian hormone status may influence the severity and/or persistence of symptoms and CREB expression.

Sex differences and hormonal regulation of opioid receptor binding and density as it relates to reproductive behaviors are well known. Several studies have identified sex differences and hormonal regulation of opioid receptor densities (Limonta et al., 1991; Maggi et al., 1991, 1993; Dondi et al., 1992; Piva et al., 1995), distribution, and signaling efficiency (Lee and Ho, 2013; Huhn et al., 2018). There is evidence demonstrating sex differences in the development of analgesic tolerance and dependence to morphine (Craft et al., 1999, 2004; Dahan et al., 2008). Sex differences in the potency of morphine seem to account for differences in tolerance to the antinociceptive effects of the drug when animals are exposed to the same dose of morphine (Barrett et al., 2001). Although males develop analgesic tolerance to chronic morphine administration more quickly than females, one study observed higher NAc glutamate levels in morphine-tolerant female rats (Mousavi et al., 2007). Increased glutamate in NAc induces tolerance by downregulating glutamate transporters (Yang et al., 2008).

In summary, studies of sex differences in opioid withdrawal are severely lacking, and the few that do exist report inconsistent results: male rodents express greater somatic symptoms of opioid withdrawal than females (Cicero et al., 2002; Towers et al., 2019); females are more sensitive than males (Ali et al., 1995; Papaleo and Contarino, 2006); no sex differences, or females more severely affected after acute morphine administration or naloxone-precipitated withdrawal (el-Kadi and SharifI, 1994; Ali et al., 1995; Craft et al., 1999; Cicero et al., 2002; Papaleo and Contarino, 2006; Bodnar and Kest, 2010). Aside from methodological differences (i.e., drug dose, exposure frequency/duration, withdrawal sign measured, etc.), these inconsistencies may be due, in part, to lack of assessment of estrous cycle phase in females (Bobzean et al., 2014b; Becker and Chartoff, 2019). Currently, most of what is currently known about opioid withdrawal has been derived from studies exclusively in males and then extrapolated to females as most withdrawal scales have been developed using male subjects (Arfken et al., 2001; Puigdollers et al., 2004). The dearth of studies characterizing opioid withdrawal in females is surprising, given reports that sex and ovarian hormone activity modulate pain sensitivity (Berkley, 1997; Gureje et al., 1998; Stoffel et al., 2003; Fillingim et al., 2009; Green et al., 2009; Mogil, 2012), pain inhibition (Negus et al., 2002; Craft, 2003; Stoffel et al., 2003), nociception processing/opioid analgesia (Mogil, 2012; Amandusson and Blomqvist, 2013), and sensitivity to stress (Hodes, 2018), all variables known to promote opioid use.

SUMMARY

The evidence identifying important differences in the patterns of drug use, abuse, and addiction between men and women implicates sex as a risk factor for developing substance use disorder and as a powerful component affecting the course of treatment. Sex differences and sensitivity to gonadal hormones within the structure and functions of the mesolimbic reward system are linked to sex differences in the neurobehavioral response to drugs of abuse. It has also been demonstrated that the underlying cause of these sex differences on DA signaling is the cyclic fluctuation in E2 levels and its downstream neurobiological effects.

The overarching goal of much of the work reviewed in this paper is to determine how hormonal cycles affect the development of addictive phenotypes. Beginning in the 1990s studies identified estrous cycle effects on DA release and uptake in the NAc and DS (Thompson and Moss, 1994; Xiao and Becker, 1994; Thompson and Moss, 1997; Cecconi et al., 2004). Since then, data have showed that striatal DA release is increased during estrous cycle phases associated with high levels of E2 (Yoest et al., 2018). Moreover, DA clearance is shown to be substantially lower during these same phases of the cycle and higher during phases with low levels of circulating E2 (Yoest et al., 2018). In addition, basal firing rates of DA neurons of the VTA vary during the different phases of the rodent estrous cycle: DA firing rates are highest in estrus, lowest in proestrus, and intermediate in diestrus (Zhang et al., 2008). More recent work, has confirmed and extended these findings by demonstrating high levels of E2 enhance DAergic responses to cocaine and cocaine-associated rewarding cues (Calipari et al., 2017). This, in turn, leads to increased VTA-NAc responses to the drug-associated cues alone even at later stages of the cycle (Calipari et al., 2017). Moreover, cues paired with cocaine during estrus

results in greater activation of striatal brain regions (Johnson et al., 2019). Taken all together, these data demonstrate a potential mechanism by which drug-induced potentiation of the activity of DA projections from the VTA to the NAc during estrus could motivate self-administration behavior and increased responsitivity to drug-associated cues during other phases of the cycle.

Over 30 years of research has established that cyclic fluctuations of E2 modulate the mesolimbic reward. Parallel research demonstrates that mesolimbic reward pathways become dysregulated under states of chronic drug use. As these two areas of research converge, it becomes readily apparent that fluctuations in levels of E2 over the hormonal cycle of the female likely modulate the drug-induced dysregulation of the mesolimbic DA reward pathway over the course of individual's drug taking career. Therefore, when considering sex differences in addiction, it is critical to consider female hormonal cycles.

AUTHOR CONTRIBUTIONS

SK and LP participated in drafting various sections of the manuscript and revising it critically for important intellectual content. LP gave final approval of the version to be submitted.

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Methylation Patterns of the *HTR2A* Associate With Relapse-Related Behaviors in Cocaine-Dependent Participants

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Relapse during abstinence in cocaine use disorder (CUD) is often hastened by high impulsivity (predisposition toward rapid unplanned reactions to stimuli without regard to negative consequences) and high cue reactivity (e.g., attentional bias towards drug reward stimuli). A deeper understanding of the degree to which individual biological differences predict or promote problematic behaviors may afford opportunities for clinical refinement and optimization of CUD diagnostics and/or therapies. Preclinical evidence implicates serotonin (5-HT) neurotransmission through the 5-HT_{2A} receptor (5-HT_{2A}R) as a driver of individual differences in these relapse-related behaviors. Regulation of 5-HT_{2A}R function occurs through many mechanisms, including DNA methylation of the *HTR2A* gene, an epigenetic modification linked with the memory of gene-environment interactions. In the present study, we tested the hypothesis that methylation of the *HTR2A* may associate with relapse-related behavioral vulnerability in cocaine-dependent participants versus healthy controls. Impulsivity was assessed by self-report (Barratt Impulsiveness Scale; BIS-11) and the delay discounting task, while levels of cue reactivity were determined by performance in the cocaine-word Stroop task. Genomic DNA was extracted from lymphocytes and the bisulfite-treated DNA was subjected to pyrosequencing to determine degree of methylation at four cytosine residues of the *HTR2A* promoter (-1439, -1420, -1224, -253). We found that the percent methylation at site -1224 after correction for age trended towards a positive correlation with total BIS-11 scores in cocaine users, but not healthy controls. Percent methylation at site -1420 negatively correlated with rates of delay discounting in healthy controls, but not cocaine users. Lastly, the percent methylation at site -253 positively correlated with attentional bias toward cocaine-associated cues. DNA methylation at these cytosine residues of the *HTR2A* promoter may be differentially associated with impulsivity or cocaine-associated

environmental cues. Taken together, these data suggest that methylation of the *HTR2A* may contribute to individual differences in relapse-related behaviors in CUD.

