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The role of alcohol in modifying behavior

Edited by
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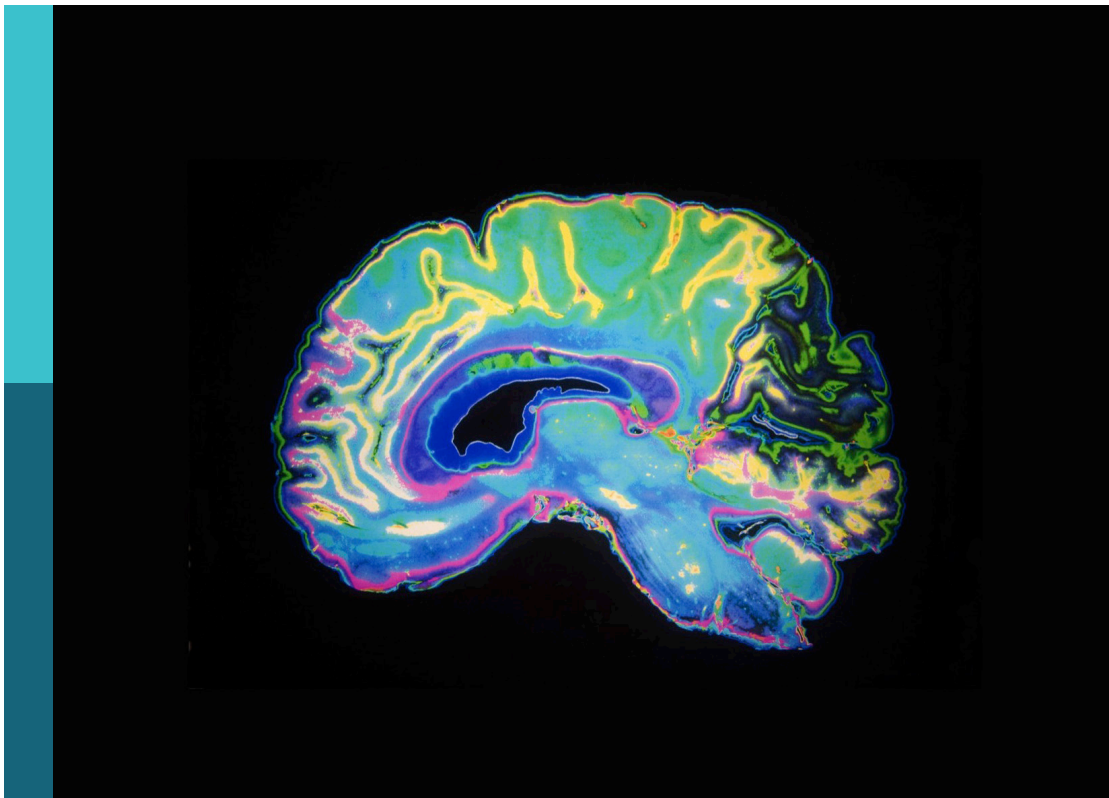

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The role of alcohol in modifying behavior

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Editorial: The role of alcohol in modifying behavior

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Editorial on the Research Topic
The role of alcohol in modifying behavior

This special topic presents experimental work on the effects of alcohol (ethanol) on the brain, and how these effects impact behavior across multiple domains. The World Health Organization (WHO) estimates that 2.3 billion people regularly consume alcohol, making alcohol one of the most widely used drugs in human society (WHO, 2022). Alcohol consumption has both acute and long-term effects on behavior. Whereas most of the acute effects are rewarding, if higher doses are consumed, negative effects including motor and cognitive impairment can occur and can be lasting. Although recreational use of alcohol can enhance sociability, excessive repeated alcohol use can lead to alcohol use disorder (AUD) and physical dependence associated with a dangerous withdrawal syndrome, such as delirium tremens. Substance use disorders are characterized by frequent comorbidity with the use of other substances, and alcohol is commonly co-used with other substances, including psychostimulants like cocaine, as well as opioids (Bobashev et al., 2018; Cicero et al., 2020). Comorbid use of substances increases the risk of adverse outcomes and relapse (Wang et al., 2017), and this complexity of the human condition requires the use of preclinical animal models to tease apart the complex effects of alcohol on behavior (Crummy et al., 2020).

Animal models play a crucial role in understanding the effects of alcohol on the brain and behavior (Mineur et al., 2022; Valyear et al., 2023). Studies have shown that a range of brain structures are involved in alcohol use including the amygdala, nucleus accumbens, and insula. Targeted stimulation and suppression of these areas of the brain is able to alter alcohol consumption. For instance, Haaranen et al. used a chemogenetic approach to alter neuronal activity in these individual brain regions, and in the specific insula outputs to the nucleus accumbens and basolateral and central subregions of the amygdala, to determine the functional role of this network on alcohol consumption in alcohol preferring Alko Alcohol (AA) rats. This type of sophisticated circuit-level analyses is necessary to understand how neural networks function to control alcohol consumption, in order to design targeted treatment strategies aimed at altering network function. The previous study found that activating the insula projections to amygdala or nucleus accumbens increased alcohol consumption, consistent with prior work demonstrating the insula is a critical driver of alcohol relapse (Campbell et al., 2019). Emerging potential new medications for treating AUD like Glucagon-Like Peptide 1 (GLP-1) may work in part by decreasing cue-associated

craving-related increases in insula activity, as systematically reviewed by Eren-Yazicioglu et al. in this special edition.

Alcohol use can be triggered by numerous factors, and stress is one of the most potent triggers for craving and relapse (Wemm et al., 2019). Interestingly, Deal et al. found that both social and non-social stressors enhance the release of catecholamines in the basolateral amygdala, and acute alcohol blunts this stress response, perhaps providing a brain-based rationale for the self-medication hypothesis (Ayer et al., 2010). This adds to a growing body of literature implicating the amygdala as an important brain site by which stress can alter alcohol seeking and use (Mineur et al., 2022). Furthermore, while the health benefits of daily exercise cannot be denied, the study by Buhr et al. suggests that it does not alter alcohol's effects on serotonin and dopamine-related turnover in the striatum and brain stem. However, alcohol drinking altered neurochemical correlates of exercise in the hypothalamus, a key component of brain networks responsible for maintaining physiological homeostasis. As demonstrated in the study by Starski et al., repeated and prolonged alcohol use can lead to allostasis and further exacerbate behavioral engagement with alcohol. Furthermore, the behavioral and brain response to stress is sexually dimorphic, and the brain response to stress and drug cues predicts subsequent relapse (Smith et al., 2023).

Genetic factors, as well as age and sex, can influence alcohol use and behavioral phenotypes associated with alcohol use. Alcohol drinking often begins in adolescence (Abela et al., 2023), and the study by Corongiu et al. demonstrates that adolescents typically drink more alcohol than adults, but that this precise phenotype interacts with genetic background. Moreover, sex can influence alcohol use and behavior, with AUD being diagnosed more often in men than women. In line with this, Bryant et al. found that male mice were more sensitive to the motivating effects of alcohol, and Landin and Chandler report that male rats exposed to alcohol during adolescence were more prone to have greater behavioral responses to threat in adulthood, although females were already predisposed to this phenotype, regardless of alcohol history. To make matters more complex, the neurobiological hallmarks of adolescent alcohol exposure may be sexually dimorphic. For example, Asarch et al. found that in male rats, mesolimbic dopamine peaks during adolescence then declines and stabilizes in adulthood, but adolescent alcohol exposure prolongs the elevated dopamine levels into adulthood, an "arrested development" phenotype not observed in female rats, whose dopamine levels are stable throughout adolescence and adulthood. On a more positive note, Rodd et al. report that negative allosteric modulators of the nicotinic α7 receptors may hold promise as prophylactics against the deleterious effects of binge alcohol use during adolescence.

Overall, the contributions to this special topic have broadened our understanding of how, where, and when alcohol acts in the brain to promote continued alcohol use, which in some individuals can lead to full blown AUD. The extensive comorbid use of alcohol with other drugs is also of growing concern and calls for novel preclinical models of polydrug use to determine the neurobiological consequences of comorbid alcohol use with other substances and to effectively screen emerging therapeutics. Continued research in this area is needed in order to develop novel treatment interventions, including prophylactics, medications, and natural remedies.

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ORIGINAL RESEARCH
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Chemogenetic Stimulation and Silencing of the Insula, Amygdala, Nucleus Accumbens, and Their Connections Differentially Modulate Alcohol Drinking in Rats

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The anterior insular cortex is hypothesized to represent interoceptive effects of drug reward in the service of goal-directed behavior. The insula is richly connected, but the insula circuitry in addiction remains poorly characterized. We examined the involvement of the anterior insula, amygdala, and nucleus accumbens, as well as the projections of the anterior insula to the central amygdala, basolateral amygdala (BLA), and nucleus accumbens core in voluntary alcohol drinking. We trained alcohol-preferring Alko Alcohol (AA) rats to drink alcohol during intermittent 2-h sessions. We then expressed excitatory or inhibitory designer receptors [designer receptors exclusively activated by designer drugs (DREADDs)] in the anterior insula, nucleus accumbens, or amygdala by means of adenovirus-mediated gene transfer and activated the DREADDs with clozapine-N-oxide (CNO) prior to the drinking sessions. Next, to examine the role of specific insula projections, we expressed FLEX-DREADDs in the efferent insula → nucleus accumbens core, insula → central amygdala, and insula → BLA projections by means of a retrograde AAV-Cre vector injected into the insula projection areas. In the anterior insula and amygdala, excitatory Gq-DREADDs significantly attenuated alcohol consumption. In contrast, in the nucleus accumbens, the Gq-DREADD stimulation increased alcohol drinking, and the inhibitory Gi-DREADDs suppressed it. The Gq-DREADDs expressed in the insula → nucleus accumbens core and insula → central amygdala projections increased alcohol intake, whereas inhibition of these projections had no effect. These data demonstrate that the anterior insula, along with the amygdala and nucleus accumbens, has a key role in controlling alcohol drinking by providing excitatory input to the central amygdala and nucleus accumbens to enhance alcohol reward.

Keywords: alcohol drinking, DREADDs, insula, amygdala, nucleus accumbens, neural circuits

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INTRODUCTION

Corticolimbic regulatory systems have long been thought to be the primary neural substrates in addictive behaviors. There is increasing evidence that also the insular cortex (insula) plays an important role in controlling drug taking and seeking. The insula is hypothesized to represent the interoceptive effects of drug taking and integrate them with emotional

and decision-making processes (Naqvi and Bechara, 2009; Droutman et al., 2015b). Lesions of insula suppressed smoking-related urges in smokers (Naqvi et al., 2007) and decreased both the conditioned drug effects and drug self-administration in various animal models of addiction (Contreras et al., 2007; Scott and Hiroi, 2011; Cosme et al., 2015; Pushparaj and Le Foll, 2015; Pushparaj et al., 2015). Drug-associated cues activated the insula, suggesting sensitized insula functions during drug craving (Claus et al., 2011; Engelmann et al., 2012). On the other hand, human addicts also displayed lower insula activation while performing decision-making and risk evaluation tasks (Li et al., 2009; Stewart et al., 2014). The apparently discordant findings show that insula may exhibit either sensitized or desensitized functions depending on the behavioral context and possibly the stage of addiction.

The role of insula in mediating drug-related responses can only be understood by studying the networks in which insula is embedded. Insula is connected extensively to the cortical and subcortical brain regions that serve sensory, emotional, and motivational functions (Droutman et al., 2015a). Goal-directed behavior is associated particularly with the anterior agranular insula that differs from the more posterior granular insula by the disappearance of the cortical layer IV (Bermudez-Rattoni, 2014). The anterior agranular insula has reciprocal connections with the anterior cingulate cortex, ventromedial prefrontal cortex, amygdala, and striatal areas, including nucleus accumbens (Shi and Cassell, 1998; Reynolds and Zahm, 2005). Activation of the anterior insula connections with the nucleus accumbens and amygdala is now emerging as an important neural correlate of drug seeking and craving. In humans, increased coupling between the anterior insula and nucleus accumbens was correlated with self-reported compulsive alcohol use (Grodin et al., 2018), and optogenetic inhibition of this projection suppressed alcohol self-administration associated with adverse effects of alcohol in rats (Seif et al., 2013). Similarly, the anterior insula influences drug seeking through connections with the amygdala, shown by increased insula–amygdala connectivity in heroin and cocaine users (Gu et al., 2010; Xie et al., 2011). In rats, relapse to methamphetamine seeking increased c-Fos expression in the central amygdala, whereas chemogenetic inhibition of the anterior insula projection to the central amygdala decreased methamphetamine seeking (Venniro et al., 2017).

Information on the role of the anterior insula connections in mediating drug intake and seeking is still scanty. We designed the present study to clarify further the role of insula and its downstream connections in the regulation of voluntary alcohol drinking during limited alcohol access that models aspects of binge drinking. We used the alcohol-preferring Alko Alcohol (AA) rats that were among the first rat lines developed for high voluntary alcohol drinking using directional selection and represent therefore an enriched genetic contribution to excessive alcohol use (Eriksson, 1968; Sommer et al., 2006). During limited access, these rats display pharmacologically relevant blood alcohol levels that induce psychomotor stimulatory effects (Päivärinta and Korpi, 1993).

Our first goal was to examine the effects of bidirectional manipulation of both the anterior insula, nucleus accumbens, and amygdala separately on alcohol drinking. Although these brain areas, particularly the nucleus accumbens and amygdala, have well-known roles in alcohol reward (Koob et al., 1998; Gilpin et al., 2015), we decided to use the possibility to both stimulate and silence them using designer receptors exclusively activated by designer drugs (DREADDs) that tap into the intracellular signaling cascades and alter neuronal excitability upon administration of clozapine-N-oxide (CNO; Urban and Roth, 2015). Our second goal was to examine the role of the anterior insula projections to the nucleus accumbens core and amygdala subdivisions. We accomplished this by using Cre-dependent DREADDs that were expressed specifically in these projections with the help of a powerful retrograde Cre-carrying adenoviral vector (Tervo et al., 2016) injected into the nucleus accumbens core, central amygdala, and basolateral amygdala (BLA). We hypothesized that bidirectional control of the anterior insula, amygdala, and nucleus accumbens excitability, added with the insula projection-specific manipulation, would give novel information on the role of these brain areas and their connectivity in mediating alcohol reward and drinking and promote ideas of circuit-based treatments for alcohol use disorders.

MATERIALS AND METHODS

Animals

One hundred and seventy-nine male AA rats, bred at the University of Helsinki (Helsinki, Finland) and approximately 10 weeks old at the start of experiments, were used for this study. Rats were maintained at the animal facility of the University of Helsinki in a temperature (20 ± 1°C) and humidity-controlled (55 ± 10%) room with a 12-h light/12-h dark cycle (lights on at 6:00) and housed in individually ventilated cages (IVCs) with access to food (SDS, Witham, UK) and water *ad libitum*. Cages were enriched with nest and bedding material, a wooden block, and a PVC tube. Experiments were authorized by the project authorization board of the Regional State Administrative Agency for Southern Finland (license number ESAVI/1172/04.10.07/2018) and followed Directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes and Finnish Act 497/2013 on the Protection of Animals Used for Scientific or Educational Purposes.

Intermittent Alcohol Drinking

To accustom rats to the taste of ethanol, rats first underwent 4 days of forced ethanol drinking. During these days, rats had access to a 10% (v/v) ethanol solution (WWR International, Fontaney-sur-Bois, France) from two 350-ml bottles equipped with stainless steel spouts but no access to water. After these 4 days, an intermittent drinking paradigm commenced. For 2 h, three times weekly (Monday, Wednesday, and Friday from approximately 10:00–12:00), rats had access to water and a 10% ethanol solution in a two-bottle choice paradigm. The fluids were offered in custom-made pipettes with stainless steel spouts that allowed measurement of fluid intake to the nearest 0.1 ml.

The side of the cage with the ethanol pipette (left or right) was alternated each drinking session to control for a possible side preference for ethanol consumption. During training, ethanol drinking levels gradually increased and reached an asymptotic level in approximately 8–10 weeks. Rats were weighed weekly for health assessment and calculation of ethanol consumption per kilogram of body weight. Before surgical procedures, each animal batch was divided into three experimental groups [one for the excitatory Gq-DREADD vector, one for the inhibitory Gi-DREADD vector, and one for the control enhanced green fluorescent protein (EGFP) vector] matched for the mean alcohol intake and its variance during the last week. The general timeline for the experiments is depicted in **Figure 1**.

Surgery

Animals were anesthetized for 4 min in 5% isoflurane (Vetflurane 1,000 mg/g; VIRBAC S.A., Carros, France) in oxygen (flowrate, 0.8–1 l/min), after which they were fixed onto a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the incisor bar set to 3.3 mm below the interaural line. Isoflurane anesthesia was continued initially at 3.5% and then at 2.5% isoflurane in oxygen. A drop of Viscotears (Novartis Healthcare A/S, Copenhagen, Denmark) was applied on eyes to prevent them from drying out. Holes were drilled onto the skull, and viral vector injections were performed using a 5-μl syringe (Hamilton 65460-02 Neuros Syringe with a 33-gauge needle, Hamilton Company, Reno, NV, USA) mounted on a stereotaxic injector (Stoelting no. 53313, Wood Dale, IL, USA). The coordinates (mm) for the bilateral injections were as follows: anterior insula, anterior posterior (AP) +3.0 from Bregma, mediolateral (ML) ± 4.2 from the sagittal suture, and ventrodorsal (VD) −6.1 from the skull surface; nucleus accumbens, AP +1.9, ML ± 1.5, VD −7.3; nucleus accumbens core, AP +1.9, ML ± 1.7, VD −7.3; amygdala, AP −2.4, ML ± 4.6, VD −8.2; central nucleus of amygdala, AP −2.2, ML ± 4.6, VD −8.2; BLA, AP −2.2, ML ± 5.1, DV −8.7. All coordinates were based on the Paxinos and Watson (2007).