Keywords: cocaine use disorder, *HTR2A*, methylation, impulsivity, attentional bias, rs6311, A-1438G

INTRODUCTION

Cocaine use disorder (CUD) continues to be a significant public health problem, with rising cocaine-related overdose deaths linked to the growing opioid crisis (1). Cocaine use begins against a background of genetics and environment, although the intricate interplay between these variables is still poorly understood and differs between individuals, presenting a barrier to understanding the origins of CUD as well as moving forward toward efficacious pharmacotherapeutics. Motor impulsivity (behavioral disinhibition) and impulsive choice (decision-making) are two dimensions of impulsivity that associate with CUD relapse-associated behaviors (2–5). Impulsivity is interlocked with cue reactivity (6, 7) which is defined as the attentional orientation toward drug-associated stimuli that predict reward (8, 9). Cocaine-dependent subjects often present with high levels of impulsivity (3, 4, 10) and cue reactivity (11–13). Furthermore, high levels of impulsivity are negatively correlated with treatment retention in cocaine-dependent individuals (10, 14). At present, CUD is a chronic relapsing disorder with no known Food and Drug Administration-approved medications.

Gene expression and function are regulated through genetic and epigenetic mechanisms essential for cellular differentiation, function, and development (15–17). DNA methylation is one of several types of epigenetic modifications which is essential in imprinting chromosomal DNA with the memory of past gene-environment interactions (15, 18). At the core of epigenetics is a series of proteins that establish DNA methylation and histone modification patterns (writers), those that interpret these patterns by selective binding (readers), and those that erase the patterns (erasers) during epigenetic reprogramming (19). Methylation occurs at CpG islands, which are stretches of DNA with a higher concentration of cytosines and guanines (18). These CpG islands are located in the promoter region of genes and DNA methylation at CpG sites generally results in inhibited gene transcription through obscured transcription factor binding sites (18, 20). Up to 70–80% of cytosines in CpG islands are subjected to methylation and altered epigenetic profiles are associated with a wide range of neuropsychiatric diseases (21, 22), including substance use disorders (23).

Studying the influence of epigenetic differences provides an opportunity to greatly improve diagnosis and treatment outcome by identifying interindividual responses to facilitate the development of precision pharmacotherapies. Epigenetics is a vital point of regulation necessary to fine tune transcriptional and translational processes such that the cell and ultimately the organism adapts to its environment. There is mounting evidence that serotonin (5-HT) neurotransmission through the 5-HT_{2A} receptor (5-HT_{2A}R) controls neural mechanisms underlying relapse-related behavioral vulnerability to cocaine, including both impulsivity and cue reactivity (for reviews) (6, 7). In preclinical models, selective 5-HT_{2A}R antagonists administered systemically consistently reduce impulsivity and cue-primed drug seeking (24–29). In particular, pharmacological and genetic association studies implicate 5-HT_{2A}R regulation of impulsive choice (30–33). Further, methylation of the *HTR2A* (human 5-HT_{2A}R gene) is implicated as a contributor to schizophrenia and mood disorders (34–36), but has not been interrogated in impulsivity or CUD relapse-related behaviors. Here, we profiled the methylation pattern of the *HTR2A* promoter from blood lymphocyte DNA collected from healthy controls and cocaine-dependent participants. Tracking epigenetic changes in the blood provides a useful clinical tool as gene targets in the periphery, like the brain, are subjected to an array of changes following exposure to environmental stimuli. We tested the hypothesis that the DNA methylation profile of the *HTR2A* promotor from peripheral lymphocytes aligns to individual differences in impulsivity and cocaine cue reactivity in healthy controls and cocaine-dependent participants.

MATERIALS AND METHODS

Participants

The sample consisted of 48 healthy controls and 53 cocaine-dependent participants recruited from ongoing studies at the University of Texas Health Science Center at Houston (n=10 healthy controls; n=10 cocaine users) or at the Institute for Drug and Alcohol Studies at Virginia Commonwealth University (VCU) (n=38 healthy controls; n=43 cocaine users) using the same diagnostic, psychometric, and advertising procedures. Participants were recruited *via* newspaper advertisements and were initially screened by a brief telephone interview. Individuals were excluded if they indicated significant psychiatric or medical conditions, including a self-reported history of severe brain injury. Following the phone screen, eligible participants attended an in-person intake assessment session, during which they completed a medical history and physical examination and were screened for psychiatric disorders using the structured clinical interview for DSM-IV (SCID-I) (37). Information about the participants' demographic and drug use history was also collected at the intake interview. All participants were urine tested for cocaine (benzoylecgonine), tetrahydrocannabinol (THC), opiates, amphetamine, methamphetamine, and benzodiazepines using integrated E–Z split key cup II (Innovacon Company, San Diego, CA) on each visit to the clinic.

Eligible participants met current DSM-IV criteria for cocaine dependence, did not meet DSM-IV current dependence criteria

for drugs other than cocaine, marijuana, nicotine, or alcohol and did not have current or past medical disorders affecting the central nervous system. The cocaine-dependent sample included both treatment-seeking (n= 41) and non-treatment-seeking (n=12) participants. The treatment-seekers were part of studies in which they received manualized cognitive behavioral therapy and were randomized to either placebo or any one or combination of the following medications: levodopa/carbidopa and/or citalopram. All data from treatment-seekers were collected at intake prior to the start of medication or behavioral therapy; therefore treatment-seekers and non-treatment-seekers were grouped together for the analyses. The healthy control group consisted of participants who had a negative urine drug screen, negative breathalyzer test, and did not have any current or past DSM-IV axis I disorders (including any substance dependence) or medical disorders affecting the central nervous system. Healthy controls were recruited *via* similar advertising procedures as the cocaine-dependent participants. Healthy controls who were also smokers (n=13) were excluded from all analyses except for genotyping due to the influence of nicotine on baseline impulsivity task performance [(38–41); Moeller laboratory, unpublished observations].