The following vectors were used for single-site DREADD and EGFP expression: the excitatory Gq-DREADD vector ssAAV-8/2-hSyn1-hM3D(Gq)-mCherry-WPRE-hGHp(A) [5.4×10^{12} vector genomes (vg)/ml], the inhibitory Gi-DREADD vector ssAAV-8/2-hSyn1-hM4D(Gi)-mCherry-WPRE-hGHp(A) (7.3×10^{12} vg/ml), and the EGFP control vector ssAAV-8/2-hSyn1-EGFP-hGHp(A) (9.4×10^{12} vg/ml). For the projection-specific expression of DREADDs, the following vectors were used: the excitatory Gq-DREADD vector ssAAV-8/2-hSyn1-dlox-hM3D(Gq)-mCherry(rev)-dlox-WPRE-hGHp(A) (4.0×10^{12} vg/ml), the inhibitory Gi-DREADD vector ssAAV-8/2-hSyn1-dlox-hM4D(Gi)-mCherry(rev)-dlox-WPRE-hGHp(A) (7.4×10^{12} vg/ml), and the EGFP control vector ssAAV-8/2-hSyn1-dlox-eGFP(rev)-dlox-WPRE-hGHp(A) (8.0×10^{12} vg/ml). The retrograde vector for Cre-recombinase expression was ssAAV-retro/2-hSyn1-chl-EGFP_2A_iCre-WPRE-SV40p(A) (8.0×10^{12} vg/ml). Finally, for tracing the anterior insula projections into the subdivisions of the amygdala and nucleus accumbens, we used the following vectors: ssAAV-retro/2-hSyn1-chl-EGFP_2A_iCre-WPRE-SV40p(A) and ssAAV-retro/2-hSyn1-chl-mCherry-WPRE-SV40p(A). The atlas coordinates for the central amygdala were AP −2.2, ML ± 4.6, VD −8.2, and for the BLA, AP −2.2, ML ± 5.1, VD −8.7. The coordinates for the nucleus accumbens core were AP +1.9, ML ± 1.7, VD −7.3, and for the shell, AP +1.9, ML ± 0.8, VD −7.8. All viral vectors were produced by the Viral Vector Facility (VVF) of the Neuroscience Center Zürich (Zentrum für Neurowissenschaften Zürich, ZNZ).

Viral vectors were injected over 3 min, with a 3-min diffusion time. The injection volume for the single-site injections was 0.75 μl, and for injections targeting specific projections, 0.6 μl. In the tracing experiments, the volume of 0.5 μl was used. Carprofen (5 mg/kg s.c., Norbrook Laboratories, Newry, UK) was administered for postoperative analgesia. After surgery, animals were returned to their home cages, and drinking sessions were resumed after two suspended sessions.

Drugs

DREADD expression was allowed to accumulate for 4 weeks before systemic saline and CNO (ab141704, Abcam, Cambridge, UK) injections. Rats were first injected i.p. with saline 60 min prior to a drinking session to habituate them to the injection procedure and to verify that the injection procedure did not cause any adverse reactions impacting alcohol drinking. After two additional sessions, rats were injected i.p. with CNO (10 mg/kg, dissolved in saline) to activate DREADDs 60 min prior to a drinking session. Ethanol and water intake during the sessions were measured. We chose the 10 mg/kg CNO dose to maximally activate both Gq- and Gi-DREADDs without disturbing motivated behaviors or locomotion, as reported previously (Farrell and Roth, 2013; Mahler and Aston-Jones, 2018; Haaranen et al., 2020). We also controlled the possible nonspecific off-target effects by including an EGFP-expressing non-DREADD group in all experiments. Each subject received CNO only once.

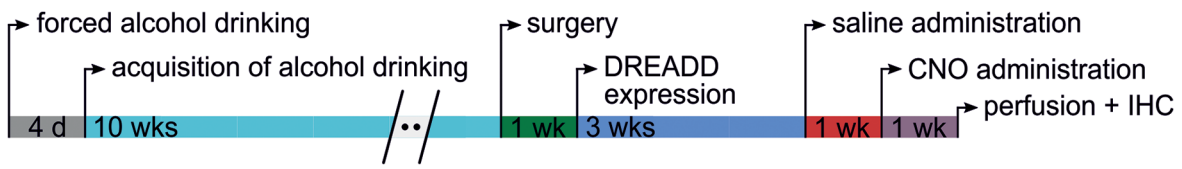

FIGURE 1 | Timeline of the experiments. Shown are the experimental phases and their approximate length each subject was exposed to during the experiments.

Immunohistochemistry

After completion of the experiments, animals were deeply anesthetized first with 5% isoflurane and then with a 120 mg/kg lethal dose of pentobarbital (Mebunat vet 60 mg/ml; Orion Pharma, Espoo, Finland). Rats were perfused transcardially with ice-cold phosphate-buffered saline (PBS, +4°C, pH 7.4) followed by 4% paraformaldehyde (PFA, +4°C, pH 7.4). The brains were removed and placed in PFA for a 24-h postfixation, after which PFA was replaced with 30% sucrose in PBS until brains were saturated (∼4 days). Brains frozen in isopentane were stored at −80°C until cutting with a freezing microtome into 40-μm coronal sections that were cryoprotected at −20°C.

DREADD expression at the injected brain areas was visualized by immunohistochemical detection of mCherry tagged to DREADDs. Brain sections were washed three times in PBS for 5 min and blocked at room temperature (RT) for 1 h in a blocking solution containing 3% bovine serum albumin (BSA), 10% donkey serum, and 0.3% Triton solubilized in PBS. Sections were incubated overnight at +4°C with the 1:800 rabbit anti-mCherry primary antibody (ab167453, Abcam, Cambridge, UK), followed by a 2-h incubation with the 1:1,000 secondary donkey antirabbit antibody (ab150076, Abcam) at RT. Sections were then mounted on microscopic slides, coverslipped with Vectashield-DAPI (Vectashield + DAPI, Vector Laboratories Inc., Burlingame, CA, USA), and imaged with a Zeiss AxioImager.Z1 upright epifluorescent microscope using the ZEN Blue software. Animals with bilateral DREADD expression at the intended brain areas were included in the data analysis. Six rats (3.4% of all subjects) were discarded due to failure to detect DREADD expression.

Statistical Analysis

We determined the sample sizes of the experiments based on similar published behavioral studies in rats. In all drinking experiments, the data were expressed as baseline alcohol intake (g/kg) compared with the intake on the CNO injection day. The baseline was determined as the mean of the saline injection day and the following baseline day preceding the CNO injection. The differences between the groups expressing EGFP, Gq-DREADDs, or Gi-DREADDs were analyzed with two-way (session, DREADD vector) repeated measures ANOVA, with repeated measures on session. Differences between the baseline and CNO days were compared with paired *t*-tests. The level for statistical significance was set at $p < 0.05$.

RESULTS

During the intermittent 2-h sessions given three times per week for 10 weeks, AA rats included in the final data analysis exhibited a significant increase in alcohol drinking, as shown by a significant effect for session ($F_{(29,4,988)} = 31.56$, $p < 0.0001$; **Figure 2**). The mean intake of all included subjects was 0.80 ± 0.02 g/kg during the 10th acquisition week. In these AA rats, we first tested the involvement of the anterior insula, nucleus accumbens, and amygdala in alcohol drinking by expressing the stimulatory Gq- and inhibitory Gi-DREADDs, as well as the EGPF protein as a control in these brain areas using adenovirus-mediated gene transfer

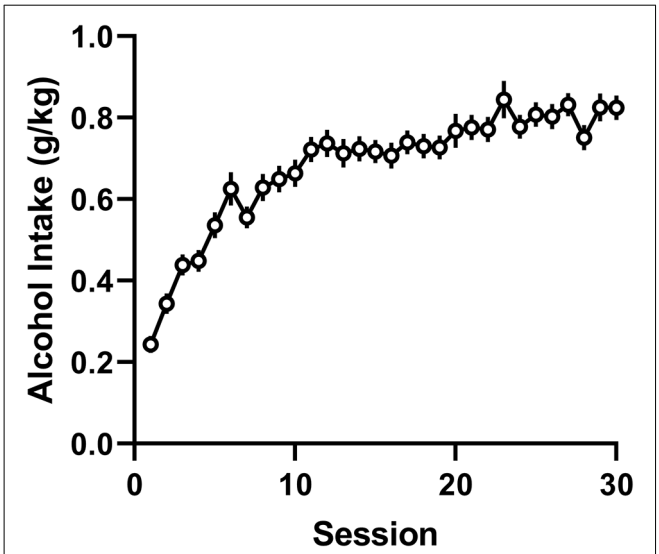

FIGURE 2 | Acquisition of alcohol drinking during intermittent 2-h sessions by alcohol-preferring Alko Alcohol (AA) rats. The data are depicted as mean (±SEM) alcohol intake (g/kg body weight in 2 h) over 30 sessions (10 weeks) by all animals included in the final data analysis ($n = 173$).

(**Figure 3**). As shown by the representative coronal sections, in the insula, the expression of Gq-DREADDs displayed by mCherry immunoreactivity was largely confined in the anterior agranular insula, encompassing both the ventral and dorsal subdivisions (**Figure 3A**). **Supplementary Figure 1** depicting the maximal and minimal mCherry expression at three Bregma levels for all experimental groups shows mCherry expression also in the granular subdivision in the more posterior insula regions. In the amygdala, DREADD expression was seen both in the central amygdala and to some degree also in the basolateral and basomedial amygdala (**Figure 3B** and **Supplementary Figure 1**), and injections into the nucleus accumbens produced DREADD expression both in the core and shell accumbal compartments (**Figure 3C** and **Supplementary Figure 1**). When we challenged these animals by giving them CNO 60 min before the onset of the alcohol drinking sessions, we saw significant changes in alcohol consumption in animals expressing the DREADDs in all three brains areas. First, as a replication of our previously published data (Haaranen et al., 2020), CNO significantly altered alcohol intake in rats expressing Gq-DREADDs in the insula, as shown by a significant effect for session ($F_{(1,27)} = 7.31$, $p = 0.012$) and a significant session × vector interaction ($F_{(2,27)} = 7.60$, $p = 0.002$). A comparison of the CNO challenge sessions with the baseline sessions indicated that the 54% decrease in drinking produced by the Gq-DREADDs was significant ($t_{(9)} = 4.00$, $p = 0.003$), with no changes in the other CNO-challenged groups. In a similar fashion, we saw a significant effect of vector ($F_{(2,25)} = 6.52$, $p = 0.005$) and a session × vector interaction ($F_{(2,25)} = 9.45$, $p = 0.001$) in rats expressing Gq-DREADDs in the amygdala. Also in these rats, the only significant change was the 60% decrease in alcohol intake produced by the Gq-DREADD stimulation ($t_{(9)} = 3.56$, $p = 0.006$). Both in the insula and amygdala,

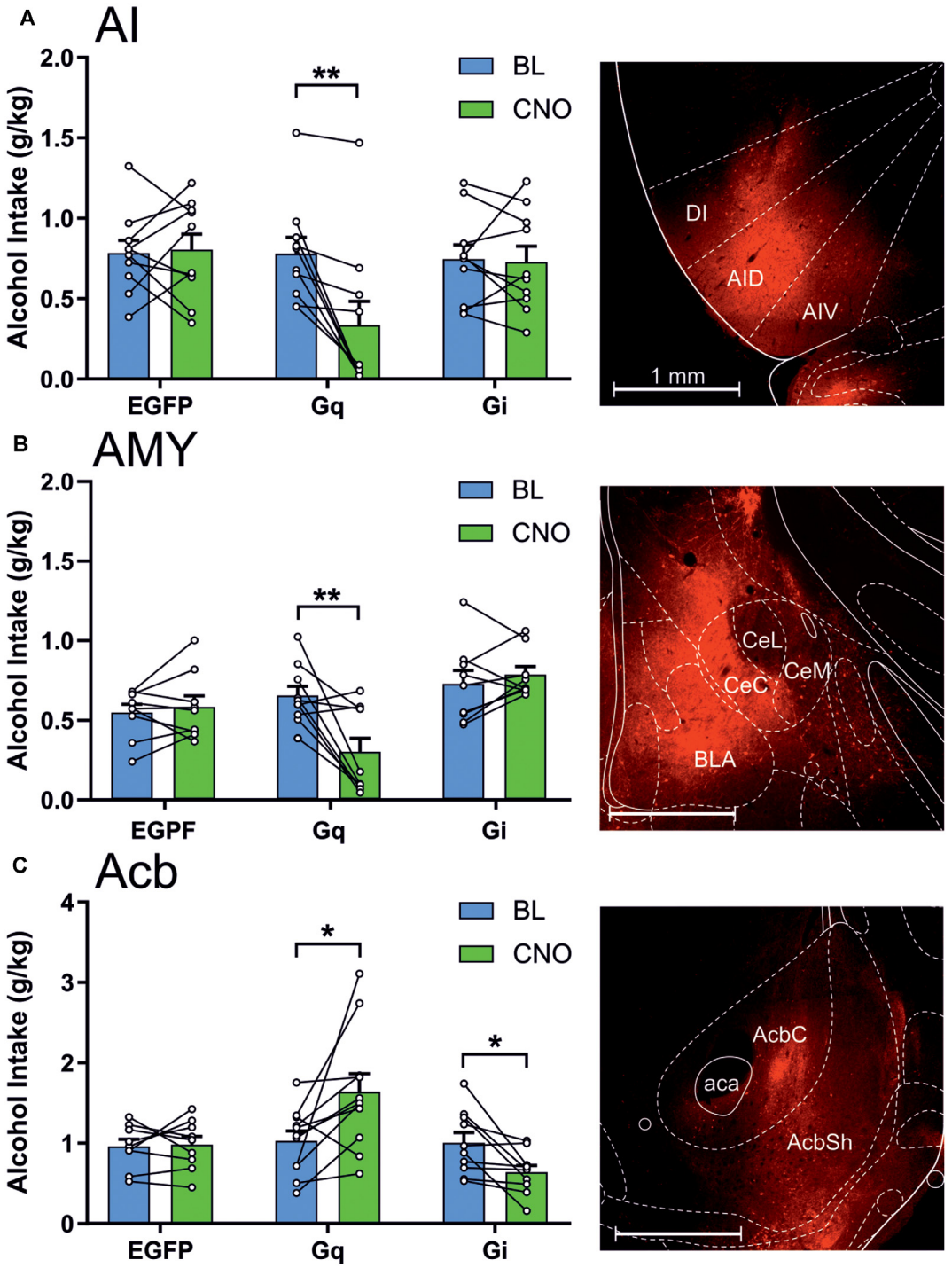

FIGURE 3 | Chemogenetic stimulation and inhibition have differential effects on alcohol drinking in the anterior insula, amygdala, and nucleus accumbens. The data are expressed as mean ± SEM alcohol intake (g/kg) during 2-h sessions during the baseline (BL) and following a clozapine-N-oxide (CNO) injection in three separate treatment groups expressing enhanced green fluorescent protein (EGFP), Gq-designer receptors exclusively activated by designer drugs (Gq-DREADDs), or Gi-DREADDs in each brain area. **(A)** EGFP (n = 10), Gq-DREADDs (n = 10), and G-DREADDs (n = 10) expressed in the anterior insula. **(B)** EGFP (n = 9), Gq-DREADDs (n = 10), and G-DREADDs (n = 9) expressed in the amygdala. **(C)** EGFP (n = 9), Gq-DREADDs (n = 11), and G-DREADDs (n = 10) expressed in the nucleus accumbens. Each histogram is accompanied by a representative brain section expressing Gq-DREADD-tagged mCherry reporter. *$p < 0.05$, **$p < 0.01$, paired t-tests comparing the BL and CNO sessions. Abbreviations used: DI, dysgranular anterior insula; AID, agranular anterior insula, dorsal; AIV, agranular anterior insula, ventral; CeC, central amygdaloid nucleus, capsular division; CeL, central amygdaloid nucleus, lateral division; CeM, central amygdaloid nucleus, medial division; BLA, basolateral amygdala; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; aca, anterior commissure.

the effects of Gq-DREADD activation were bimodal, i.e., three rats in the insula experiment and four rats in the amygdala experiment exhibited no effects or a minor decrease in alcohol drinking. However, when we inspected the extent of mCherry expression in these subjects, they did not differ from subjects with the largest suppression, suggesting that the behavioral effects produced by Gq-DREADD activation were not correlated with DREADD expression. Chemogenetic manipulation altered alcohol drinking also in rats expressing DREADDs in the nucleus accumbens, as revealed by a significant effect of vector ($F_{(2,27)}$ = 5.88, p = 0.008) and a significant session × vector interaction ($F_{(2,27)}$ = 8.83, p = 0.001). In contrast to the insula and amygdala manipulation, in the nucleus accumbens, the effects were bidirectional. We found both a significant 59% increase in alcohol drinking by the stimulatory Gq-DREADDs ($t_{(10)}$ = 2.67, p = 0.024) and a 36% decrease by the inhibitory Gi-DREADDs ($t_{(9)}$ = 2.99, p = 0.015), indicating that the failure to see effects by Gi-DREADD-mediated neuronal inhibition in the insula and amygdala was not due to the Gi-DREADD vector used.

We also recorded the volume of water offered concomitantly with alcohol during the drinking sessions. Generally, the rats consumed only small amounts of water, from 1 to 3 ml during 2 h, and therefore, any decreases in water consumption would be difficult to record. We observed no significant changes in water intake, indicated by the lack of significant session × vector interactions (insula, $F_{(2,27)}$ = 0.40, p = 0.67; amygdala, $F_{(2,25)}$ = 1.67, p = 0.21; nucleus accumbens, $F_{(2,27)}$ = 2.26, p = 0.12).

We next investigated how the efferent projections of the anterior insula to the amygdala and nucleus accumbens regulate alcohol consumption. Projection-specific DREADD expression was accomplished by injecting the Cre-dependent DREADD vectors into the anterior insula and injecting a powerful retrograde AAV-retro-Cre vector either to the central nucleus of the amygdala, BLA, or nucleus accumbens core (**Figure 4** and **Supplementary Figure 2**). When we challenged the animals expressing DREADDs in the projections to the amygdala, only the projection to the central amygdala was involved in alcohol drinking, shown by a significant session × vector interaction ($F_{(2,22)}$ = 8.47, p = 0.002), whereas the projection to the BLA had no role (session × vector interaction, $F_{(2,27)}$ = 1.01, p = 0.38). A more detailed analysis showed that, in the central amygdala projection, the Gq-DREADDs produced a significant 27% increase in alcohol drinking ($t_{(8)}$ = 5.08, p = 0.001), whereas the inhibition of this projection had no effect. In addition, the insula projection to the nucleus accumbens core affected alcohol consumption, shown by a significant session × vector interaction ($F_{(2,27)}$ = 3.36, p = 0.050). Similar to the central amygdala projection, only the Gq-DREADDs altered alcohol drinking by producing a 47% increase ($t_{(9)}$ = 3.70, p = 0.005). Projection-specific manipulation produced no significant effects on water consumption (session × vector interactions, insula to central amygdala, $F_{(2,22)}$ = 0.00, p = 0.99; insula to BLA, $F_{(2,27)}$ = 1.47, p = 0.25; insula to nucleus accumbens core, $F_{(2,27)}$ = 0.07, p = 0.93).

Projection-specific DREADD expression suggested that the anterior insula neurons sending projections to the central amygdala and BLA, as well as the nucleus accumbens, consist of overlapping neuronal populations. To visualize these populations in more detail, we injected either mCherry- or GFP-expressing AAV2-retro vectors into the central or basolateral amygdaloid nuclei, as well as the core and shell accumbal compartments (**Figures 5A–F**). The central amygdala-projecting neurons comprised 58% of the labeled amygdala-projecting neurons, whereas 34% of neurons projected to the BLA (**Figure 5C**). The amygdala-projecting neuros exhibited equal numbers in the dorsal agranular insula, but the neurons projecting to the central amygdala were more numerous in the ventral insula. The anterior insula sections showed that the nucleus accumbens core- and shell-projecting anterior insula neurons exhibited the same distribution pattern in the insula, localized mainly in the dorsal and ventral agranular anterior insula, with very few neurons in the dysgranular insula (**Figure 5E**). However, quantification of the labeled neurons revealed that the core-projecting neurons were more abundant (64% of neurons) than the shell-projecting neurons (29%; **Figure 5F**), in accordance with earlier findings (Reynolds and Zahm, 2005). In all cases, projection neuron locations were largely confined to cortical layers II and V.

DISCUSSION

In the present work, we used both excitatory and inhibitory DREADDs for examining the role of the forebrain circuitry comprised of the anterior insula, amygdala, and nucleus accumbens in voluntary alcohol drinking in alcohol-preferring rats. We found that chemogenetic stimulation of the anterior insula and amygdala robustly suppressed alcohol consumption, whereas inhibition of these brain areas had no effect. In contrast, nucleus accumbens stimulation increased alcohol drinking, and the inhibition of this area decreased it. Projection-specific chemogenic manipulation of the insula efferents showed that the excitatory insula inputs to the central amygdala and nucleus accumbens core stimulated alcohol drinking, whereas inhibition of these efferents failed to affect it. Together, these data show the importance of both the anterior insula, amygdala, and nucleus accumbens in the regulation of alcohol drinking and suggest that the excitatory input from the anterior insula to the central amygdala and nucleus accumbens core enhances the reinforcing properties of alcohol. We have previously shown that chemogenetic stimulation of the anterior insula also suppressed sucrose consumption (Haaranen et al., 2020), warranting further studies to test the effects produced by DREADDs in the nucleus accumbens, amygdala, and the insula projections to these areas on consummatory behaviors in general.

In a replication of our previous study, we found that anterior insula stimulation by the Gq-DREADDs reduced alcohol consumption (Haaranen et al., 2020). The simple limited access alcohol drinking model does not allow us to analyze which aspects of drinking behavior were affected by insula stimulation apart from the consumed alcohol volume. Insula mediates taste processing and the interoceptive effects of alcohol (Maffei et al.,

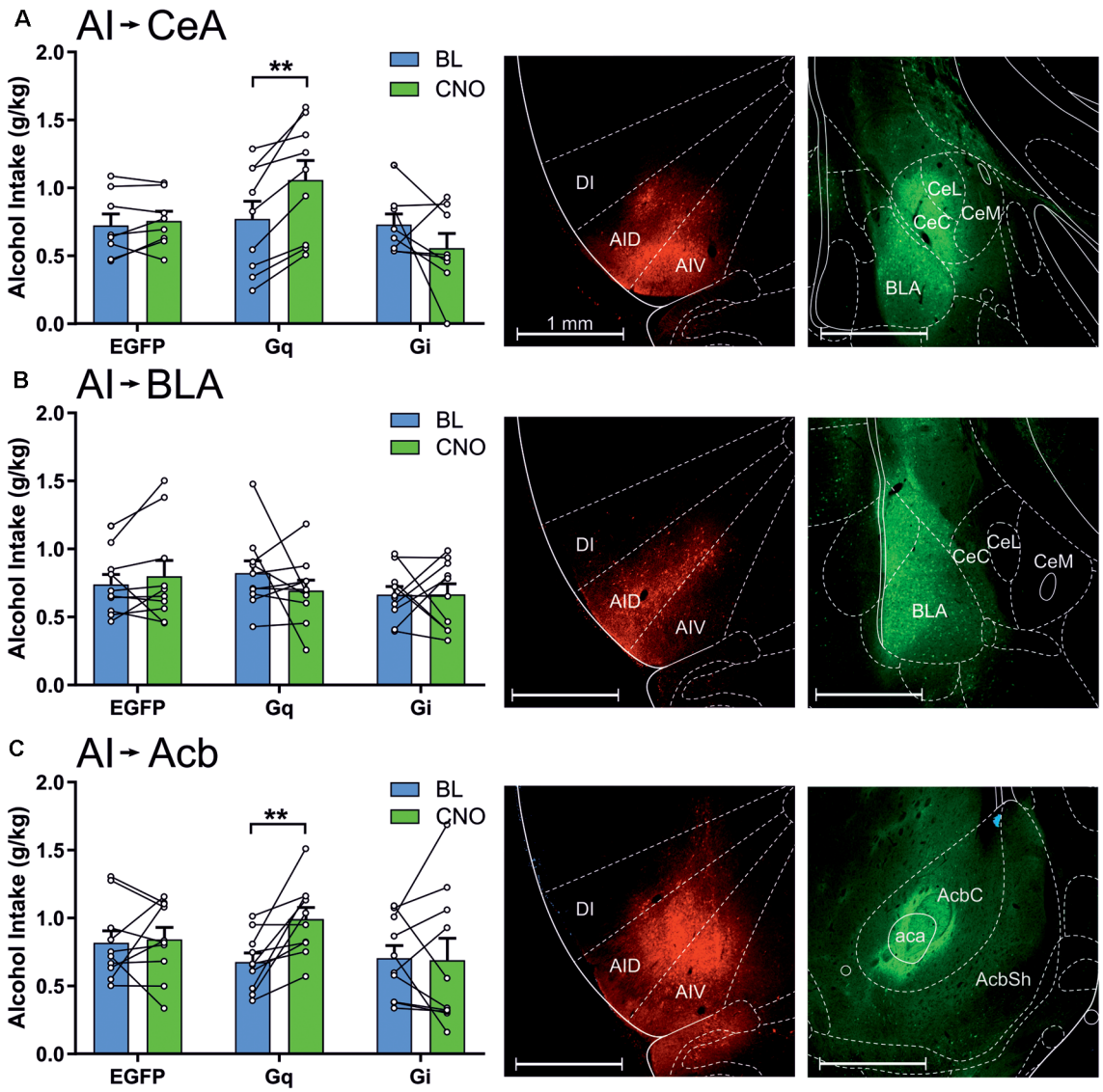

FIGURE 4 | Chemogenetic stimulation of the anterior insula efferents to the central amygdala and nucleus accumbens core increases alcohol drinking. The data are expressed as mean ± SEM alcohol intake (g/kg) during 2-h sessions during the BL and following a CNO injection in three separate treatment groups expressing EGFP, Gq-DREADDs, or Gi-DREADDs in each insula projection. **(A)** EGFP (n = 8), Gq-DREADDs (n = 9), and G-DREADDs (n = 8) expressed in the anterior insula efferents to the central amygdala. **(B)** EGFP (n = 10), Gq-DREADDs (n = 10), and G-DREADDs (n = 10) expressed in the insula efferents to the BLA. **(C)** EGFP (n = 10), Gq-DREADDs (n = 10), and G-DREADDs (n = 10) expressed in the insula efferents to the nucleus accumbens core. Each histogram is accompanied by representative brain sections expressing Gq-DREADD-tagged mCherry reporter in the anterior insula and the AAV2-retro-Cre-tagged GPF in the central amygdala, BLA, and nucleus accumbens core. $^{**}p < 0.01$, paired t-tests comparing the BL and CNO sessions. Abbreviations used: DI, dysgranular anterior insula; AID, agranular anterior insula, dorsal; AIV, agranular anterior insula, ventral; CeC, central amygdaloid nucleus, capsular division; CeL, central amygdaloid nucleus, lateral division; CeM, central amygdaloid nucleus, medial division; BLA, basolateral amygdala; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; aca, anterior commissure.

2012; Jaramillo et al., 2016), but it is unlikely that these factors contributed to suppressed drinking because many animals hardly sampled alcohol after insula stimulation. This could suggest that insula stimulation causes aversive states or general malaise. We have recently demonstrated with c-Fos mapping and pharmacological magnetic resonance imaging that anterior insula stimulation activates also the more posterior insula areas (Haaranen et al., 2020). Optogenetic or chemogenetic stimulation of the posterior insula induced freezing and escape movements, and stimulation of posterior insula projections to the central amygdala caused aversive states and avoidance behavior (Schiff et al., 2018; Gehrlach et al., 2019). In our experiments, however, anterior insula stimulation failed to produce freezing or escape reactions, and both locomotor activity and water drinking induced by mild water deprivation remained intact (Haaranen et al., 2020), suggesting that the stimulation effects were

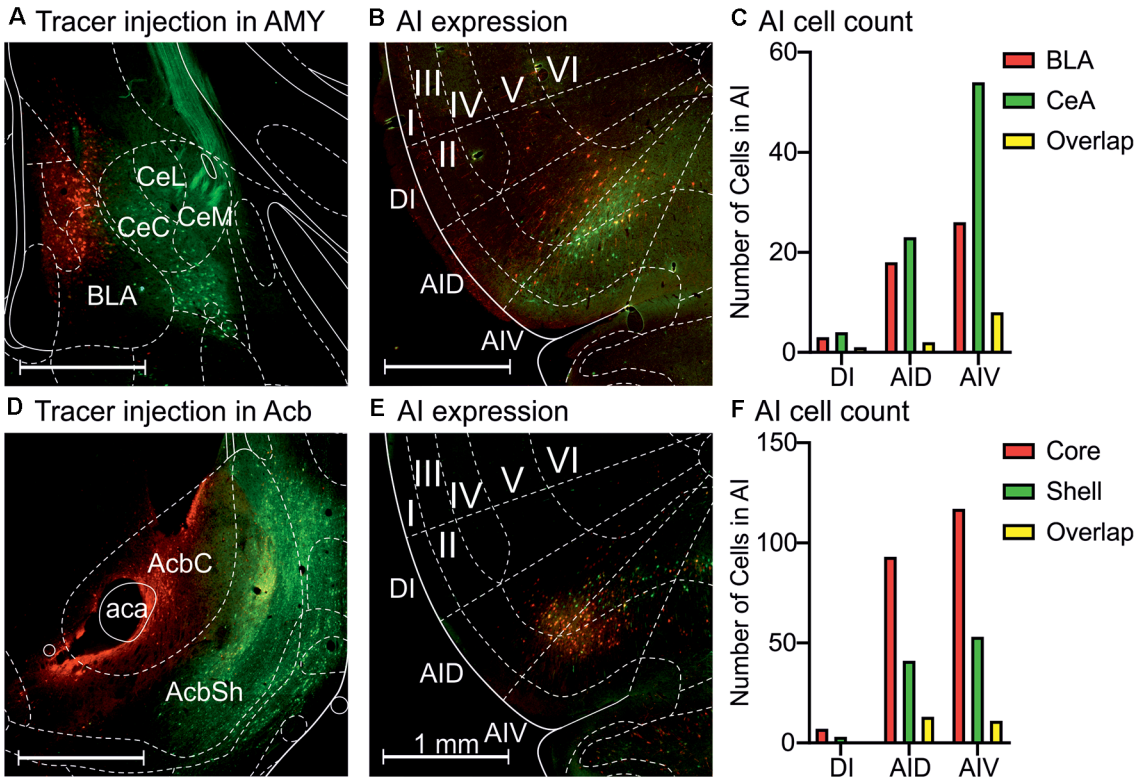

FIGURE 5 | Retrograde tracing with AAV2-retro vectors shows populations of anterior insula neurons projecting to the amygdala and nucleus accumbens subdivisions. **(A)** Starter neurons in the central amygdala (GFP) and BLA (mCherry). **(B)** Retrogradely labeled neurons in the agranular insula. **(C)** Quantification of the DI, AID, and AIV neurons projecting to central (CeA) and basolateral (BLA) amygdala. **(D)** Starter neurons in the nucleus accumbens core (mCherry) and shell (GFP). **(E)** Retrogradely labeled neurons in the agranular insula. **(F)** Quantification of the DI, AID, and AIV neurons projecting to nucleus accumbens core and shell. Abbreviations used: DI, dysgranular anterior insula; AID, agranular anterior insula, dorsal; AIV, agranular anterior insula, ventral; CeC, central amygdaloid nucleus, capsular division; CeL, central amygdaloid nucleus, lateral division; CeM, central amygdaloid nucleus, medial division; BLA, basolateral amygdala; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; aca, anterior commissure. The Roman numerals refer to cortical layers I–VI.

related to hedonic processes, in accordance with earlier studies mapping appetitive processes to anterior insula regions (Peng et al., 2015). Indeed, anterior insula electric stimulation given during presentation of liquid reward-associated conditioned stimuli attenuated appetitive approach behavior in primates (Saga et al., 2018).

The failure to see changes in alcohol drinking by insula inhibition by Gi-DREADDs appears inconsistent in the light of previous data showing that insula lesions or inactivation decreased drug consumption and their conditioned effects in various animal models (Scott and Hiroi, 2011; Contreras et al., 2012; Cosme et al., 2015; Pushparaj and Le Foll, 2015; Pushparaj et al., 2015). The fact that the same Gi-DREADDs expressed in the nucleus accumbens decreased alcohol intake in this study shows that these DREADDs were fully functional, suggesting that the efficacy of inhibitory DREADDs to alter behavior may depend on the neuron type or brain area in which they are expressed. In addition, the crucial difference between lesioning or inactivation methods and chemogenetic inhibition is that the latter results only in a partial suppression of neuronal firing (Chang et al., 2015; Smith et al., 2016). There is also evidence that DREADD-induced neuronal stimulation may produce much more robust effects than inhibition (Chang et al., 2015). Finally, one of the possible factors for determining the effects of neuronal inhibition in the anterior insula is the behavioral context of inhibition. For example, insula inhibition may preferentially suppress drug seeking or consumption in contexts associated with adverse consequences (Seif et al., 2013; Campbell et al., 2019).

As our DREADD injections into the amygdala produced DREADD expression in the central as well as the basolateral and basomedial amygdala subdivisions, DREADD-induced behavioral effects cannot be conveniently ascribed to any individual amygdala subdivision. The largely GABAergic central nucleus is divided further in the central lateral (CeL) and central medial (CeM) nuclei. The CeL receives projections from various cortical and subcortical brain areas through the lateral amygdala and projects to the CeM that receives projections also from the BLA, which is a cortical-like structure with glutamatergic projection neurons and GABAergic interneurons (Duvarci and Pare, 2014). The CeM is the main output nucleus of the amygdala, projecting to behavioral and physiological effector regions (Gilpin et al., 2015).

Amygdala chemogenetic stimulation suppressed alcohol drinking in a remarkably similar manner as in the anterior insula, an effect reminiscent of central amygdala ibotenic acid lesions, blockage of $GABA_A$ receptors, or ablation of neurotensin neurons (Hyytiä and Koob, 1995; Möller et al., 1997; Torruella-Suarez et al., 2020). All central amygdala subdivisions harbor GABAergic neurons defined by the expression of specific molecular markers. Both the CeM and CeL contain corticotropin-releasing hormone (CRH) neurons. Their chemogenetic activation increased anxiety-like behavior in mice, possibly through CRH projections to the locus coeruleus, periaqueductal gray, parabrachial nucleus, and bed nucleus of stria terminalis (BNST; Paretkar and Dimitrov, 2018), whereas their inactivation decreased escalation of alcohol drinking and somatic withdrawal symptoms induced by dependance, mediated by CRH projections to the BNST (de Guglielmo et al., 2019). In the CeL, on the other hand, optogenetic activation of protein kinase C-δ-expressing neurons attenuated food intake and water drinking (Cai et al., 2014). Similarly, deep brain electrical stimulation (DBS) into the central amygdala stopped rats working for sucrose pellets and consuming them (Ross et al., 2016), in parallel with our findings. Both DBS and chemogenetic stimulation may exert supraphysiological effects, and it could hypothesized that they severely disrupt functions of the intrinsic amygdala circuitry encoding reward and therefore suppress behavior in a manner of lesioning the area (Grill et al., 2004; Ross et al., 2016).

In addition, the BLA has been implicated in reward-related behaviors, often by loss-of-function studies (Wassum and Izquierdo, 2015). The BLA exhibits an intermingled distribution of both positive and negative valence-coding neurons that have different projection targets. For example, neurons projecting to the nucleus accumbens mediate preferentially reward-predicting cues, whereas neurons projecting to the CeM are involved in aversive outcomes (Beyeler et al., 2018). BLA lesions did not affect alcohol drinking (Möller et al., 1997), but optogenetic stimulation of the BLA projection to the nucleus accumbens shell attenuated cued alcohol seeking as well as alcohol drinking (Millan et al., 2017). Recent optogenetic experiments suggested that stimulation of the BLA, while enhancing conditioned approach and appetitive conditioning, does not affect primary reward (Servonnet et al., 2020). In contrast, optogenetic stimulation of a genetically distinct subset of central amygdala neurons was reinforcing (Kim et al., 2017), which is consistent with our data that stimulation of the anterior insula projections into the central amygdala but not the BLA increased alcohol drinking. This finding is in agreement with earlier data that alcohol consumption is positively correlated with neural activation in the central amygdala (Sharko et al., 2013) and that relapse to methamphetamine seeking is associated with an activation of the anterior insula projection to the central amygdala but not to the BLA (Venniro et al., 2017). Finally, these findings suggest a dichotomy of the functional roles of the projections that the central amygdala receives from the anterior and posterior insula. The anterior insula projections appear to promote drug seeking and reward, whereas the posterior insula projections are associated with avoidance behavior and aversive states (Schiff et al., 2018; Gehrlach et al., 2019).