All participants were free of alcohol at the time of testing as determined by a breathalyzer (Intoximeters, Inc., St. Louis, MO). Female participants were excluded if they had a positive urine pregnancy test. All participants were compensated for their participation. Participants were fully informed of the nature of the research and provided written consent for their involvement in accordance with the Declaration of Helsinki. The studies from which participant data were included were approved by the University of Texas Health Science Center at Houston, VCU, and University of Texas Medical Branch Institutional Review Boards.

Barratt Impulsiveness Scale (BIS-11)

The BIS-11 is one of the most commonly used questionnaire-based measures of trait impulsivity (42). The BIS-11 is a 30-item self-report scale with three oblique second order factors: (1) attentional/cognitive impulsivity, measuring tolerance for cognitive complexity, and persistence; (2) motor impulsivity, measuring the tendency to act on the spur of the moment; and (3) non-planning impulsivity, measuring the lack of sense of the future. Items were rated from 1 (absent) to 4 (most extreme); total scores are a summation of attention, motor, and non-planning attributes, and ranged from 30–120, with non-psychiatric controls generally scoring 50–60. The BIS-11 questionnaire was completed by most participants except for two participants from the healthy controls and four participants from the cocaine users, these individuals were not included in the BIS-11 analyses.

Adjusting Delay Discounting Task

The adjusting delay discounting task (43, 44) is designed to measure discounting rate when participants are presented with the possibility of receiving a hypothetical reward determined using a choice algorithm. The task (as previously described) (39) is presented on a computer screen displaying two large command buttons, one on the left and one on the right side of the screen, in which the choices are presented. The left button always displays an immediate adjusting reward, and the right button displays a delayed reward. Participants were exposed to a series of choices with varying reward magnitudes and delay periods during which indifference points between the adjusted immediate reward and the delayed reward were recorded for each set of delays. Participants were randomly assigned to complete the assessment in either ascending or descending order of delays. Choice presentations ended once indifference points were determined for each magnitude at each delay. The indifference points were the analyzed for each participant using a nonlinear regression and the following equation: $V = 1/(A+kD)$ (44, 45), where V is the indifference point, A is the amount of the delay reward, and D is the delay. The result of the regression is the best-fitting k, this is a free parameter related to the rate of discounting. The natural logarithm of transformation, log10k, was used to normalize the distribution of k across participants for further statistical analyses. The adjusting delay discounting task was completed by most participants except for 14 healthy controls and 15 cocaine users; these individuals were excluded from the delay discounting analyses.

Cocaine-Word Stroop Task

The cocaine-word Stroop task was designed to measure attentional bias to cocaine-related stimuli (38, 46–50). As previously described (38, 47, 48, 50), each analyzed session began with a block of 60 practice trials, two blocks of 30 trials with cocaine-related words, and two blocks of 30 trials with neutral words. Trials with correct responses and reaction times larger than 200 msec were used to calculate mean reaction times (38, 47, 48, 50). Attentional bias was operationalized as the difference between the reaction times (in msec) observed in trials with cocaine-related words and trials with neutral words, calculated for each subject and averaged across subjects (51). A correct response was defined as responding to the word color on an appropriately colored response button. Accuracy was assessed as the ratio of correct trials to total trials within each block type. The cocaine-word Stroop task was completed by healthy controls and most cocaine users except for five; these individuals were excluded from the attentional bias analyses.

DNA Collection and Isolation

Venous blood (10 ml) from each subject was centrifuged at 2,000 rpm for 30 min (Eppendorf North America, Inc., NY). The buffy coat, containing lymphocytes and platelets, was removed, and stored in 2.0 ml cryogenic vials at -80°C. DNA was isolated from the buffy coat using the Puregene Kit (Qiagen Inc., CA) according to manufacturer's recommendations. Purified DNA for each subject was dissolved in 0.25 ml of DNA hydration solution. An aliquot of each DNA sample (50 μl) was transferred to a 96-well plate for pyrosequencing analysis by the Assay Development Service Division at the University of Texas Medical Branch.

HTR2A Pyrosequencing

The methylation state of CpG sites within the *HTR2A* promoter was evaluated through bisulfite conversion of genomic DNA with subsequent pyrosequencing. Briefly, DNA was subjected to bisulfite conversion using an Epitect bisulfite kit (Qiagen) following the manufacturer's recommendations. CpG sites were then interrogated with a PCR – pyrosequencing approach (52). Bisulfite-converted DNA was used as template in PCR reactions consisting of 12.5 µl of PyroMark Master Mix containing coral load reagent (Qiagen) or iQ supermix™ (Bio-Rad, Hercules, CA, assay CpG 102) that was mixed within a 25 µL PCR reaction containing 200 nM of both biotinylated and standard primers, and nuclease-free water. Thermocycling was completed using a Bio-Rad C1000™ thermocycler. Generated biotinlyated PCR products were pyrosequenced and methylation state quantified using PyroMark Gold reagents on a PyroMark Q96 ID platform using CpG Software (Qiagen). Sequencing primer concentrations varied from 0.3–0.45 µM.

HTR2A Genotyping

There is a single nucleotide polymorphism (SNP) of the *HTR2A* (rs6311; G A) at site -1438 that results in the loss of a CpG site (52, 53). Genotyping of site -1438 was accomplished using the same pyrosequencing workflow with the exceptions of bisulfite conversion and CpG software analysis. The forward primer (5'AAACACTGTTGGCTTTGGATGG3'), reverse primer (5' Biotin-TATGTCCTCRGAGTGCTGTGA3'), and sequencing primer (5'TTGGATGGAAGTGCC3') were designed in house. Polymorphism status was determined through PyroMark Q96 software. Individuals homozygous for the rs6311 SNP do not possess the -1439 CpG methylation site, therefore, homozygous individuals from the healthy controls (n=5) and cocaine users (n=8) were excluded from analyses of the -1439 site.