The opposite effects of DREADD-induced stimulation in the anterior insula and the anterior insula projections into the central amygdala appear perplexing, but there could be various reasons for this discrepancy. Because we used the panneuronal human synapsin 1 (hSyn) promotor for expressing the DREADDs, it is possible that Gq-DREADDs were also present in cortical inhibitory interneurons. Their activation could lead to suppression of large ensembles of insula projection neurons. In addition, a general insula stimulation could also target the somas of neurons projecting to other areas than the ones examined in our experiments, and these projections could have inhibitory effects on alcohol drinking. As mentioned above, anterior insula stimulation is also propagated to the posterior insula, and although we do not favor posterior insula-mediated aversion as the explanation for reduced alcohol consumption in our study, we cannot exclude the possibility that interactions between the anterior and posterior insula could produce behavioral effects.

In contrast to the other brain areas examined, chemogenic manipulation of the nucleus accumbens produced bidirectional effects on alcohol drinking. Inhibition of the nucleus accumbens with Gi-DREADDs decreased alcohol drinking, consistent with previous studies with chemogenetic inhibition, transient inactivation, or electrolytic lesions in limited-access alcohol drinking paradigms both in mice and rats (Hodge et al., 1995; Dhaher et al., 2009; Cassataro et al., 2014; Wilden et al., 2014). Stimulation of the nucleus accumbens or the anterior insula projection to the nucleus accumbens core increased alcohol consumption, which replicates the enhancement of reward-related behavior produced by optogenetic stimulation of either the nucleus accumbens or the excitatory accumbal inputs from the ventral hippocampus, prefrontal cortex, or BLA (Britt et al., 2012). Our data therefore suggest that the glutamatergic projection from the anterior insula is one of the various inputs contributing to the total accumbal excitatory input that enhances reward seeking. This finding is also agreement with data from human heavy drinkers showing increased task-dependent connectivity between the anterior insula and nucleus accumbens associated with behavioral compulsivity (Grodin et al., 2018). Given that the insula projection to the nucleus accumbens is only one of the many excitatory inputs increasing accumbal glutamate release, the lack of effect by silencing this projection could be expected. In agreement with this notion, optogenetic inhibition of this projection suppressed only consumption of quinine-adulterated but not unadulterated alcohol (Seif et al., 2013), suggesting that silencing is efficient only for aversion-resistant alcohol consumption. However, chemogenetic inhibition on the insula–accumbens projection reduced alcohol self-administration, when CNO was administered intracranially into the nucleus accumbens (Jaramillo et al., 2018a,b), which may have led to a higher local CNO concentration at Gi-DREADDs than systematically given CNO in our experiments and thereby enhanced neuronal silencing.

To conclude, our present study produced two main outcomes. First, we showed using chemogenetic tools that manipulation

of the anterior insula, amygdala, or nucleus accumbens alone is sufficient to alter voluntary alcohol drinking. Second, we provided evidence that excitatory projections from the anterior insula to the nucleus accumbens core and central amygdala enhance alcohol reward. Particularly in the central amygdala with various subdivisions and neuron types identified by molecular markers, the exact circuitry targeted by chemogenetic manipulation remains to be elucidated. All in all, these data suggest that the anterior insula is one of the forebrain hubs regulating alcohol reward through various downstream targets, including the central amygdala and nucleus accumbens.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Project authorization board of the Regional State Administrative Agency for Southern Finland.

AUTHOR CONTRIBUTIONS

PH and MH were responsible for the study concept and design and drafted the manuscript. MH, AS, and VJ contributed to the acquisition of animal data, immunohistochemical analysis, microscopy, and provided critical revision of the manuscript for important intellectual content. All authors contributed to the article 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.2020.580849/full#supplementary-material.

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SYSTEMATIC REVIEW
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Can GLP-1 Be a Target for Reward System Related Disorders? A Qualitative Synthesis and Systematic Review Analysis of Studies on Palatable Food, Drugs of Abuse, and Alcohol

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The role of glucagon-like peptide 1 (GLP-1) in insulin-dependent signaling is well-known; GLP-1 enhances glucose-dependent insulin secretion and lowers blood glucose in diabetes. GLP-1 receptors (GLP-1R) are also widely expressed in the brain, and in addition to its role in neuroprotection, it affects reward pathways. This systematic review aimed to analyze the studies on GLP-1 and reward pathways and its currently identified mechanisms.

Methods: "Web of Science" and "Pubmed" were searched to identify relevant studies using GLP-1 as the keyword. Among the identified 26,539 studies, 30 clinical, and 71 preclinical studies were included. Data is presented by grouping rodent studies on palatable food intake, drugs of abuse, and studies on humans focusing on GLP-1 and reward systems.

Results: GLP-1Rs are located in reward-related areas, and GLP-1, its agonists, and DPP-IV inhibitors are effective in decreasing palatable food intake, along with reducing cocaine, amphetamine, alcohol, and nicotine use in animals. GLP-1 modulates dopamine levels and glutamatergic neurotransmission, which results in observed behavioral changes. In humans, GLP-1 alters palatable food intake and improves activity deficits in the insula, hypothalamus, and orbitofrontal cortex (OFC). GLP-1 reduces food cravings partially by decreasing activity to the anticipation of food in the left insula of obese patients with diabetes and may inhibit overeating by increasing activity to the consumption of food in the right OFC of obese and left insula of obese with diabetes.

Conclusion: Current preclinical studies support the view that GLP-1 can be a target for reward system related disorders. More translational research is needed to evaluate its efficacy on human reward system related disorders.

Keywords: GLP-1, reward, food intake, mood, cocaine, amphetamine, alcohol, nicotine

INTRODUCTION

GLP-1 is an incretin hormone, derived from preproglucagon and released mostly by the L-cells of intestines (Lovshin and Drucker, 2009). Through its peripheric effects as inducing insulin secretion from pancreatic beta cells, gut emptying, and inhibiting glucagon secretion; its analogs are used in the treatment of type 2 diabetes mellitus (T2DM) (Zander et al., 2002). GLP-1 can cross the blood-brain barrier and access the nervous system. It can also be produced by neurons and microglial cells (Kappe et al., 2012). It acts through G-protein coupled GLP-1 receptors (GLP-1R), that act via the activation of adenylyl cyclase (Drucker et al., 1987) and protein kinase A, which induces gene transcription (Drucker, 2006). A recent study on detailed localization and characterization of GLP-1R in the brain reported that GLP-1Rs are highly expressed in mostly GABAergic neurons within the lateral septum (LS), hippocampus, bed nucleus of the stria terminalis (BNST), and amygdala (Graham et al., 2020).

GLP-1 analogs can decrease apoptosis, increase cell viability, neurogenesis, reduce inflammation, and decrease oxidative stress. GLP-1 reduces apoptosis by increasing levels of anti-apoptotic proteins as bcl-2 and decreasing levels of pro-apoptotic proteins as cytochrome c, caspase3, and bax. GLP-1 restores neuronal growth and increases cell viability by elevating cAMP, PKA, and CREB levels and altering the phosphorylation levels of GSK-3b, AKT, ERK, and mTOR, which results in other downstream changes important for cell survival. GLP-1 also promotes neurogenesis by stimulating neurotrophic factors as GDNF, VEGF, and BDNF and reduces inflammation by decreasing TNF-a, IL-6, IL-10, and microglial activation (Erbil et al., 2019).

In addition to its role in neuroprotection, current evidence showed that GLP-1 affects reward pathways. GLP-1Rs are widely expressed in areas of the mesolimbic reward pathway that receive direct projections from the nucleus tractus solitarius (NTS) (Alhadeff et al., 2012). GLP-1Rs are located as opposed to dopamine terminals in the caudal and rostral lateral septum (LS) (Reddy et al., 2016), and GLP-1 antagonist was shown to reduce lithium chloride-induced suppression of Nucleus accumbens (NAc) phasic dopamine release (Fortin et al., 2016). Furthermore, the expression of GLP-1R was highest in the LS compared to all other regions, and these GLP-1 neurons were colocalized with dopamine receptor and calbindin-expressing cells in the LS (Graham et al., 2020). A recent study on the effect of GLP-1 on dopamine activity showed that while GLP-1 increased DA uptake, DA clearance, and DAT surface expression in the striatum in rats (Jensen et al., 2020). Due to its effect on ventral tegmental area (VTA) and striatal dopamine levels, it is suggested that both peripheral and central GLP-1 regulates hunger, satiety, and body weight (Kenny, 2011). GLP-1R in the mesolimbic reward system specifically influences the control of hedonic eating (Hernandez and Schmidt, 2019). Moreover, in humans, exenatide, a GLP-1 receptor agonist, was shown to increase brain responses to palatable food consumption and decrease brain responses to the anticipation of palatable food consumption compared to placebo, along with significantly reduced food intake in obese patients with and without T2DM (van Bloemendaal et al., 2015a).

In addition to altering food satiety signals, GLP-1 is suggested to modulate "satiety" in drugs of abuse and alter reward-related changes in multiple drugs of abuse such as cocaine (Hernandez et al., 2018, 2019), alcohol (Egecioglu et al., 2013c), nicotine (Egecioglu et al., 2013a), and amphetamine (Egecioglu et al., 2013b).

Even though the role of GLP-1 on neuroprotection is well-known and new clinical trials have been started for its repurposing in neuroprotection, the role of GLP-1 on reward systems and its potential clinical use for reward modulation still need further studies. Therefore, in this systematic review, it was aimed to analyze the studies on GLP-1 and reward, establish a comprehensive framework on the reward-related effects of GLP-1, and answer its potential use for targeting reward system related pathologies.

METHODS

Search Strategy and Study Selection

This study was conducted in line with the suggested PRISMA (Moher et al., 2009) and MOOSE (Stroup et al., 2000) guidelines. Web of Science and Pubmed were searched as the databases to reach relevant articles that include "glucagon-like peptide-1," "GLP-1," "glucagon-like peptide-1 receptor" or "GLP-1R" in their title, abstract, or as a keyword. "Reward" was not used as a keyword at this step. The search was done on September 5, 2018, and updated twice on December 27, 2019, and November 20, 2020. Duplicates between the two databases were removed in EndNote. Overall, 26,539 articles on Web of Science and Pubmed were identified. All articles were downloaded as abstracts. The methodological steps used were similar to our previous study (Erbil et al., 2019). Three authors synchronously probed the abstracts for identifying possible articles that included relevant information with a particular emphasis on GLP-1 and its effect on palatable food intake and addiction-related studies to cover reward system related disorders. Abstracts not about psychiatry, neurology, or neuroscience were excluded. In the identification step, original articles, in addition to reviews, were evaluated in full text to identify references that may have been missed by our search strategy. Reviews were screened for their references, and related citations not found by our initial search were included in the systematic review. In the screening step, two authors synchronously read all abstracts for inclusion. At this level, the main focus was on DPP-IV inhibitors, GLP-1R agonists, and antagonists. In case abstracts were inconclusive, the full article was accessed to study their relevance. Poster and conference proceedings and articles in other languages than English were excluded as quality criteria. Another author checked the final lists. Both preclinical and clinical studies were included to present a translational view and the current status of the research on this topic.

Data Extraction

Studies were grouped for models of palatable food intake and drugs of abuse, considering different pharmacological pathways that they might be associated with. For preclinical studies, mouse/rat line, design of the study, GLP-1R

agonist or DPP-IV inhibitor name, dose, administration method and duration, experimental groups in the study, molecular/behavioral/electrophysiological assessments, and main results were extracted. For human studies, the study sample size and design, GLP-1R agonist/antagonist or DPP-IV inhibitor name, dose, administration method and duration, assessments, and main results were extracted. Studies that focused on behavior but did not assess their relationship with GLP-1, such as behavioral or molecular change, were excluded. At least two authors have checked all steps.

Quality Assessment and Evaluation of Findings

As a quality assessment, it was expected for articles to fully explain the design and usage of GLP-1, along with the design of molecular and behavioral outcome measures. Results were given as organized tables for each topic, and studies were given in chronological order in the tables. Studies that included data on more than one reward-related system as Sirohi et al. (2016), where the effect of GLP-1 agonists on palatable food intake, alcohol, and amphetamine was reported, and Suchankova et al. (2015), where the effect of GLP-1 on alcohol was assessed in both humans and mice, are presented in all related tables. Results are demonstrated first on the effects of GLP-1 on rewarding behaviors, secondly, its neuroanatomical explanations, and lastly, molecular mechanisms. A meta-analysis and publication bias analysis could not be conducted due to the heterogeneity of the study designs.

RESULTS

Description of the Included Studies and the Study Characteristics

After identification, screening, and data extraction, 100 studies were included for the systematic review. The PRISMA flow diagram of the search strategy can be found in **Figure 1**. Full details of the evaluated studies are given in **Supplementary Tables**, with a classification based on the rewarding target, rat or mice group, and chronological order. Summaries are presented for each rewarding category below. Neuroanatomical associations studies for each substance and schematic drawing of reversed reward responses after GLP-1 modulations are given in **Figure 2**.

Preclinical Studies About Palatable Food Intake and GLP-1

All preclinical studies that assessed palatable food intake and its association with GLP-1 are given in **Supplementary Table 1**. In summary, evidence in rats and mice points out that GLP-1 analogs mainly decrease palatable food intake (Mathes et al., 2012; Hansen et al., 2014; Wright and Rodgers, 2014) in doses that do not affect blood glucose levels (Asarian et al., 1998; Yamaguchi et al., 2017; Maske et al., 2018; Gabery et al., 2020; Vestlund et al., 2020). Behavioral investigations of GLP-1 and palatable food intake also reported that injection of exendin-4 (Ex-4) (1 or 3.2 μg/kg IP doses only and not higher doses) also changed acquisition of food aversion learning (Liang et al., 2013) and that liraglutide (10 mg/kg IP) decreased rewarding cue responses in both chow and western diet (Jones et al., 2019). Below, neuroanatomical effects and molecular pathways associated with the GLP-1 control of palatable food intake are described.

Neuroanatomical Pathways About GLP-1 and Palatable Food Intake

Many studies investigated the role of GLP-1R modulation in different neuroanatomical areas on palatable food intake (**Supplementary Table 1**). Here, each regional effect is summarized. The first study on the topic by Asarian et al. (1998) suggested that intracerebroventricular infusions of GLP-1 inhibit sham feeding by decreasing the orosensory positive feedback that drives licking. Vagal afferents damaged by surgery protected against Ex-4 mediated conditioned flavor avoidance, and it was suggested that PVN activation by GLP-1 might be dependent on vagal afferents (Labouesse et al., 2012).

Lateral hypothalamus (LH) is a core region for palatable food intake (López-Ferreras et al., 2018). Previous eating habits as chronic consumption of palatable foods, modeled with cafeteria diet in mice, changed GLP-1 mRNA expression in the hypothalamus and led to less responsiveness to Ex-4 in food reward behaviors (Mella et al., 2017). LH injections of GLP-1/estrogen reduced food intake more than GLP-1 or estrogen-only, which showed that this effect might be sex-biased (Vogel et al., 2016).

Moreover, NAc and VTA are the core cites for reward processing and are mostly studied for this topic (Dickson et al., 2012; Dossat et al., 2013). IP or VTA injection of Ex-4 decreased ghrelin-induced food intake (Howell et al., 2019). When Ex-4 is injected into VTA, it both decreased palatable food intake, 24 h of chow intake, and body weight; however, when injected into the core or shell of NAc, it did not affect normal chow but decreased high-fat food intake (Alhadeff et al., 2012). In mice, highly palatable high-fat food intake was reduced by the activation of GLP-1 neurons (Wang et al., 2015). When NAc core is studied, anorexia observed after NAc GLP-1 injections were suggested not to be due to viscerosensory stress or nausea but due to increased satiety. Intra-lateral ventricle (LV) GLP-1 injections of 1 and 3 μg intra-LV GLP-1 injections reduced food intake, whereas lower doses had no effect (Dossat et al., 2011). Intra-NAc core Ex-4 also successfully reduced μ-opioid receptor agonist (DAMGO) induced palatable food intake, and combination of intra-NAc core Ex-9 and DAMGO increased length of food intake (Pierce-Messick and Pratt, 2020). Viral modulation of GLP-1 expression on NTS (Alhadeff et al., 2017) or modulation of NTS by Ex-4 injection in rats (Alhadeff and Grill, 2014) or activation of NTS GLP-1 neurons in mice (Wang et al., 2015) decreased palatable food intake. When only chow was presented, intra-NTS Ex-4 also reduced chow intake along with bodyweight but did not change locomotor activity or induce a pica response, which is a model of nausea (Richard et al., 2015). Similar to LH, the effects on NTS was also found to be regulated by estrogen. NTS injections of the GLP-1/estrogen reduced food intake and

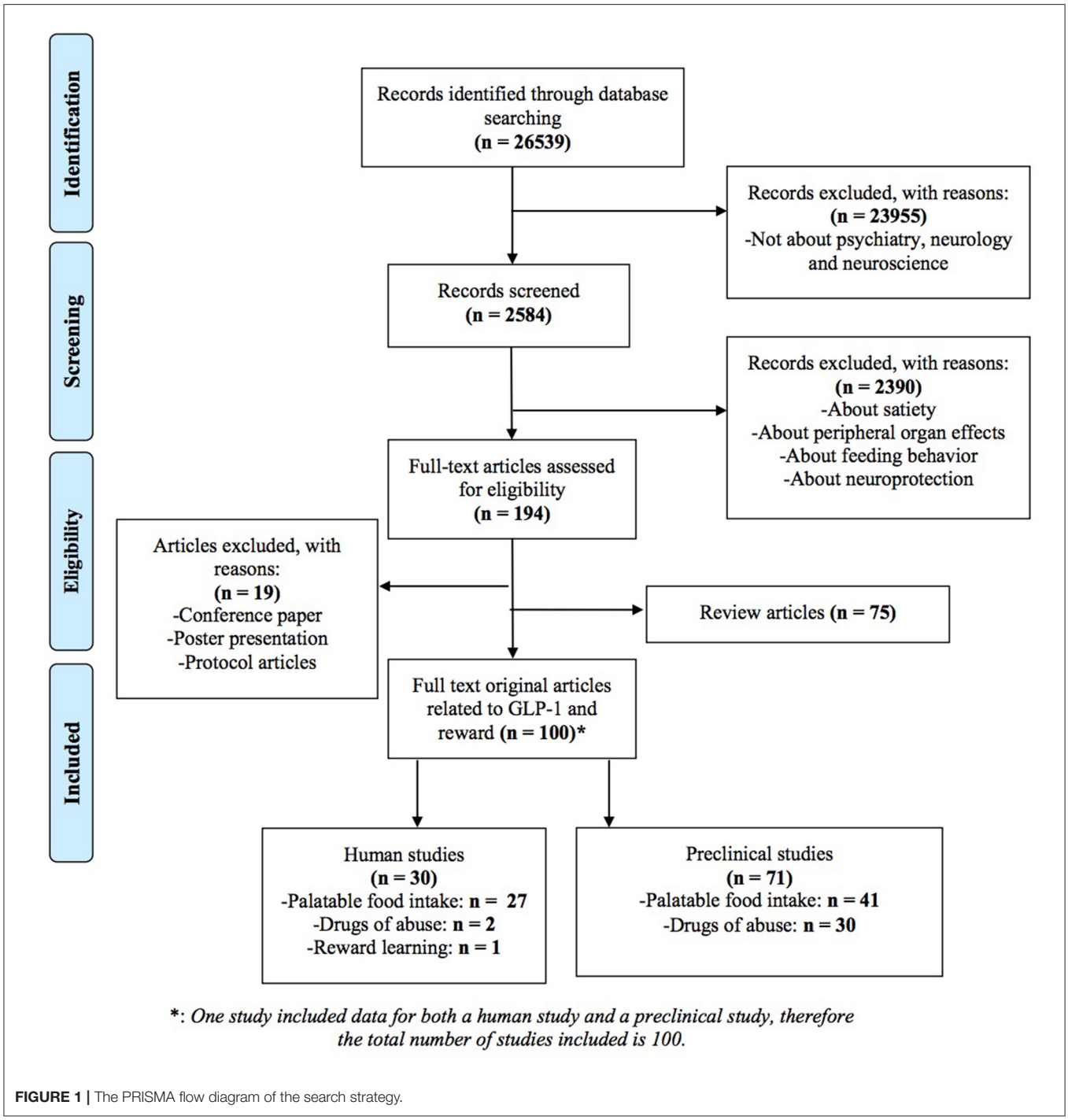

FIGURE 1 | The PRISMA flow diagram of the search strategy.

caused a trend toward decreased bodyweight more than GLP-1 or estrogen alone (Vogel et al., 2016). NTS GLP-1 neurons project on the lateral parabrachial nucleus (LPBN), and Ex-4 injection of LPBN neurons showed similar effects on both chow and high-fat food intake (Alhadeff et al., 2014). Furthermore, semaglutide induced neuronal activation overlapped with meal-termination neuronal pathways controlled by PBN neurons (Gabery et al., 2020).

Expression of supramammillary nucleus (SuM) GLP-1R is comparable with the expression in the LH and NAc, but lower than that found in the NTS (López-Ferreras et al., 2019). Activation of GLP-1R by infusion of Ex-4 (0.01 and 0.03 μg) into the SuM or AAV virus in male or female rats was found to be potently anorexigenic. SuM GLP-1R controlled food reward predominantly in male rats and with a lower degree in females (López-Ferreras et al., 2018). Also, SUM injection of

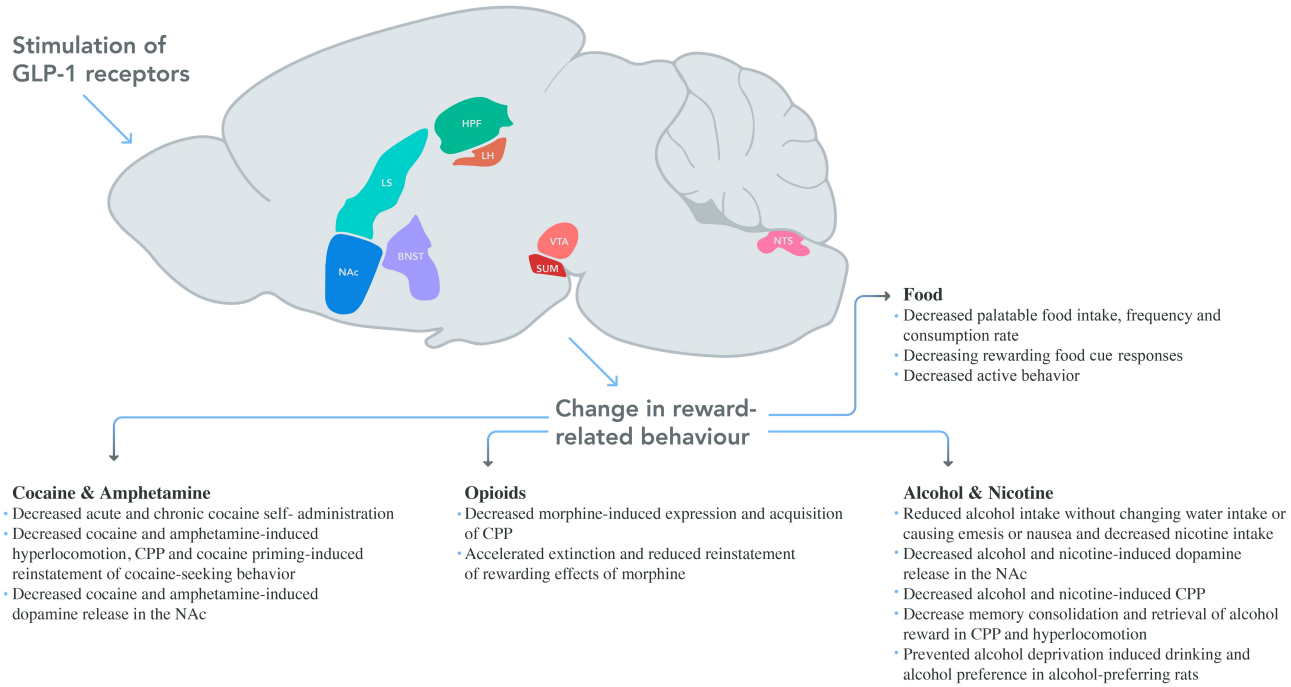

FIGURE 2 | Schematic drawing of reversed reward responses after GLP-1 modulation. In addition to the systematic injection of GLP-1 agonists' effects on the brain; LH, VTA, NAc, NTS, SUM, and LS were studied for palatable food, whereas VTA, NAc, and NTE were studied for alcohol, NAc was studied for amphetamine, NAc and LS were for cocaine and NTS was for nicotine use behavior. Areas were drawn based on Allen Mouse Brain Atlas (NAc, nucleus accumbens; LS, lateral septum; HPF, hippocampal formation; LH, lateral hypothalamus; VTA, ventral tegmental area; SUM, supramammillary nucleus; NTS, nucleus tractus solitarius; BNST, bed nucleus of the stria terminalis).

GLP-1/estrogen reduced food reward and food intake more than GLP-1 or estrogen alone (Vogel et al., 2016).

The intra-BNST GLP-1 decreased chow intake at a dose-dependent magnitude of effect, while exendin9-39 (Ex-9) reversed it. GLP-1 injection into the BNST less effectively suppressed feeding in high-fat diet (HFD) at 1 and 2 h (more significant at higher doses), and Ex-9 did not change intake in HFD mice (Williams et al., 2018).

The LS is a relay center for connections from the CA3 of the hippocampus to the VTA. Hippocampal formation (HPFv) Ex-4 decreased chow intake, meal size but did not affect meal frequency, and HPFv Ex-9 increased food intake only at the 6th hour. HPFv Ex-4 increased preference for the chow diet over the Western diet (Hsu et al., 2015). Ventral hippocampal field CA1 (vCA1) Ex-4 reduced food intake and operant responding for palatable food and this was modulated via vCA1 to medial PFC (mPFC) projections (Hsu et al., 2018). Intra-dLS GLP-1 decreased active lever presses; however, intra-LS Ex-4 with Ex-9 (at subthreshold doses) did not change chow intake, indicating GLP-1 in LS do not control *ad libitum* feeding. Ensure intake (large meal) before test sessions decreased subsequent chow intake, which was reversed with intra-LS Ex-9 (Terrill et al., 2019). In rats as well, intra-LS Ex-4 decreased overnight chow and HFD intake. The average dark-phase meal size and average light-phase meal size were decreased after intra-LS Ex-4 (Terrill et al., 2016).

The effect of GLP-1 on food intake can also be related to the circadian rhythm. Animal models show that blood GLP-1 levels can vary during the day based on mealtimes, and this variation may be independent of the insulin variation (Dailey et al., 2012). GLP-1 is not mainly dependent on Melanocortin-3 or 4 receptors (MC3R or MC4R), or any other postulated Agouti-related protein (AgRP) sensitive pathway for their function for food intake behavior (Edwards et al., 2000). Night/light eating changes also were defined by Alhadeff et al. (2017).

Molecular Pathways About GLP-1 and Palatable Food Intake

Evidence suggests a significant role of GLP-1 in modulating *dopaminergic circuits* and molecular synthesis of dopamine. Mesolimbic tyrosine hydroxylase (TH) and dopamine receptor 1 (D1R) gene expression were significantly decreased in chronic HFD-fed rats; Ex-4 and food restriction reduced these decreased expressions (however, only D1R changes reached significance) (Yang et al., 2014). In mice, GLP-1/dexa reduced the expression of reward-related genes in the NAc such as D1R, dopamine receptor 2 long isoform (D2rlg), kappa opioid receptor (Kor), glucocorticoid receptor (Gr), others such as TH and dopamine transporter (DAT) were also decreased but non-significantly (Decarie-Spain et al., 2019). Intra-NTS Ex-4 in rats increased dopamine-B-hydroxylase expression in NTS, an enzyme for noradrenaline synthesis, and GLP-1 fibers and noradrenergic

neurons were found to be colocalized in the NTS (Richard et al., 2015). In the VTA, intra-NTS changed TH (Mietlicki-Baase et al., 2013; Richard et al., 2015) and D2 expression levels (Richard et al., 2015). In a more detailed analysis of dopaminergic receptors, central Ex-4 injection (0.3 μg) increased the levels of dopamine metabolites, DOPAC and HVA, as well as dopamine turnover in the amygdala (Anderberg et al., 2014). Central Ex-4 decreased food intake at the 1st hour, without affecting food motivated operant behavior, by increasing amygdala dopamine, and it was not related to D2/D3 receptor blockage. In contrast, the 24-h chow-intake reduction produced by Ex-4 was significantly attenuated by the D2/D3 receptor blockade. Ex-4 significantly reduced operant behavior for a sucrose reward, and this reduction was not attenuated by the D2/D3 receptor blockade (Anderberg et al., 2014). Ex-4 also decreased sucrose induced licking behavior, and the magnitude of cue evoked phasic dopamine activity; this response of dopamine was significantly associated with subsequent sucrose directed behavior (Konanur et al., 2020).

Another neurotransmitter system GLP-1 modulates is the *glutamatergic neurotransmission*. In mice, Ex-4 application to TH+ VTA-to-NAc projecting DA neurons suppressed AMPA-R-mediated excitatory post-synaptic potentials (EPSCs) without changing NMDA EPSCs (Wang et al., 2015). In another study by Mietlicki-Baase et al. (2013) in rats, intra-VTA Ex-4 was found to reduce high-fat food intake (from 3 to 24 h), and these reductions were suppressed by AMPA/kainate receptor antagonist, CNQX. NMDA-R was not involved in food intake and the meal size suppressive effects of intra-VTA Ex-4. Ex-4 increased sEPSC frequency in VTA dopamine without changing sEPSC decay, time, peak amplitude, and charge transfer, which indicated a presynaptic GLP-1R activation on glutamatergic terminals in AMPA/kainate receptors. When these interactions were studied for NAc (Mietlicki-Baase et al., 2014), Ex-4 treatment did not alter dopamine release in NAc core slices. Ex-4 increased the frequency of NAc core medium spiny neuron (MSN) mEPSCs but did not affect kinetics or amplitude, indicating a presynaptic effect of GLP-1R activation. Ex-4 bath application decreased the paired-pulse ratio (PPR) of evoked EPSCs, further supporting a presynaptic effect by increasing the probability of glutamate release. Ex-4 suppressed action potential (AP) firing on PVT-to-NAc projecting neurons (Ong et al., 2017). Ex-4 induced suppression of AP firing was smaller and delayed when given with synaptic blockers (CNQX for glutamate receptors, PTX for GABA receptors). Ex-4 caused hyperpolarization of PVT-to-NAc neurons in the presence of synaptic blockers. However, in mice, GLP-1 reversibly depolarized or hyperpolarized BNST neurons, which was the opposite of when dopamine was applied, indicating excitatory and inhibitory responses in BNST (Williams et al., 2018).

In addition to other neuromodulators affected by GLP-1 agonists, Ex-4 increased pro-opiomelanocortin (POMC) expression while decreasing neuropeptide Y (NPY), and this effect was independent of the reduction of food intake and body weight (Yang et al., 2014). Estrogen receptors and GLP-1R are co-localized in areas involved in reward behavior regulation such as the VTA and NAc; central ERα signaling might be modulating the actions of GLP-1 on food-reward behavior (Richard et al., 2016).

Preclinical Studies About Drugs of Abuse and GLP-1

Among the drugs of abuse that have been studied, cocaine (Egecioglu et al., 2013b; Graham et al., 2013; Harasta et al., 2015; Sørensen et al., 2015; Reddy et al., 2016; Schmidt et al., 2016; Sirohi et al., 2016; Fortin and Roitman, 2017; Hernandez et al., 2018, 2019; Bornebusch et al., 2019; You et al., 2019; Łupina et al., 2020), amphetamine (Lautar et al., 2005; Erreger et al., 2012; Egecioglu et al., 2013b; Sirohi et al., 2016), opioids (Bornebusch et al., 2019; Łupina et al., 2020; Zhang et al., 2020), alcohol (Egecioglu et al., 2013c; Shirazi et al., 2013; Sirohi et al., 2016; Sørensen et al., 2016; Vallöf et al., 2016, 2019a,b, 2020; Thomsen et al., 2017, 2019; Abtahi et al., 2018; Dixon et al., 2020), and nicotine (Egecioglu et al., 2013a; Tuesta et al., 2017) were found. All preclinical studies that assessed the association between GLP-1 and drugs of abuse are presented in **Supplementary Table 2**. Findings from each drug of abuse are summarized below.

Cocaine

Studies show a link between GLP-1 levels, GLP-1R modulation, and cocaine use (**Supplementary Table 2**). GLP-1 levels increased after cocaine use (You et al., 2019). Ex-4 significantly reduced cocaine-induced conditioned place preference (CPP) even at the lowest dose without affecting locomotor activity or causing aversion (Graham et al., 2013). Ex-4 decreased acute and chronic cocaine self-administration and D1R agonist-induced hyperlocomotion along with cocaine-induced c-fos expression and dopamine release in the striatum in mice (Sørensen et al., 2015). Ex-4 decreased cocaine-induced hyperlocomotion and CPP along with accumbal dopamine release without affecting spontaneous locomotor activity and accumbal dopamine release in normal conditions (Egecioglu et al., 2013b; Sørensen et al., 2015). On the first day of extinction, GLP-1 levels were increased to the same level seen in the cocaine self-administration period; however, it was normalized on the 14th day of extinction (You et al., 2019). Low doses of peripheral Ex-4 (0.1 and 0.2 μg/kg) reduced cocaine priming-induced reinstatement of cocaine-seeking behavior dose-dependently but did not affect chow food intake, meal patterns, and body weight (Hernandez et al., 2018, 2019). When intra-VTA was injected, Ex-9 inhibited the effects of peripheral Ex-4 (Hernandez et al., 2018), and VTA GLP-1R knockdown (KD) significantly increased cocaine intake (Schmidt et al., 2016). Moreover, intra-VTA Ex-4 (0.05 μg/kg) also reduced cocaine-seeking dose-dependently but did not affect sucrose seeking (Schmidt et al., 2016; Hernandez et al., 2018). Extinction following cocaine exposure diminished preproglucagon (PPG) expressions in the NTS (Hernandez et al., 2018). Ex-4 reduced cocaine-induced increase in dopamine concentration in the NAc core but not the shell, without affecting the dopamine reuptake (Fortin and Roitman, 2017). Cocaine priming-induced reinstatement of cocaine-seeking behavior was attenuated in rats Ex-4 administered into the NAc core (0.005 and 0.05 μg/500 nl) and shell (only the higher dose) without again changing sucrose

seeking. Ex-4 increased MSN AP frequency in the NAc core and shell following the extinction of cocaine self-administration without changing sEPSC frequency, paired-pulse ratio (PPR), or sEPSC. These results were not associated with GLP-1R expression levels in the NAc after voluntary cocaine intake (Hernandez et al., 2019).

Cocaine-experienced rats had greater plasma corticosterone levels, and corticosterone administration into the hindbrain fourth ventricle reduced cocaine self-administration dose-dependently without changing sucrose intake, and these effects were inhibited by GLP-1 antagonist (Schmidt et al., 2016).

In the LS slices, GLP-1 increased DAT surface expression (increased transport capacity), and DA uptake and GLP-1 antagonist reversed these effects. Systematic administration of Ex-4 of 2.4 μg/kg decreased LS activity and cocaine-induced extracellular DA release in the LS along with reduced expression of retrograde messenger 2-AG and arachidonic acid (AA), which serves as a reducing agent of DAT function (Reddy et al., 2016). Also, GLP-1R mRNA expression was high in the GABAergic neurons of the dorsal lateral septum (dLS). In the dLS, GLP-1R deficient mice neurons were shown to be more excitable. GLP-1R deficient mice had increased cocaine-induced CPP and cocaine-induced locomotor activity. GLP-1R gene delivery to the dLS of GLP-1R deficient mice resulted in reduced cocaine-induced CPP and locomotor activity without changing anxiety behaviors (Harasta et al., 2015).