Statistical Analysis

All statistical analysis were performed using GraphPad Prism software Version 7.02 or IBM® SPSS® Statistics package Version 1.0.0.1298. All individuals were pyrosequenced, however, for some individuals, the reaction was not possible for certain CpG sites; for these sites, these individuals were not included in the analysis. A Mann Whitney test was used to compare percent methylation between healthy controls and cocaine users. A two-way ANOVA with Sidak's multiple comparisons test was used to determine differences in the BIS-11 scores between healthy controls and cocaine users. A Students *t*-test was used to determine if delay discounting rates (log10k values), age and years of education were significantly different between healthy controls and cocaine users. A Friedman test with Dunn-Bonferroni multiple comparisons was carried out to compare the percent methylation of the four CpG sites within healthy controls or within cocaine users. A repeated measures ANOVA with Bonferroni multiple comparisons was performed to assess within subject differences for BIS-11 measures of healthy controls or cocaine users. All correlations were generated using the nonparametric Spearman's correlation. A nonparametric partial correlation was used for analyses corrected for age and years of cocaine use. A Chi-square test with Fisher's exact test was used to determine differences in sex, race, smoking, and marijuana use in healthy controls versus cocaine users. The Chi-square test with Fisher's exact test was performed to determine if allelic frequency of the rs6311 SNP was different between healthy controls and cocaine users. The alpha level for all analyses was set at p=0.05.

RESULTS

Assessment of *HTR2A* Methylation Profile in Healthy Controls and Cocaine-Dependent Participants

A careful analysis of the literature identified four key methylation sites within the *HTR2A* promoter that correspond to potential transcription factor binding sites: -1439, -1420, -1224, and -253 (**Figure 1A**). The first CpG site identified is located at -1439 (**Figure 1A**, maroon line); this is a binding site for the polyoma enhancer activator 3/early region 1A enhancer-binding protein (PEA3/E1AF) transcription factors (**Figure 1A**, blue box) (54–56). The rs6311 SNP in the *HTR2A* gene (**Figure 1A**, maroon line) at site -1438 removes the -1439 CpG methylation site and introduces an E47 transcription factor binding site (**Figure 1A**; green box) (52, 53). In carriers of the rs6311 SNP at site -1438, the guanine (G) is converted to an alanine (A), and hence the -1439 CpG island is missing (**Figure 1A**, maroon line). In both healthy controls and cocaine users, there was a mix of individuals who were heterozygous and homozygous for the rs6311 SNP. Using a Chi-squared analysis with Fisher's exact test, the allelic frequency of the rs6311 SNP (A-1438G) was not significantly different between cocaine users (G/G: n=21; A/G: n=24; A/A: n=8) versus healthy controls (G/G: n=18; A/G: n=25; A/A: n=5) (n.s.). Individuals heterozygous for the rs6311 SNP lose one of the CpG sites thereby reducing their maximum percent methylation to 50% as compared to homozygous for the wild type G allele at the -1439 CpG site. Individuals homozygous for the rs6311 SNP have no -1439 CpG site and were excluded from the percent methylation analyses for CpG site -1439. The next CpG site under study is at -1420 (**Figure 1A**, red line) to which the progesterone receptor (PR) and glucocorticoid receptor (GR) bind (52, 57) (**Figure 1A**, blue box). The -1224 CpG site (**Figure 1A**, red line) corresponds to the binding site of specificity protein 1 (Sp1) (**Figure 1A**, blue box) (52, 54). The final CpG site -253 (**Figure 1A**, red line) is in a known silencer region that spans from nucleotides -120 to -578 (**Figure 1A**, tan box) of the *HTR2A* promoter (54).

Demographic data for cocaine users and healthy controls were collected, including age, sex, race, years of education as well as drug use. The sex and race of healthy controls and cocaine users did not differ (n.s.; **Table 1**). Cocaine users were significantly older with fewer years of education and greater smoking and marijuana use ($p < 0.05$, **Table 1**). In healthy controls, age correlated significantly with percent methylation at

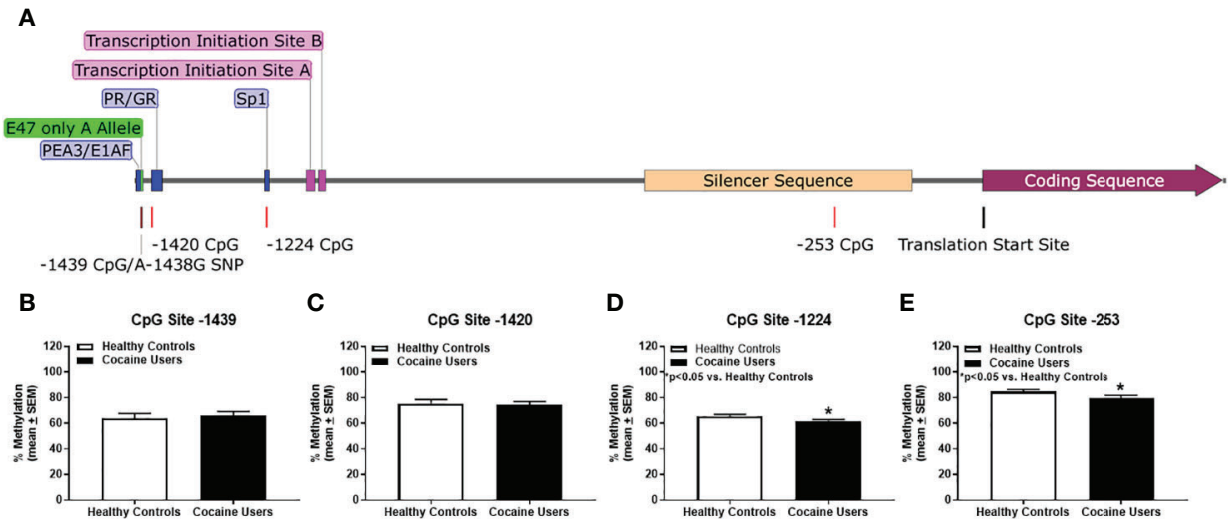


FIGURE 1 | Cocaine users demonstrate hypomethylation of the *HTR2A* promoter versus healthy controls. **(A)** *HTR2A* gene is represented as the gray line and contains the promoter region, exon 1, exon 2, and the initial segment of the coding sequence of the gene. Transcription factor binding sites are represented in blue and green. CpG islands and SNPs are represented as red lines and transcription initiation sites are in pink. Translation start site is represented as a black line. The silencer sequence is annotated in tan and the coding sequence is in maroon. Comparison of percent methylation between healthy controls and cocaine users for **(B)** CpG site -1439, **(C)** -1420, **(D)** -1224 and **(E)** -253. *p < 0.05 versus healthy controls.

TABLE 1 | Demographic analyses of healthy controls and cocaine users.

Characteristics	Healthy Controls (N=48)	Cocaine Users (N=53)	*p value vs Healthy Controls
Age (mean ± SD)	33.04 ± 10.93	45.87 ± 7.75	***<0.0001***
Female (%)	47.92%	35.85%	0.233
African American (%)	79.17%	88.68%	0.276
Years of Education (mean ± SD)	14.18 ± 2.26	12.35 ± 1.81	***<0.0001***
Smokers (%)	27.08%	88.10%	***<0.0001***
Marijuana Users (%)	14.58%	56.60%	***<0.0001***

*p < 0.05 versus healthy controls is bolded and italicized.
Recruitment site: Healthy Controls-University of Texas Health Science Center (n = 10), Virginia Commonwealth University (n=38); Cocaine Users-University of Texas Health Science Center (n = 10); Virginia Commonwealth University (n = 43).

TABLE 2 | Correlational analyses of demographics with percent methylation of *HTR2A* promoter CpG sites.

Healthy Controls

Characteristics	Site -1439	Site -1420	Site -1224	Site -253
Age (R Value, P value)	-0.086, 0.573	-0.111, 0.460	***-0.312, <0.05***	***-0.336, <0.05***
Years of Cocaine Use (R value, P Value)	–	–	–	–
Years of Smoking (R value, P Value)	–	–	–	–

Cocaine Users

Characteristics	Site -1439	Site -1420	Site -1224	Site -253
Age (R Value, P value)	-0.078, 0.678	-0.249, 0.156	***-0.407, <0.05***	-0.269, 0.118
Years of Cocaine Use (R value, P Value)	0.035, 0.822	0.082, 0.587	0.039, 0.779	***-0.293, <0.05***
Years of Smoking (R value, P Value)	-0.102, 0.593	-0.045, 0.813	***-0.365, <0.05***	-0.171, 0.319

*p < 0.05 for correlation is bolded and italicized.

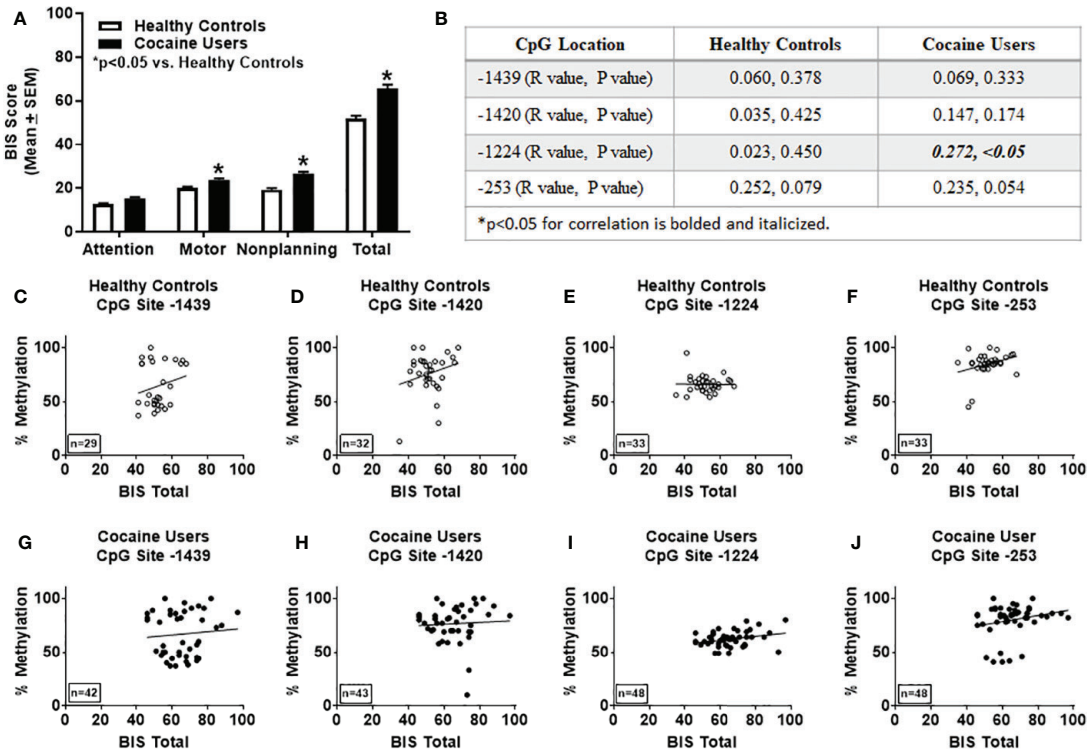
site -1224 (R=-0.312, p < 0.05; **Table 2**) and -253 (R=-0.336, p < 0.05; **Table 2**), but not at CpG sites -1439 and -1420 (n.s.; **Table 2**). Age significantly correlated with percent methylation at site -1224 (R=-0.407, p < 0.05; **Table 2**), but was not significant at CpG sites -1439, -1420, and -253 (n.s.; **Table 2**) for cocaine users. Years of cocaine use significantly correlated with percent methylation at site -253 (R=-0.293, p < 0.05; **Table 2**), but was not significant at CpG sites -1439, -1420, and -1224. Years of smoking significantly correlated with percent methylation at site -1224 (R=-0.635, p < 0.05; **Table 2**), but not at CpG sites -1439, -1420, and -253 for cocaine users.

Within subject comparisons of healthy controls indicated a significant difference for percent methylation between the CpG sites [$\chi^2(3) = 38.725$; $p < 0.05$]. Dunn-Bonferroni multiple comparisons tests indicated significant differences for percent methylation at all sites ($p < 0.05$) *except* between sites -1224 and -1439 CpG as well as between sites -1420 and -253 for healthy controls. Within subject comparisons of cocaine users detected a significant difference for percent methylation between the CpG sites [$\chi^2(3) = 27.98$; $p < 0.05$]. Dunn-Bonferroni multiple comparisons tests indicated significant differences for percent methylation between sites -1224 and -1420 ($p < 0.05$) as well as between sites -1224 and -253 ($p < 0.05$) and no significant

differences between any other CpG sites for cocaine users. Overall percent methylation between healthy controls and cocaine users was determined for each CpG site of interest (**Figures 1B–E**). There was no significant difference between healthy controls and cocaine users for CpG sites -1439 (n.s.; **Figure 1B**) and -1420 (n.s.; **Figure 1C**). Cocaine users displayed hypomethylation at CpG sites -1224 ($p < 0.05$, **Figure 1D**) and -253 ($p < 0.05$, **Figure 1E**) versus healthy controls.