Amphetamine

Ex-4 (2.4 μg/kg) decreased amphetamine-induced hyperlocomotion, CPP, and accumbal dopamine release without affecting spontaneous locomotor activity and accumbal dopamine release in normal conditions (Egecioglu et al., 2013b). It reduced basal and amphetamine-induced locomotor activity in rats in very high doses (30 μg/kg). Therefore, GLP-1 agonists were suggested as potential targets for psychostimulant abuse (Erreger et al., 2012). This finding was also supported by the use of DPP-IV inhibitor, AMAC, which dose-dependently decreased amphetamine-induced hyperactivity (Lautar et al., 2005). In GLP-1R KD Nestin mice, Ex-4 blockage of amphetamine-induced CPP was not observed (Sirohi et al., 2016).

Opioids

Linagliptin, a DPP-IV inhibitor, inhibited the expression and acquisition of morphine-induced CPP along with accelerating the extinction and reducing reinstatement of the rewarding effects of morphine (but only at the lower dose), without affecting the locomotor activity in rats (Łupina et al., 2020). Contrary to this finding, in mice, Ex-4 did not impact morphine-induced CPP, morphine withdrawal, or hyperlocomotion. Ex-4 also did not reduce remifentanil (a synthetic opioid) self-administration (Bornebusch et al., 2019). However, both systematic and intra-NAc shell Ex-4 successfully decreased oxycodone self-administration and cue priming-induced reinstatement of oxycodone seeking-behavior in mice without causing adverse feeding behaviors or changing analgesic effects of oxycodone (Zhang et al., 2020).

Alcohol

In rats, Ex-4 successfully reduced ethanol intake dose-dependently and reduced spontaneous locomotion (Bornebusch et al., 2019). Ex-4 reduced alcohol intake and alcohol-seeking behavior in rats after 8 months of alcohol use (Egecioglu et al., 2013c). Ex-4, at doses not affecting baseline locomotor activity, reduced alcohol-induced locomotor behavior, and accumbal dopamine release evoked by ethanol. Ex-4 (3.2 μg/kg) decreased intravenous ethanol self-administration but did not change palatable liquid food intake (Sørensen et al., 2016). In alcohol dependent mice, an exenatide analog, AC3174, significantly reduced voluntary ethanol intake (Suchankova et al., 2015). Liraglutide and exenatide (to a lesser extent) reduced alcohol intake without affecting water intake or causing emesis or nausea in monkeys (Thomsen et al., 2017, 2019). Acute liraglutide attenuated the alcohol-induced increase in accumbal dopamine, decreased alcohol-induced CPP, and prevented alcohol deprivation-induced drinking. After 12 weeks of alcohol consumption, both acute and chronic liraglutide administration reduced alcohol intake (along with food intake). In alcohol-preferring rats, chronic liraglutide decreased operant alcohol self-administration (Vallöf et al., 2016). Long-term treatment (9 or 5 weeks) of dulaglutide reduced ethanol intake and ethanol preference in both male and female rats and altered levels of dopamine, serotonin, and noradrenalin in the amygdala of male rats and dopamine, DOPAC, and noradrenaline levels in the striatum of female rats (Vallöf et al., 2020).

When neuroanatomical associations of this behavioral effect were analyzed, intra-NTS Ex-4 (0.05 μg per side) decreased alcohol-induced locomotor behavior, accumbal dopamine release, and memory consolidation of alcohol reward in mice, whereas a lower dose (0.025 μg per side) was ineffective. Ex-9 injected into the NTS blocked the reducing effect of systematic Ex-4 on locomotor activity in mice (Vallöf et al., 2019b). In another study, both peripheral GLP-1 and Ex-4 decreased alcohol intake, and GLP-1 reduced alcohol preference in the CPP test in rats, whereas Ex-9 increased alcohol intake (Shirazi et al., 2013). Ex-4 alone, or in combination with the ghrelin antagonist, in the NAc shell but not core, reduced alcohol intake in a time-dependent manner (Abtahi et al., 2018). Ex-4 into the NAc shell blocked alcohol-induced locomotor behavior, memory retrieval of alcohol reward in CPP, and decreased alcohol intake without affecting water intake and body weight. GLP-1R expression in the NAc shell (but not in the PFC, VTA, amygdala, hippocampus, and striatum) was also increased in high alcohol-consuming mice. Ex-4 into the posterior VTA reduced alcohol-induced locomotor behavior but did not change CPP or alcohol intake, and Ex-4 into the anterior VTA did not alter locomotor activity or CPP. Ex-4 into the laterodorsal tegmental area blocked alcohol-induced locomotor behavior and reduced alcohol intake but not CPP (Vallöf et al., 2019a). Intra-VTA Ex-4 significantly decreased alcohol self-administration without affecting food intake or locomotor activity, and this was more prominent in alcohol-preferring rats. However, intra-VTA Ex-4 did not change extinction following the reacquisition of alcohol self-administration or motivation for alcohol (Dixon et al., 2020).

Nicotine

Ex-4, at doses not affecting baseline locomotor activity, reduced nicotine-induced locomotor behavior, accumbal dopamine release, and blocked nicotine-induced CPP in mice. Ex-4 also abolished nicotine-induced locomotor sensitization (Egecioglu et al., 2013a). Also, Ex-4 (10 μg/kg) and sitagliptin decreased nicotine intake, whereas GLP-1R KO mice increased nicotine intake, but neither changed food responses. Chemogenetic activation of NTS GLP-1 neurons also decreased nicotine intake. NTS GLP-1 neurons activated medial habenular (MHb) projections to the interpeduncular nucleus (IPN), and GLP-1 application into the IPN decreased nicotine intake and attenuated nicotine reward without causing malaise; Ex-9 into the IPN reversed these effects. Neither changed food intake nor food reward (Tuesta et al., 2017).

Human Studies About Palatable Food Intake, Cocaine, and GLP-1

All human studies of palatable food intake and GLP-1 are summarized in **Supplementary Table 3**. Only two studies assessed drugs of abuse and GLP-1 in humans. The one study on cocaine showed that GLP-1 levels were significantly reduced after cocaine injections in cocaine users, which might be precipitating further cocaine intake, and subjective "anxiety" was a positive predictor of post-cocaine GLP-1 concentrations (Bouhlal et al., 2017). Another study on alcohol use disorder (AUD) found that 168Ser allele (rs6923761) was associated with AUD in humans. Furthermore, 168Ser allele was associated with increased neuronal activity in the globus pallidus, an area crucial for reward processing, for high monetary reward during an incentive delay task (Suchankova et al., 2015). One study evaluated GLP-1s effect on binge episodes in T2DM patients with a history of binge eating disorder (Da Porto et al., 2020).

As indirect evidence about palatable food intake and GLP-1, blood levels of GLP-1 were analyzed in relation to food intake. GLP-1 levels did not correlate with the food wanting task scores after a 4-course meal either in staggered or non-staggered intake models. However, staggered meal intake resulted in higher GLP-1 levels and satiety, along with lower ghrelin and desire for food scores than non-staggered meal intake (Lemmens et al., 2011). Higher GLP-1 plasma levels before *ad libitum* food intake were associated with lower intake of carbohydrates and simple sugar but not total food intake (Basolo et al., 2019). When exposed to palatable food compared to non-palatable food, GLP-1 levels did not change significantly in obese patients (Rigamonti et al., 2015). Supporting this finding, acute exenatide decreased sodium and total food intake without affecting salt craving scores in healthy, obese, and T2DM obese groups. Prolonged liraglutide did not change sodium or total food intake along with salt craving scores (Smits et al., 2019).

Exenatide increased cerebral glucose metabolic rate (CMRglu) in regions related to glucose homeostasis regulation (frontal, occipital, temporal, parietal lobes, limbic system, insula, and putamen) and food reward (orbitofrontal lobe, thalamus, anterior, and posterior cingulate). It decreased CMRglu in the hypothalamus, along with reduced plasma insulin levels (Daniele et al., 2015). In a small sample of male patients, resting-state data also showed that co-administration of intragastric glucose and GLP-1 antagonist resulted in higher resting state functional connectivity (rsFC) between the hypothalamus and the left lateral orbitofrontal cortex (OFC), between the right NAc and the right lateral OF, and lower rsFC between the midbrain and the right caudate nucleus. Glucose ingestion only decreased prospective food consumption and increased sensations of fullness compared to baseline. These changes were not observed after co-administration with GLP-1 antagonists (Meyer-Gerspach et al., 2018). In a small sample of female patients, exenatide increased functional connectivity between left NTS and left hypothalamus and thalamus in obese and between right NTS and left thalamus in both obese and lean individuals. Exenatide also induced a positive correlation between hunger ratings and NTS functional connectivity in both obese and lean, with more significance in the obese (Coveleskie et al., 2017). In Roux-en-Y gastric bypass (RYGB) surgery patients, octreotide (a somatostatin analog) suppressed post-prandial plasma GLP-1 levels, which correlated with palatable food reward in a progressive ratio task, food appeal scores, and increased activation of important reward system areas such as NAc, caudate, anterior insula, and amygdala. These results were not seen in gastric banding (BAND) surgery patients or controls (Goldstone et al., 2016). Furthermore, Ex-9 resulted in decreased connectivity in the right middle frontal gyrus and right caudate nucleus pre-RYGB surgery and reduced connectivity in the right OFC post-RYGB surgery (van Duinkerken et al., 2020). In addition, Ex-9 more significantly increased the connectivity in the left lateral occipital cortex post-RYGB compared to pre-RYGB, and this was associated with a greater decrease in BMI, savory food appetite scores, and hunger scores (van Duinkerken et al., 2020).

Plasma GLP-1 levels showed a positive correlation with regional cerebral blood flow (rCBF) in the left dorsolateral prefrontal cortex (dlPFC) and hypothalamus independent of sex, age, adiposity, insulin, glucose, and free fatty acids (Pannacciulli et al., 2007). In a long-term lifestyle intervention, when GLP-1 and dlPFC activity co-occurred, it successfully predicted subsequent weight loss. However, neither GLP-1 nor dlPFC predicted weight loss individually (Maurer et al., 2019). Post-prandial GLP-1 levels increased from pre to post-RYGB and SG surgeries. In RYGB, this increase in GLP-1 correlated with decreased activity in the inferior temporal gyrus and right middle occipital gyrus and increased activity in the right medial PF gyrus/paracingulate cortex to high-energy vs. low-energy visual and auditory food cues (Baboumian et al., 2019).

Combined infusion of GLP-1 and PYY in fasted subjects reduced energy intake and decreased brain activity in the amygdala, caudate, insula, NAc, OFC, and putamen to food vs. non-food cues (De Silva et al., 2011). In lean controls, higher sugar intake was associated with reduced GLP-1 response to glucose intake. These responses showed negative correlations with dorsal striatum reactivity to food cues without having any correlations with NAc activity (Dorton et al., 2018). Colonic propionate injection, a short-chain fatty acid which acutely increased plasma GLP-1 and PYY levels, reduced brain activity in

the caudate and NAc to high-energy food pictures in healthy lean men. Even though these changes were associated with decreased high-energy food appeal, they were independent from changes of GLP-1 or PYY levels (Byrne et al., 2016).

Independent of other metabolic and hormonal factors, exenatide decreased activation of the bilateral insula, left putamen, and right OFC to both food and high-calorie food vs. non-food pictures in T2DM patients and of the right insula and left OFC to high-calorie vs. non-food pictures in obese patients along with reduced food intake (van Bloemendaal et al., 2014). Exenatide decreased brain activation to the anticipation of palatable food in bilateral OFC of lean subjects, bilateral putamen, left insula, and left amygdala of the obese with T2DM, which might reduce food cravings. Exenatide increased brain activation to the consumption of palatable food in the right caudate nucleus of lean subjects, in the right OFC of obese subjects and left insula, bilateral putamen, and left amygdala of obese with T2DM, which might inhibit overeating. Ex-9 reversed these effects (van Bloemendaal et al., 2015a). Emotional eating was negatively correlated with exenatide-induced reductions of activation in the amygdala of obese and in the insula of T2DM patients, which showed that effects of GLP-1 were less significant on patients with emotional eating (van Bloemendaal et al., 2015b). The increase in GLP-1 levels after an oral glucose load was negatively correlated with food cue-induced OFC activity in both lean and obese individuals, and this was independent of changes in insulin, glucose concentrations, gender, BMI, and age. Only lean individuals showed associations between post-prandial insulin and OFC activations (Heni et al., 2015).

T2DM patients showed increased activity in the bilateral insula, left amygdala, right OFC in response to high-energy food cues during the fasting condition. Meal intake decreased bilateral insula activation, and GLP-1R antagonist reversed the meal-induced reductions in the bilateral insula activity in response to high-energy food, in addition to leading to increased hunger scores (Jennifer et al., 2015). In obese T2DM patients, GLP-1R agonist, liraglutide, decreased activation in the bilateral insula after fasting and left putamen after the post-prandial condition in the fMRI task while viewing both food and high-calorie food pictures at 10 days after treatment. This effect was not observed after 12 weeks of treatment (Jennifer et al., 2016). T2DM patients showed reduced activation in the right insula in response to palatable food compared to lean controls, and liraglutide increased the activation in the right insula and caudate nucleus at 10 days after treatment, but these effects were not observed after 12 weeks. Liraglutide decreased brain activity in the parietal cortex, insula, and putamen of the T2DM patients. While on liraglutide, the hunger and appetite scores of T2DM patients were positively correlated with activations in the precuneus, cuneus, parietal cortex, and nausea scores negatively correlated with activations in the cuneus, precuneus, cingulate cortex, and some parts of the PFC in response to highly desirable vs. less desirable food cues (Farr et al., 2016a). Short-term liraglutide administration in T2DM patients increased GLP-1, gastrointestinal peptide (GIP), and decreased percent change of leptin levels (Farr et al., 2016b). Liraglutide, when administered for a more extended period (5 weeks) at the highest dose, increased OFC activation to food vs. non-food cues when corrected for BMI/weight in obese patients (Farr et al., 2019). These results further support ten Kulve (Jennifer et al., 2016) as longer-term treatments of GLP-1 failed to reach significance in the weight loss process (ten Kulve et al., 2016). When compared to pre-surgery, GLP-1R antagonist administration more significantly increased activation in the caudate nucleus to visual food cues and in the insula to gustatory food cues after RYGB, indicating an effect of surgery on the actions of GLP-1R blockage (Jennifer et al., 2017).

DISCUSSION

To our knowledge, this study is the most up to date and comprehensive translational review of the effect of GLP-1 on reward. Our current review demonstrates that GLP-1 not only decreases palatable food intake, but it can also decrease cocaine, amphetamine, alcohol, and nicotine use in animal models (**Supplementary Tables 1, 2**). A limited number of human studies also support the central regulatory role of GLP-1 on reward pathway functional connectivity (**Supplementary Table 3**).

This review summarizes that the effect of GLP-1 is not just through its peripheric effects, but also through its central effects on reward processing as assessed by injection of GLP-1 analogs or GLP-1 itself to intra-NTS, NAc, VTA, LH, lPBN, SUM, BNST, LS for palatable food intake, intra-VTA, NAc, and LS for cocaine, intra-NAc for amphetamine, intra-NAc, NTS, dLS for alcohol, and intra-NTS and IPN for cocaine. It is shown to be increasing dopamine-B-hydroxylase levels, decreasing extracellular dopamine release, increasing DAT surface expression, increasing dopamine turnover, and suppressing projections to NAc, GLP-1R agonists might be decreasing palatable food or drug-induced phasic dopaminergic signaling in reward-related areas and therefore decreasing conditioned consummatory behavior.

As an example pathway to decrease conditioned consummatory behavior, VTA GLP-1R signaling potentially controls for palatable food intake by modulating the rewarding value of the ongoing meal while having fewer effects on between-meal satiety processes. Focusing on the molecular systems behind the dopaminergic modulation, it was shown that GLP-1R signaling in the VTA alters presynaptic modulation of glutamatergic excitation of dopamine neurons via AMPA/kainate but not NMDA receptors. AMPA/kainate but not NMDA receptors also mediate the effects of intra-NAc GLP-1R activation. On the opposite, in mice, GLP-1 reversibly depolarized or hyperpolarized BNST neurons, which was the opposite of when dopamine was applied, which indicated excitatory and inhibitory responses in BNST (Williams et al., 2018; **Supplementary Tables 1, 2**). When these effects were studied using cocaine, Ex-4 increased MSN action potential (AP) frequency in the NAc core and shell following the extinction of cocaine self-administration without changing sEPSC frequency or PPR or sEPSC, which indicated that increased excitability of MSNs in NAc might be contributing to suppressive effects of

Ex-4 and this is a mechanism independent of the presynaptic stimulation (Hernandez et al., 2019). As a gap in the literature, many of the preclinical studies on drugs of abuse were based on behavioral data and the modulation of GLP-1 in only specific brain regions; further studies are needed that evaluate the molecular and electrophysiological background of these relationships, and that discriminate between the molecular effects of different substances.

As another significant finding of this study, current literature points that while GLP-1 treatment significantly decreases addictive-like behavioral effects on cocaine, alcohol, and nicotine, the evidence for opioid addiction is contradictory. One study reported that Ex-4 fails to reduce morphine-induced CPP, morphine withdrawal, or hyperlocomotion in mice, which indicates that GLP-1 may not be beneficial in the treatment of opioid addiction as it is in other drugs of abuse (Bornebusch et al., 2019). However, a recent study found that Ex-4 successfully decreases oxycodone self-administration and oxycodone-seeking behavior in mice without changing food intake or causing aversive behaviors (Zhang et al., 2020). These results are also replicated when Ex-4 is directly injected into the NAc shell (Zhang et al., 2020). This new data on opioids can serve as an indicator of using GLP-1 to reduce opioid reinforcement and drug relapse. The differences between studies can be due to species, strain, type of assays, or dose range; new and more standardized studies on opioids are necessary to better understand this relationship.

Some new studies also suggest the role of GLP-1 in the control of sexual behaviors. Both systematic and intra-NTS Ex-4 results in decreased sexual interaction behaviors in sexually naïve male mice (Vestlund and Jerlhag, 2020b). Furthermore, investigating specific regions important in reward neurocircuitry, Ex-4 injection into posterior VTA or NAc shell reduces pre-sexual interaction behaviors, and intra-LDTg Ex-4 reduces all phases of sexual interaction behaviors (Vestlund and Jerlhag, 2020a). Ex-4 into anterior-VTA does not result in significant changes in sexual behavior. These results support the role of GLP-1 in social rewarding behaviors and further highlight its effect on reward-related regions.

In terms of human studies, they have the disadvantage of having small sample sizes. Also, they focus mainly on the palatable food intake, except for one on cocaine. They lack evaluation of the mediators as chronic stress and intellectual level on the outcome. Here, the translation of preclinical studies to human studies is limited. When clinical trial registries are also investigated, it can be seen that upcoming studies also focus on diabetes or obesity. Studies are still limited on different drugs of abuse, alcohol, or nicotine.

As a summary of human studies, GLP-1 analogs modulated palatable food intake in humans. In T2DM patients, GLP-1 analogs modulated palatable food intake in humans. In T2DM patients, the insula has increased activity during fasting and less activity in response to palatable foods, while GLP-1R agonists reversed these activity differences. GLP-1 and dlPFC connectivity coupling also play a role in regulating palatable food intake (**Supplementary Table 3**). However, current evidence may indicate a role of GLP-1 in the induction of weight loss without affecting the long-term weight loss process. Plasma GLP-1 levels may correlate more with carbohydrate intake rather than total food wanting. However, in obese patients, the expected fluctuation of GLP-1 levels in response to food consumption may be lost, and weight control surgeries may restore these levels. When exposed to palatable food compared to non-palatable food, GLP-1 levels were not changed significantly in obese patients. These findings indicate that palatable food intake might be partially promoted due to insufficient GLP-1 response (Rigamonti et al., 2015; **Supplementary Table 3**). GLP-1 may change the functional connectivity of the hypothalamus and alter eating. As seen in animals, GLP-1R agonists reduce food cravings and increased brain activation to the consumption of palatable food in the right OFC of obese subjects and the left insula, bilateral putamen, left amygdala of obese patients with T2DM.

In our systematic review, we have observed that different substances such as palatable food or drugs of abuse have different effects on specific brain regions based on the dose, administration route, timing, satiety, along with other environmental factors. Hunger ratings were not associated with either GLP-1 or dorsal striatum activity. As a result of increased simple sugar intake, reduced GLP-1 mediated satiety signaling might result in sustained salience of food cues via dorsal striatum activity even after food intake and contribute to overeating and weight gain. In lean individuals, abnormal activities were not observed in the NAc as it did in the obese; therefore, hunger ratings might not be associated with GLP-1 levels in lean individuals.

The current literature on GLP-1 and reward has several limitations. Preclinical studies explored the short-term effects of GLP-1 analogs; however, knowledge about their long-term benefits is limited and needs to be investigated. A few clinical studies by Jennifer et al. (2016) and ten Kulve et al. (2016) found that longer-term GLP-1R agonist treatments may not be useful in the weight loss process as the changes seen after short-term treatment such as improvement of activity deficits in the insula and caudate nucleus in response to palatable food did not persist during the long-term treatment (12 weeks). Further studies with longer assessment periods are needed for both preclinical and clinical studies because of these contrasting findings. As another limitation, different GLP-1 analogs could not be distinguished for their effect on reward pathways since most of the studies used either Ex-4, liraglutide, linagliptin, or Ex-9 as an antagonist and did not compare them to each other. Previous literature on GLP-1 and neuroprotection (Erbil et al., 2019) showed that different GLP-1 analogs might diverge on their neuroprotective effect, and it was mostly time and dose-dependent as higher doses showed greater reversal of neurodegenerative processes and dual combinations were more effective in general. This effect might also be observed in the regulation of reward pathways, which needs to be further assessed.

This systematic review brings two possible future perspectives. Firstly, amphetamine administration results in hyperlocomotion, risk-taking behavior, and impaired cognition in mice resembling mania symptoms in bipolar disorder. The effect of GLP-1R agonists on reversing amphetamine-induced locomotion

and that liraglutide may act similar to lithium in terms of reversing cognitive deficits and liraglutide decreased lithium-induced weight gain brought the idea that GLP-1 analogs can serve as a potential target for psychostimulant abuse and treatment for bipolar disorder since it can reverse several aspects of mania and drug-induced side effects in bipolar disorder (Chaves Filho et al., 2020). Along the same line, Ex-4 decreases amphetamine-induced locomotion without changing anxiety or aversive behaviors in rats (Erreger et al., 2012) along with reducing amphetamine-induced CPP and accumbal dopamine release (Egecioglu et al., 2013b). As limitations, both studies mentioned above use relatively high doses of GLP-1R agonist (30 and 2.4 μg/kg, respectively) and thus need further research.

Secondly, GLP-1 could have a significant role in psychological stress responses, in addition to mood disorders. GLP-1 could be a mediating molecule between mood, feeding, and reward-related behaviors. It has been shown by the cited studies above that GLP-1R exists on reward pathways, in addition to the amygdala, dorsal raphe, LS, hippocampus, and also BNST. These pathways also regulate mood and stress responses. A study showed that central GLP-1 administration increases anxiety-like behavior (Möller et al., 2002). GLP-1R KD of BNST neurons, specifically in rats, resulted in decreased anxiety-like behavior and stress-induced hypophagia (Zheng et al., 2019). Plasma corticosterone levels of GLP-1R KD were also increased in response to acute stress, while baseline corticosterone levels were similar between GLP-1R KD and control, indicating that actions of GLP-1 may be partially by limiting acute stress-induced plasma corticosterone production. In humans, one study reported that GLP-1R gene expression in the dlPFC was significantly different between healthy controls and mood disorder patients after controlling for age, sex, ethnicity, and BMI (Mansur et al., 2019). Emotional eating is negatively correlated with exenatide-induced reductions of activation in the amygdala of obese patients and in the insula of T2DM patients, which indicates an attenuated effect of GLP-1 on patients who suffer from emotional eating (van Bloemendaal et al., 2015b). Another study on GLP-1 polymorphisms found that two GLP-1 polymorphisms, A allele in rs10305492 and C allele in rs1042044, show associations with response bias in a probabilistic reward task (PRT), a laboratory-based measurement of anhedonia (Yapici-Eser et al., 2020). Based on these studies, future studies (both preclinical and clinical) should also assess mood as a construct of reward pathway associated with GLP-1.

In conclusion, GLP-1Rs are located in areas important for reward, and they can significantly alter palatable food or drug-induced dopamine levels and glutamatergic neurotransmission, which results in decreased palatable food intake and substance use. Preclinical evidence is accumulating, and translational research is highly required to increase knowledge on the effect of GLP-1 on reward systems.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

HY-E and CYE-Y designed the study, wrote the protocol, and wrote the first draft of the manuscript. CYE-Y, AY, and RD managed the literature searches and analyses. All authors contributed to and have approved the final manuscript.

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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.2020.614884/full#supplementary-material

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The Influence of Moderate Physical Activity on Brain Monoaminergic Responses to Binge-Patterned Alcohol Ingestion in Female Mice

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Monoamine neurotransmitter activity in brain reward, limbic, and motor areas play key roles in the motivation to misuse alcohol and can become modified by exercise in a manner that may affect alcohol craving. This study investigated the influence of daily moderate physical activity on monoamine-related neurochemical concentrations across the mouse brain in response to high volume ethanol ingestion. Adult female C57BL/6J mice were housed with or without 2.5 h of daily access to running wheels for 30 days. On the last 5 days, mice participated in the voluntary binge-like ethanol drinking procedure, "Drinking in the dark" (DID). Mice were sampled immediately following the final episode of DID. Monoamine-related neurochemical concentrations were measured across brain regions comprising reward, limbic, and motor circuits using ultra High-Performance Liquid Chromatography (UHPLC). The results suggest that physical activity status did not influence ethanol ingestion during DID. Moreover, daily running wheel access only mildly influenced alcohol-related norepinephrine concentrations in the hypothalamus and prefrontal cortex, as well as serotonin turnover in the hippocampus. However, access to alcohol during DID eliminated wheel running-related decreases of norepinephrine, serotonin, and 5-HIAA content in the hypothalamus, but also to a lesser extent for norepinephrine in the hippocampus and caudal cortical areas. Finally, alcohol access increased serotonin and dopamine-related neurochemical turnover in the striatum and brainstem areas, regardless of physical activity status. Together, these data provide a relatively thorough assessment of monoamine-related neurochemical levels across the brain in response to voluntary binge-patterned ethanol drinking, but also adds to a growing body of research questioning the utility of moderate physical activity as an intervention to curb alcohol abuse.

Keywords: alcohol abuse, drinking in the dark, exercise, physical activity, monoamine, rodent models, binge drinking, voluntary ethanol consumption

INTRODUCTION

Alcohol abuse can cause debilitating health issues, despite being amongst the top preventable contributors to worldwide death. Alcohol abuse has also been linked to over 200 diseases including drug dependence, hypertension, type 2 diabetes, and dementia. Thus, there is a need to identify the neurophysiological underpinnings of alcohol abuse to develop effective interventions that curtail its consequences. Evidence indicates that regularly engaging in moderate physical activity may have tremendous therapeutic value for substance use disorders, and therefore may be a beneficial intervention to curb alcohol abuse (Goodwin, 2003). Indeed, individuals that regularly exercise are less likely to engage in substance abuse or experience relapse during recovery from drug dependence (Goodwin, 2003; Smith and Lynch, 2012). Moreover, mounting evidence from rodent models of drug abuse also suggests that exercise therapy may aid in recovery from cocaine, morphine, and methamphetamine dependence (Cosgrove et al., 2002; Miladi-Gorji et al., 2012; Engelmann et al., 2014). However, the impact of physical activity status on alcohol abuse, in particular, remains less clear, as reports with human subjects and rodent models have demonstrated varied outcomes (Giesen et al., 2015; Leasure et al., 2015; Manthou et al., 2016; Horrell et al., 2020). Yet still, regular exercise can promote adaptations in mood regulation and reward neuropathways that also overlap with the motivation to ingest excessive amounts of alcohol (Werme et al., 2002b; Greenwood et al., 2011; Herrera et al., 2016; Robison et al., 2018). Therefore, the possibility remains that these exercise-induced adaptations may influence the motivation to misuse alcohol.

Considerable evidence suggests that the activity of monoamine neurotransmitters, including serotonin (5-HT), dopamine (DA), and norepinephrine (NE) in brain reward, motor, and limbic systems, play key roles in the motivation to drink alcohol (Camarini et al., 2010; Fitzgerald, 2013; Belmer et al., 2016). Therefore, interventions that change the activity of monoamine neurotransmitters may hold therapeutic value for alcohol abuse. Acute bouts of physical activity can stimulate transient 5-HT, DA, and NE activity in brain reward and limbic system structures (Freed and Yamamoto, 1985; Bailey et al., 1992; Dunn et al., 1996; Gomez-Merino et al., 2001; Lin and Kuo, 2013). Furthermore, long-term exercise produces adaptations to the availability of receptors that can modulate the release of monoamines, like the serotonin 1A receptor of the raphe nuclei and dopamine 2 receptor in the striatum (Gilliam et al., 1984; MacRae et al., 1987; Greenwood et al., 2003; Clark et al., 2014; Bauer et al., 2020). Such exercise-induced adaptations may change monoamine neurotransmitter responses during alcohol ingestion, thereby altering the risk for its misuse. However, whether or not physical activity status can influence the activity of monoamines during alcohol ingestion remains unknown.

"Drinking in the dark" (DID) is a rodent model of binge-like ethanol drinking that has contributed greatly to our understanding of the physiological underpinnings of alcohol abuse (Sprow and Thiele, 2012). The National Institute on Alcohol Abuse and Alcoholism defines alcohol binge drinking as a pattern of ethanol ingestion that produces blood alcohol concentrations (BAC) greater than 0.08%. C57BL/6J mice consume high amounts of ethanol under the DID paradigm, reliably reaching BACs greater than 0.1% within a 2-to-4-h period of ethanol access. This results in behavioral signs of intoxication including ataxia and anxiolysis (Rhodes et al., 2005, 2007; Barkley-Levenson and Crabbe, 2015). Thus, the DID model is not only an excellent resource to identify correlates of changes to brain monoaminergic activity that are associated with motivation to consume high levels of alcohol, but also to understand how physical activity status may influence monoamine responses to episodes of binge-like drinking. However, brain monoaminergic activity during voluntary ethanol drinking to binge-like levels has not been comprehensively investigated using rodent models. Furthermore, only a few studies have investigated the interaction between physical activity status and motivation to ingest alcohol in animal models, while typically examining alcohol preference using a two-bottle choice, instead of binge-like drinking (Werme et al., 2002a; Pichard et al., 2009; Darlington et al., 2014; Booher et al., 2019). Thus, there is a need for a more comprehensive examination of how monoaminergic activity becomes influenced during voluntary binge-like alcohol drinking, and how physical activity status might alter such responses. Identifying the effects of binge-like ethanol drinking on the levels of monoamines and metabolites, as well as how physical activity may interact with such responses could provide insights into new approaches that reduce the risk of alcohol abuse.

The purpose of the current study was to investigate the influence of physical activity status on mouse brain monoaminergic responses to binge-like ethanol drinking using the DID paradigm. Brain area-specific markers of monoaminergic activity were investigated immediately following access to ethanol after five consecutive days of DID in mice that were or were not granted daily running wheel access. Monoamines were analyzed by measuring concentrations of the neurotransmitters 5-HT, DA, and NE, along with the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), and DA pre-cursor levodopa (L-DOPA) using Ultra-High-Performance Liquid Chromatography (UHPLC). Monoamine concentrations were assessed in the prefrontal cortex, hypothalamus, striatum, hippocampus, brainstem, and caudal cortical region, as these areas comprise limbic, reward, and motor pathways that contribute to the misuse of alcohol and cognitive dysfunction related alcohol abuse (Koob, 2014). The results of this study not only provide a relatively thorough assessment of monoamine-related neurochemical changes across the brain in response to voluntary binge-patterned ethanol drinking but may also have implications for the utility of moderate physical activity as an intervention to curb alcohol abuse.

MATERIALS AND METHODS

Rodent Housing

Six-week-old female C57BL/6J mice (Jackson Laboratory) were individually housed upon arrival at standard laboratory

conditions with *ad libitum* access to food and water, except during DID sessions, in which 20% ethanol replaced water in the cages where mice were assigned to the alcohol drinking condition. Female mice were chosen for this study because previous work has suggested they drink more ethanol and run further on wheels than male mice (Lightfoot et al., 2004; Rhodes et al., 2007), which we hypothesized would result in a greater likelihood of detecting effect sizes sufficient to observe differences in the dependent measures between groups. Mice were given a 1-week acclimation period to adjust to a reversed light-dark cycle before experimental procedures (described below). All procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, USA, 2011) and were approved by the Iowa State University Institutional Animal Care and Use Committee. All possible efforts were made to minimize the number of animals used and their suffering.

Wheel Running Paradigm

Mice were randomly placed in one of four groups: no access to running wheels with water during DID (sed/water; $n = 9$), no access to running wheels with alcohol during DID (sed/alcohol; $n = 9$), daily running wheel access with water during DID (run/water; $n = 9$), or daily running wheel access with alcohol during DID (run/alcohol; $n = 9$). Alcohol was only present during a short period following wheel access on the last 5 days (see "Drinking in the Dark" section). At the start of the dark cycle, mice designated for running wheel access were moved from their home cage to a cage with a 4-inch diameter voluntary running wheel for 2.5 h, to encourage moderate physical activity. Wheel rotations were recorded *via* Starr Life Sciences VitalView software. Sedentary mice were also moved from their home cage to a temporary standard cage during this period to control for the change in environment and handling. Mice were returned to their home cages after 2.5 h. This process was repeated daily for approximately 35 days.

Drinking in the Dark

On day 30, a DID protocol was administered to alcohol-consuming groups for alcohol self-administration over 5 days (Rhodes et al., 2005). After the 2.5-h running period, mice were returned to their home cages and left undisturbed for 30 min before the start of the DID procedure. For DID, water bottles were removed and replaced with 10 ml graduated sipper tubes containing 20% ethanol or water. Mice were allowed to freely consume 20% ethanol for 2 h each day for 4 days and 4 h on the fifth and final day. Four hours of DID was performed on the final day because we hypothesized that longer access to alcohol would yield more drinking and a greater likelihood of observing changes to neurochemical concentrations (Rhodes et al., 2007). The volume of fluid in sipper tubes was recorded every 30 min during DID. Mice were sacrificed *via* rapid decapitation. Brain extraction and dissection occurred immediately following the final DID session. Brain areas containing the prefrontal cortex, remaining caudal cortical area, hypothalamus, cerebellum, striatum, hippocampus, and caudal brainstem areas were rapidly microdissected on a glass plate placed over ice. Microdissection of specific brain areas was completed as follows and considered the stereotaxic coordinates detailed in Paxinos and Franklin's "Mouse Brain in Stereotaxic Coordinates fifth edition." Using a flat edge razor blade the olfactory bulbs were removed and disposed of followed by a coronal cut approximately 1.97 mm rostral to bregma to excise the prefrontal cortex area. Vascular tissue (i.e., The circle of Willis) surrounding the hypothalamus area was removed using forceps before extraction of the hypothalamus between approximately 0.13 mm and −2.69 mm anteroposterior to bregma. At approximately 0.83 mm caudal to bregma a razor blade was used to separate the striatum from the remaining brain tissue. The cortical area surrounding the striatum was carefully removed and placed into a vial for caudal cortical tissue. The cerebellum was severed from its point of attachment to the rest of the brainstem using forceps and collected. A mid-sagittal cut through the remaining brain tissue then allowed for access to the hippocampal area which was then collected (excluding cortical tissue) from each hemisphere. The brainstem area was then separated from the remaining cortical tissue at approximately −2.69 mm, −8.15 mm to bregma. The remaining cortical tissue was then added to the vial for caudal cortical tissue. Microdissected brain areas were placed in pre-weighed cryovials containing 0.2 M perchloric acid, flash-frozen with liquid nitrogen, weighed again to obtain sample weights (see **Supplementary Table 2.1**), and then stored at −80°C in an Ultra-Low freezer until UHPLC processing.