Levels of Impulsivity Correlate With *HTR2A* Promoter Percent Methylation at Site -1224 in Cocaine Users

Overall levels of impulsivity were determined using the BIS-11 (2). Within subject analyses of healthy controls for BIS-11 (all measures) indicated that the assumption of sphericity was violated [$\chi^2(5)=12.69$; $p < 0.05$; Mauchly's test], therefore degrees of freedom were corrected using Huynh-Feldt estimates of sphericity ($\epsilon=0.895$). The results show that there was a significant difference between measures of BIS-11 of the healthy controls, [$F(2.69, 85.92)=776.10$, $p < 0.05$]. Bonferroni correction for multiple comparisons showed significant difference between all measures of impulsivity ($p < 0.05$) except for BIS-11 motor vs BIS-11 non-planning for healthy controls. Within subject analyses of cocaine users for BIS-11 (all measures) indicated that the assumption of sphericity was violated [$\chi^2(5)=48.88$; $p < 0.05$; Mauchly's test], therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\epsilon=0.614$). The results show that there was a significant difference between measures of BIS-11 of the cocaine users [$F(1.84, 86.59)=1062.95$, $p < 0.05$]. Bonferroni correction for multiple comparisons showed significant difference ($p < 0.05$) between all measures of the BIS-11 for cocaine users. Although levels of attention were not different between groups, cocaine users showed significantly higher scores on the BIS-11 for motor, non-planning, and total scores versus healthy controls ($p < 0.05$, **Figure 2A**). Total BIS-11 scores positively correlated with percent methylation at CpG site -1224 in cocaine users only ($R=0.272$, $p < 0.05$, **Figures 2B, I**). As noted above, cocaine users were significantly older than healthy controls (**Table 1**); after correcting for age, the correlation between total BIS-11 scores and percent methylation at CpG site -1224 trends toward significance ($R=0.237$, $p=0.054$). No significant correlations were detected between percent methylation at CpG sites -1439, -1420 or -235 and total BIS-11 scores in the healthy controls or cocaine users (n.s.; **Figures 2B–H, J**).



CpG Location	Healthy Controls	Cocaine Users
-1439 (R value, P value)	0.060, 0.378	0.069, 0.333
-1420 (R value, P value)	0.035, 0.425	0.147, 0.174
-1224 (R value, P value)	0.023, 0.450	***0.272, <0.05***
-253 (R value, P value)	0.252, 0.079	0.235, 0.054
*p<0.05 for correlation is bolded and italicized.		

FIGURE 2 | Levels of impulsivity correlate with percent methylation at site -1224 of the *HTR2A* promoter in cocaine users. **(A)** Impulsivity was determined using the Barratt Impulsiveness Scale (BIS-11). *$p < 0.05$ versus healthy controls. **(B)** Correlations and p values of percent methylation of the *HTR2A* promotor with total BIS scores at specific sites within the *HTR2A* promoter are represented in the table. Graphical representation of the correlations between percent methylation of the *HTR2A* promotor with total BIS-11 scores at specific sites within the *HTR2A* promoter for healthy controls **(C–F)** and cocaine users **(G–J)**. The number of individuals used in each correlation are indicated on the bottom left of the graph.

Delay Discounting Rates Correlate With *HTR2A* Promotor Percent Methylation at Site -1420 in Healthy Controls

Using the delay discounting task to measure impulsive choice, a key facet of overall impulsivity (2, 6, 58, 59), correlational analyses between delay discounting scores and BIS-11 for healthy controls [total (R=0.1342, n.s.), attention (R=-0.1671, n.s.), motor (R=0.2233, n.s.), non-planning (R=0.1586, n.s.)] or cocaine users [total (R=-0.0239, n.s.), attention (R=0.0335, n.s.), motor (R=-0.1351, n.s.), non-planning (R=0.0229, n.s.)] were not significantly correlated. These data suggest that the BIS-11 and delay discounting task are independent measures of impulsivity (60). Cocaine users showed a significant preference for the smaller immediate reward over the larger delayed reward as compared to the healthy controls ($p < 0.05$, **Figure 3A**), corroborating previous findings (5). Discounting rates negatively correlated with percent methylation at CpG site -1420 in healthy controls only (R=0.4259, $p < 0.05$, **Figures 3B, D**), which remained significant after correcting for age (R=-0.504, $p < 0.05$). There were no significant correlations between percent methylation and the discounting scores at CpG sites -1439, -1224, or -253 in the healthy controls or cocaine users detected (n.s.; **Figures 3B, C, E–J**).

Attentional Bias Correlates With *HTR2A* Promotor Percent Methylation at Site -253 in Cocaine Users

Healthy controls (11.31 ± 8.8 sec) showed less attentional bias in the cocaine word Stroop task versus cocaine users (33.19 ± 6.6 sec; $p < 0.05$). Levels of attentional bias of cocaine users in the cocaine-word Stroop task positively correlated with percent methylation at CpG site -253 (R=0.4065, $p < 0.05$, **Figures 4A, E**) and remained significant after correcting for age and years of cocaine use (R=0.410, $p < 0.05$). There were no significant correlations observed between percent methylation and attentional bias at CpG sites -1439, -1420, or -1224 in cocaine users (n.s.; **Figures 4A–D**).

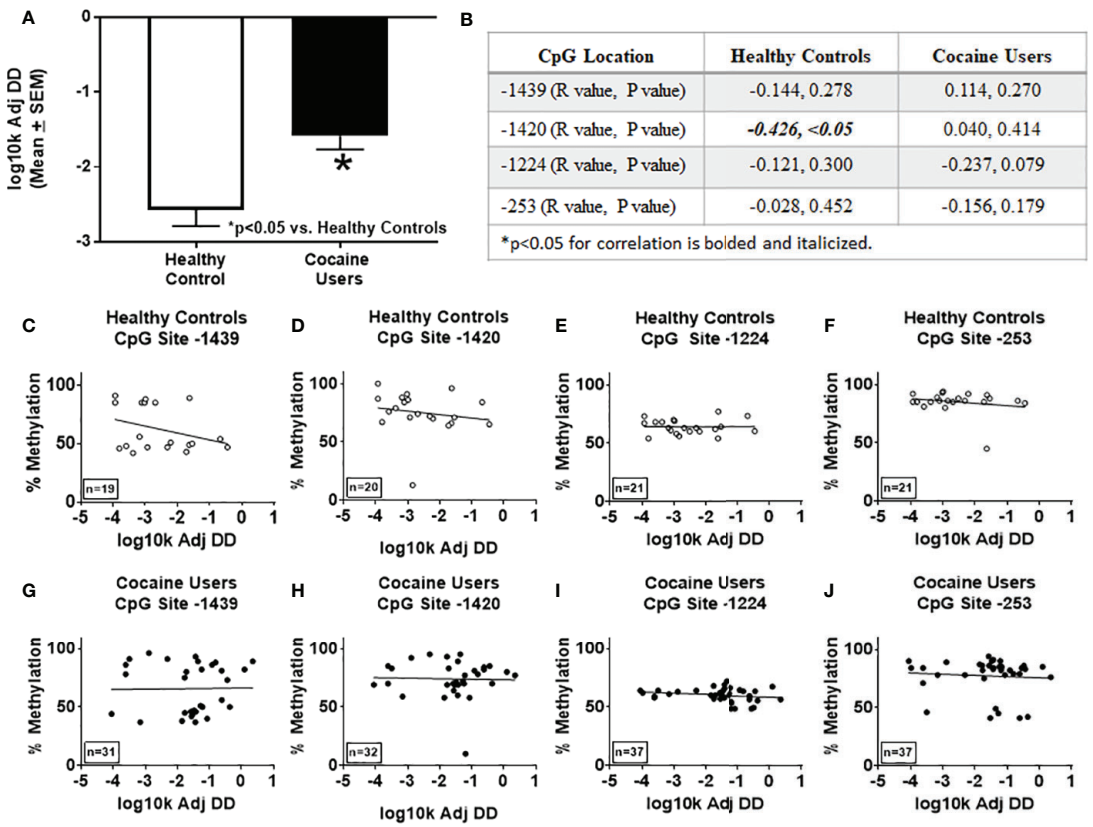


FIGURE 3 | Delay discounting rates correlate with percent methylation at site -1420 of the *HTR2A* promotor in healthy controls. **(A)** Impulsive choice was determined using the delay discounting task in healthy controls and cocaine users. *$p < 0.05$ versus healthy controls. **(B)** Correlations and p values of percent methylation of the *HTR2A* promoter with the delay discounting rates at specific sites within the *HTR2A* promoter are represented in the table. Graphical representation of the correlations between percent methylation of the *HTR2A* promotor and delay discounting rates at specific sites within the *HTR2A* promoter for healthy controls **(C–F)** and cocaine users **(G–J)**. The number of individuals used in each correlation are indicated on the bottom left of the graph.

A

CpG Location	Cocaine Users
-1439 (R value, P value)	0.128, 0.213
-1420 (R value, P value)	0.156, 0.162
-1224 (R value, P value)	0.063, 0.335
-253 (R value, P value)	***0.407, <0.05***

*p<0.05 for correlation is bolded and italicized.

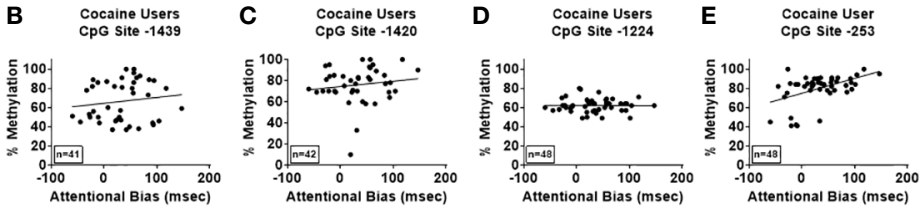


FIGURE 4 | Attentional bias correlates with percent methylation at site -253 of the *HTR2A* promotor in cocaine users. Attentional bias was determined using the cocaine-word Stroop task in cocaine users. **(A)** Correlations and p values of percent methylation of the *HTR2A* promotor with mean reaction times (msec) at specific sites within the *HTR2A* promoter are represented in the table. **(B–E)** Graphical representation of the correlations between percent methylation of the *HTR2A* promotor and attentional bias at specific sites within the *HTR2A* promoter for cocaine users. The number of individuals used in each correlation are indicated on the bottom left of the graph.

DISCUSSION

We discovered in the present study that cocaine users exhibited higher levels of impulsivity as measured by the BIS-11 and delay discounting task, as expected. Cocaine users exhibited hypomethylation at CpG sites -1224 and -253 but not at CpG sites -1439 and -1420 versus healthy controls. The percent methylation of CpG site -1224, but not -1439, -1420, or -235, of the *HTR2A* promoter trended towards a positive correlation with total BIS-11 scores in cocaine users. A negative correlation between delay discounting rates and percent methylation at site -1420 was observed in healthy controls. In addition, levels of attentional bias positively correlated with percent methylation of CpG site -253, but not other sites, in cocaine users. We also determined that the rs6311 SNP was present equally in the healthy controls and cocaine users. Taken together, this study provides evidence that individual differences in CUD relapse-related behaviors associate with the pattern of methylation within the *HTR2A* promoter.

Cytosine methylation patterns vary substantially across different cells of the same organism, and these patterns can change over time within the same cell. Of note, the association between methylation at site -1224 and total BIS-11 levels trended towards significance after correction for age, suggesting age may have a small impact on the association. Chronological age has been shown to influence DNA methylation in lymphocytes, with increasing age typically associated with DNA hypomethylation (61, 62). The CpG site -1224, which positively correlated with total impulsivity levels in cocaine users, is found in the Sp1 transcription factor binding site (52, 54). Interestingly, methylation of Sp1 binding sites increases with cocaine exposure (63), which results in reduced Sp1 binding (64) and decreased transcription of its target genes (63). Thus, the regulatory role of this CpG site over the Sp1 transcription factor is an excellent future candidate for determining gene-environment interactions for cocaine-dependent individuals with high trait impulsivity.

Interestingly, the pattern of methylation detected herein differed between the two measures of impulsivity and is most likely a reflection of the tools employed, that is a subjective (BIS-11) versus objective (delay discounting) measure. The BIS-11 is a onetime questionnaire designed to elicit individual reporting of their past acts of impulsiveness (2). In contrast, the delay discounting task is an active measure of impulsive choice or the tendency to prefer smaller, immediate rewards over larger, delayed rewards (2, 6, 58, 59). Results from the delay discounting task indicated a distinct pattern of *HTR2A* promoter methylation as compared to the BIS-11. A negative correlation between methylation at the CpG site -1420 and delay discounting rates for healthy controls, even after correcting for age, was detected, suggesting the pattern of methylation at CpG site -1420 could be disrupted in cocaine users with high impulsive choice. The CpG site -1420 for the *HTR2A* is found in the binding site for the GR and PR (52, 57). Glucocorticoids and progesterone levels are elevated after administration of cocaine (65, 66) resulting in increased progesterone receptor DNA binding following acute cocaine injection (66). Further, genetic removal or pharmacological inhibition of the GR results in reduced cocaine self-administration in rodents (67). Thus, we hypothesize that dysregulation of methylation in high impulsive choice cocaine users versus healthy controls at site -1420 could be the result of altered glucocorticoid and progesterone levels associated with cocaine use (65, 66) resulting in increased receptor DNA binding (66). Future studies are warranted to explore the hypothesis that specific types of impulsivity (e.g., impulsive action, impulsive choice) may present with their own unique gene-environment interactions.

Attentional bias in cocaine users, i.e. cocaine cue reactivity, positively associated with methylation at CpG site -253 within a known silencer region of the *HTR2A* promoter (63). In general, methylation of silencers prevents repressor proteins from binding and potentially abolish their repressive function on gene transcription (Jones, 2001). Removal of the *HTR2A* silencer region robustly increased promoter activity as determined by reporter assays *in vitro* (54). Broadly, these data align with preclinical studies reporting a putative hyperfunctional 5-HT_{2A}R system in relapse-related behaviors (for reviews) (6, 7). Studies to investigate the causal relationship between epigenetic reprogramming of the peripherally and centrally localized 5-HT_{2A}R and CUD relapse-like related behaviors are warranted.

The rs6311 SNP results in the loss of the -1439 CpG site and introduces a new transcription factor binding site for E47 (52, 53). E47 is a member of the helix-loop-helix transcription factor family which upon binding can result in increased promoter activity of the *HTR2A* (68). Further, this family of transcription factors play a critical role in corticogenesis (69). The rs6311 SNP also associates with levels of disease severity (70), drug response (71), and is found at higher frequencies in schizophrenic patients (72). The rs6311 SNP also associates with early onset obsessive-compulsive disorder (73) and impaired impulse control in individuals diagnosed with schizophrenia (73). While this SNP actually removes the -1439 CpG island, impacts transcription levels of the *HTR2A* gene and is associated with a number of other neuropsychiatric disorders, we did not identify a bias for cocaine users to express this SNP to a greater degree than healthy controls. The prevalence of the rs6311 SNP is approximately 44% in the global population (1,000 Genomes). However, we may have had too small a sample size to detect allelic differences between the healthy controls and cocaine users.

Limitations of this study are in the sample size and the need to replicate these findings in additional cohorts measuring the same behaviors of impulsivity (both subjective and objective) and attentional bias. Furthermore, while our targeted gene and transcription factor binding profile is key to implicating molecular regulation of the *HTR2A* in relapse-related behaviors in cocaine-dependent individuals, it is wholly conceivable that an unidentified gene by environment interaction and/or the highly polygenic nature of psychiatric disorders exists, such that the methylation profile of the *HTR2A* may aggregate with an unidentified genetic/epigenetic target to contribute to phenotypic variation (74, 75). Further, a major part of epigenetic regulation is the impact on gene and protein levels. There is evidence that methylation of the *HTR2A* promoter associates with levels of *HTR2A* mRNA expression in postmortem brain (21, 36), suggesting that changes in methylation may influence expression of the *HTR2A* gene and ultimately protein levels. Finally, lymphocytes have been used to demonstrate aberrant global and/or site-specific DNA methylation in several psychiatric disorders (e.g., bipolar disorder, schizophrenia) (76, 77) justifying our initial approach to measure DNA methylation in blood of individuals characterized for clinical and behavioral impulsivity or cocaine cue reactivity. DNA methylation in the peripheral blood cells of several neuropsychiatric disorders have also been shown to mirror changes in the brain (78–82). Additionally, there is evidence that percent methylation of the *HTR2A* promoter from human peripheral leukocytes associates with methylation from the human temporal cortex (36). This suggests that peripheral blood lymphocytes can be used as marker of neuropsychiatric disorders, even where the direct cause to neural changes in the brain is, yet, unclear. Thus, future studies are required to compare DNA methylation in blood versus brain ideally in humans, although there is an obviously significant barrier to this research. We can, however, compare blood and brain global and site-specific DNA methylation patterns in well-controlled rodent studies.

The discovery and validation of biological and phenotypic individual differences in relapse-related behavioral vulnerability would greatly improve objective risk assessment of disease progress, predict response to treatments, and subgroup patients to receive a more optimized treatment regimen for this multifaceted disease (for review) (83). Currently, a key tool used to predict patient response to treatment is the cocaine selective severity assessment (CSSA), which is an 18-item questionnaire that measures levels of cocaine withdrawal (84–86). The CSSA does not account for the complex nature of CUD with its interlocking phenotypes of impulsivity and cocaine cue reactivity. Other measures such as neuroimaging, metabolomics, transcriptomics, genetics, and epigenetics could be used to supplement the current CSSA questionnaire and further stratify cocaine-dependent individuals into specific treatment subgroups. As DNA methylation is a memory of past gene-environment interactions, consideration of individual differences in targeted gene methylation profiles might allow identification of those individuals who have the highest risk for relapse-related behaviors, and subsequently lead to rational behavioral or pharmacotherapeutic strategies for minimizing damage where abstinence is not successful.

DATA AVAILABILITY STATEMENT

The sequencing data of site -1438 (rs6311) presented in this study is publicly available and can be found here: https://www.ncbi.nlm.nih.gov/clinvar/, SCV001142620. The remaining raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to all qualified researchers.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Boards at the University of Texas Health Science Center at Houston, Virginia Commonwealth University, and the University of Texas Medical Branch. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML performed the data analyses and drafted the manuscript. DR performed behavioral assessments. AM and RP designed and performed the pyrosequencing experiments. KC, FM, and NA conceptualized the project, oversaw experimental design/interpretation/analyses, and wrote/edited the manuscript.

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