Western Blot Analysis

The amount of protein synthesis enzyme, phosphorylated p70S6K1, was measured in the gastrocnemius muscle of all mice, as a marker of muscle adaptation to running wheel access (Drummond et al., 2009). Immediately after decapitation, the gastrocnemius was extracted and flash-frozen with liquid nitrogen then stored at −80°C until the time of analysis. Western blot analysis followed previously described methods (Valentine et al., 2018). Approximately 15–25 μg of protein were separated by 4–15% gradient Stain-Free Criterion TGX gel electrophoresis (Bio-Rad, Hercules, CA, USA), and transferred onto a polyvinylidene difluoride membrane (MilliporeSigma, Burlington, MA, USA). Gels were activated according to Bio-Rad's Stain-Free protocol, and total protein was quantified to normalize signal intensity for the protein of interest in each lane to the total protein. The membrane was then blocked in Tris-buffered saline (pH 7.5) containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature. Next, strips were incubated overnight in primary antibodies at a 1:2,000 dilution. The primary antibodies were directed against p70S6K1 (#2708, Cell Signaling Technology, Danvers, MA, USA) and phospho-p70S6K1 (#9234, Cell Signaling Technology, Danvers, MA, USA), an enzyme involved in the synthesis of proteins related to training adaptations in the muscle. Membranes were then washed with TBST, incubated in horseradish peroxidase-conjugated secondary antibodies against rabbit (Cell Signaling Technology, Danvers, MA, USA) at a 1:5,000 dilution, and

washed with TBST and Nano-pure water. The membranes were imaged using an enhanced chemiluminescence solution. Bands were captured with the ChemiDoc™ XRS Imaging System (Bio-Rad, Hercules, CA, USA), and densitometry was performed using Image Lab V6.0 (Bio-Rad, Hercules, CA, USA).

High-Performance Liquid Chromatography

Immediately following dissection, brain tissue samples were preserved in 0.2 M HCLO4 and stored at −80°C until UHPLC preparation. Brain regions were homogenized using the Omni Bead Ruptor system. Sample homogenates were then centrifuged for 30 min at 3,000 *g*. An Ultra-High-Performance Liquid Chromatography with electrochemical detection (UHPLC-ECD) system was used to determine neurochemical and metabolite concentrations as described previously (Villageliú et al., 2018). A Dionex Ultimate 3000 autosampler system queued samples before injection. Separation of neurochemical target compounds was completed *via* a 150 mm long Hypersil BDS C18 column (Thermo Scientific, Sunnyvale, CA, USA) using 0.6 ml min^{-1} flow rate of 10% buffered acetonitrile MD-TM mobile phase (Thermo Scientific, Sunnyvale, CA, USA). The column used had a diameter of 3 mm. Particle and pore size was 3 μm and 130 Å, respectively. Electrochemical detection was achieved with 6041RS glassy carbon electrode at 400 mV with a limit of detection set at 5× the signal-to-noise ratio. Neurochemical standards were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Statistical Analysis

Statistical analysis was performed using SAS Enterprise Guide 7.1 and GraphPad Prism 8.2.0. A repeated-measures ANOVA was performed on daily running distance with week, access to running wheels, and access to alcohol during DID as factors. A repeated-measures ANOVA was also performed on daily ethanol intake over each session of DID procedures. Change in body mass during the study (i.e., week 1 to week 5) and phosphorylated p70SK1 levels in the gastrocnemius muscle was analyzed using a two-way ANOVA, with access to wheels and alcohol during DID as factors.

Neurochemical measurements obtained from UHPLC were corrected for gram weight of total tissue (i.e., μg of neurochemical/gram of tissue). The ratio of metabolite to respective neurotransmitter concentration [i.e., 5-HIAA/5-HT, (HVA + DOPAC)/DA) was also calculated as a measure of neurotransmitter turnover (Nissbrandt and Carlsson, 1987; Phillips et al., 1989)]. Two-way ANOVAs were performed to compare neurochemical concentrations or turnover in each brain area with exercise (sedentary vs. running) and DID treatment (water vs. alcohol) as factors. All ANOVAs with statistically significant interactions or main effects for both factors (i.e., sedentary vs. running, and water vs. alcohol) were followed by *post hoc* analyses using pairwise *t*-tests with Fisher's least significant difference corrections for multiple comparisons. Neurochemical concentrations that were greater than two standard deviations from the mean were excluded from analysis, as noted in the degrees of freedom reported for ANOVAs.

For neurochemical data, the results were only reported for statistically significant values to conserve space. Group means (±SEM) and test statistics for all neurochemicals across each brain region can be located in **Supplementary Tables 1.1–1.9**.

RESULTS

Wheel Running, Body Mass Change, and Associated Muscle Adaption

The average daily running distance gradually increased from 0.88 km and plateaued at approximately 1.8 km per day (±SEM = 0.05). Throughout the entire study, the average distance ran for mice with access to water was 1.75 km (±SEM = 0.03), whereas mice with access to alcohol ran 1.85 km per day (±SEM = 0.04; see **Figure 1A**). Alcohol access ($F_{(1,32)} = 5.76$, $p = 0.022$) and running wheel access ($F_{(1,32)} = 8.98$, $p = 0.005$) influenced the amount of mass gained by mice throughout the study (see **Figure 1B**). *Post hoc* analysis revealed that sedentary mice with access to alcohol gained more mass than running mice with access to water ($p = 0.0006$) or ethanol ($p = 0.02$).

Finally, mice with wheel access had an increase of phosphorylated p70S6K1 in the gastrocnemius as compared to mice that did not receive access to alcohol ($F_{(1,30)} = 5.49$, $p = 0.026$; see **Figure 1C**). Indeed, the *post hoc* analysis revealed that wheel running mice with access to water during DID had greater muscle p70S6K1 protein density than sedentary mice with access to water ($p = 0.026$) and wheel running mice with access to ethanol ($p = 0.034$).

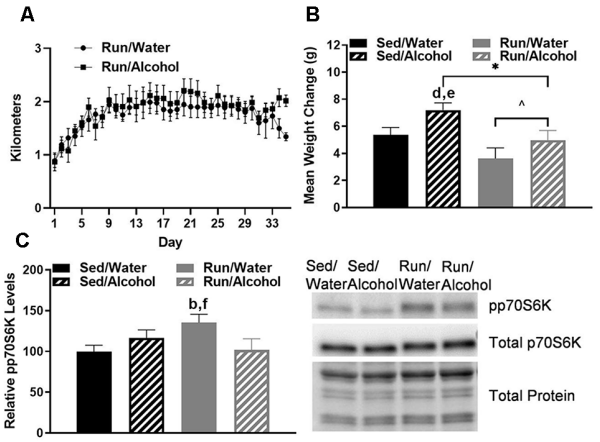

FIGURE 1 | Daily running distance and related phosphorylated p70S6K marker for muscle protein synthesis. **(A)** Average daily running distances (±SEM) for mice throughout the study. **(B)** Average weight change throughout the study, and **(C)** western blot analysis containing the relative optical density for phosphorylated p70S6K corrected for total protein in mouse gastrocnemius muscle (left) and representative blot images (right). $^{*}P < 0.05$ main effect alcohol access, $^{\wedge}P < 0.05$ main effect physical activity status, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol. Data represent +SEM.

Drinking Data

Physical activity status did not influence the amount of fluid ingested relative to bodyweight for either mice that had access to water or alcohol ($F_{(1,32)}$ = 0.00, p = 0.94; see **Figure 2A**). However, mice with access to ethanol consumed greater volumes of fluid compared to those with access to water during DID ($F_{(3,32)}$ = 7.293, $p < 0.0007$). Mice consumed almost double the volume of ethanol or water on day 5 during the 4-h DID session, when compared to the first 4 days that consisted of 2-h DID sessions ($F_{(4,123)}$ = 32.26, $p < 0.0001$; see **Figure 2A**). The amount of ethanol ingested on day 5 of DID was 8.82 g/kg (±SEM = 0.8) for sedentary mice and 9.36 g/kg (±SEM = 0.4) for mice with access to running wheels (see **Figure 2B**). Finally, wheel access did not influence the amounts of fluids ingested independent of fluid type ($F_{(1,32)}$ = 2.87, p = 0.11), or while considering day and fluid type ($F_{(4,123)}$ = 0.52, p = 0.72).

Striatum Area

The striatal area had the most statistically significant changes to neurochemical concentrations, which were primarily a result of alcohol access during DID. Ethanol ingestion robustly influenced markers of DA-related neurochemical activity. Indeed, mice that received access to ethanol had a greater (DOPAC + HVA)/DA ratio ($F_{(1,32)}$ = 9.86, $p < 0.0036$) in the striatum, suggesting an overall increase in DA turnover as a result of alcohol ingestion (see **Figure 3A**). With regards to individual neurochemicals, access to ethanol during the DID period increased the levels of DA metabolites, DOPAC ($F_{(1,32)}$ = 4.22, $p < 0.05$) and HVA ($F_{(1,32)}$ = 13.53, $p < 0.0009$; see **Figures 3B,C**). Running and access to alcohol interacted to change L-DOPA ($F_{(1,28)}$ = 4.30, $p < 0.0475$) and DA ($F_{(1,32)}$ = 5.33, p = 0.0276) concentrations in the striatum. *Post hoc* analysis revealed that running mice with access to ethanol had greater L-DOPA than their sedentary mice counterparts (p = 0.0365), as well as running mice that had access to water (p = 0.0023; see **Figure 3D**). Furthermore, DA content was lower in sedentary mice that received alcohol compared to those that had water during DID (p = 0.0377; see **Figure 3E**).

In addition to changes in DA related neurochemicals, mice that received access to ethanol had an increased 5-HIAA/5-HT ratio ($F_{(1,32)}$ = 7.01, p = 0.0125), suggesting alcohol ingestion also augmented 5-HT turnover in the striatum (see **Figure 3F**). Finally, a statistically significant interaction was observed between alcohol and exercise conditions for NE concentrations ($F_{(1,32)}$ = 10.69, p = 0.0026), whereby *post hoc* analysis revealed running mice with alcohol access had lower NE compared to running mice with water access (p = 0.0014) and sedentary mice with ethanol access (p = 0.04). Moreover, for mice that had access to water, running increased NE concentrations in the striatum compared to the sedentary condition (p = 0.0170; see **Figure 3G**).

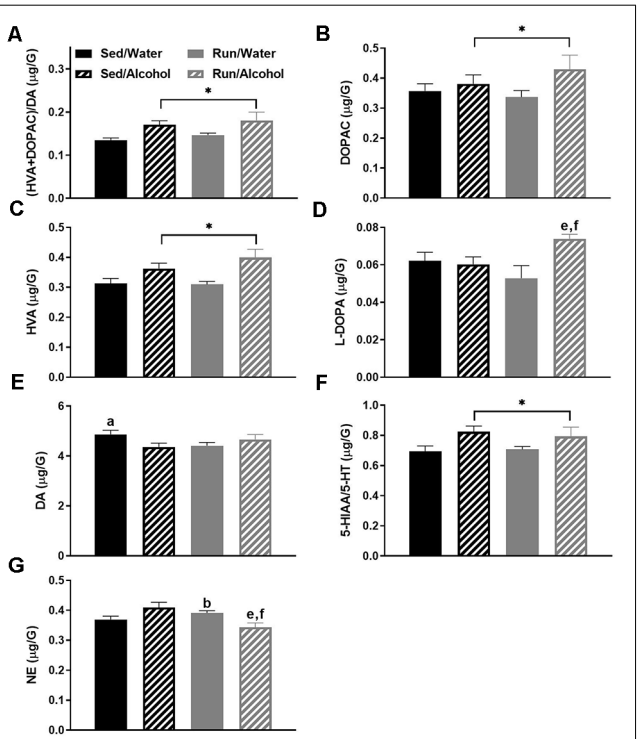

FIGURE 3 | Neurochemical levels in the mouse striatum area immediately following the final DID session. Ratio of (HVA + DOPAC)/DA **(A)**, DOPAC concentrations **(B)**, HVA concentrations **(C)**, L-DOPA concentrations **(D)**, DA concentrations **(E)**, ratio of 5-HIAA/5-HT **(F)**, NE concentrations **(G)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

Hypothalamic Area

The hypothalamus had five statistically significant neurochemical changes. The most striking of which was observed with the interactions between physical activity status and ethanol access for hypothalamic 5-HT ($F_{(1,32)}$ = 7.97, p = 0.0081), 5-HIAA ($F_{(3,32)}$ = 4.05, p = 0.0152), and NE ($F_{(1,32)}$ = 4.18, p = 0.0436) content. Overall, access to ethanol eliminated exercise-induced reductions of 5-HT, 5-HIAA, and NE concentrations in the hypothalamus. Indeed, running mice with access to water had lower 5-HT concentrations than sedentary mice with access to water (p = 0.0071) or alcohol (p = 0.0481), as well as running mice with access to alcohol (p = 0.0033; see **Figure 4A**). Paralleling 5-HT levels, running mice with access to water also had lower 5-HIAA concentrations

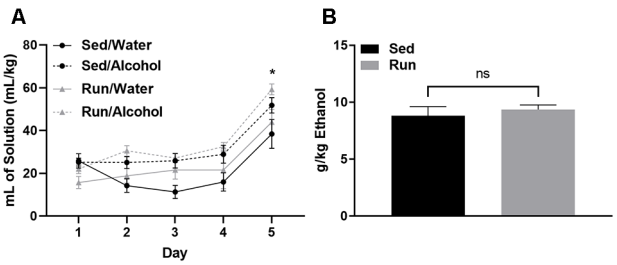

FIGURE 2 | Ethanol consumption during the drinking in the dark protocol. Comparison between the average amount of fluid ingested (±SEM) over the 5-day DID procedure (relative to body mass) for mice that had access to alcohol and water **(A)** and total ethanol intake (relative to body mass) during the final DID session **(B)**. $^{*}P < 0.05$ Main effect of day, $^{ns}P > 0.05$ between Run/Alcohol and Sed/Alcohol. Data represent means ±SEM.

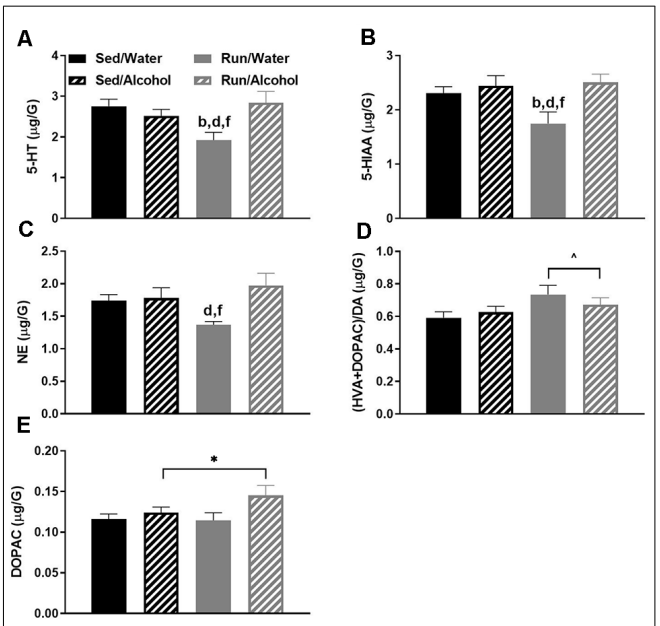

FIGURE 4 | Neurochemical levels in the mouse hypothalamus area immediately following the final DID session. 5-HT concentrations **(A)**, 5-HIAA concentrations **(B)**, NE concentrations **(C)**, ratio of (HVA + DOPAC)/DA **(D)**, and DOPAC concentrations **(E)**. Animals that ran and were exposed to DID had increased DOPAC levels **(E)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{\wedge}P < 0.05$ main effect physical activity status, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

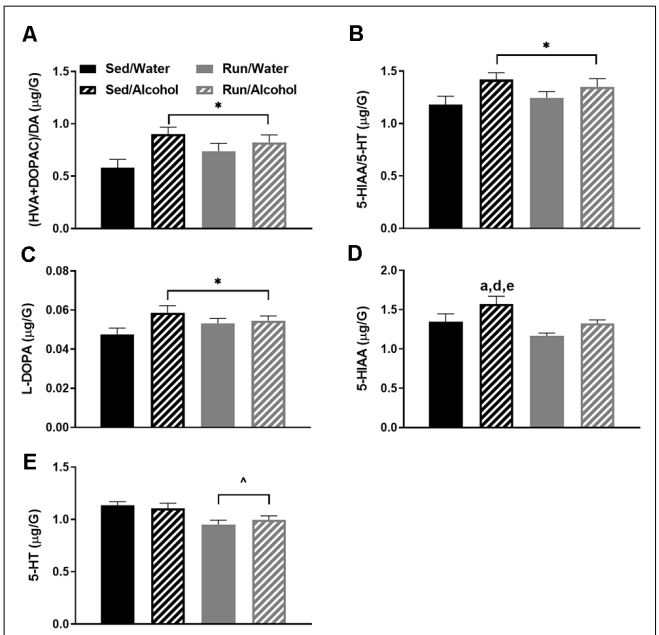

FIGURE 5 | Neurochemical levels in the mouse brainstem area immediately following the final DID session. The ratio of (HVA + DOPAC)/DA **(A)**, the ratio of 5-HIAA/5-HT **(B)**, L-DOPA concentrations **(C)**, 5-HIAA concentrations **(D)**, and 5-HT concentrations **(E)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{\wedge}P < 0.05$ main effect physical activity status, $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol.

than their ethanol drinking counterparts ($p = 0.0038$), as well as sedentary mice that had access to water ($p = 0.0272$) and ethanol ($p = 0.0074$; see **Figure 4B**). Finally, running mice with access to water also had lower NE concentrations than their ethanol drinking counterparts did ($p = 0.0031$), as well as sedentary mice that had access to ethanol ($p = 0.0375$; see **Figure 4C**). A statistically non-significant trend between sedentary and running mice with access to water was observed for NE ($p = 0.0609$).

Additionally, some changes reflecting DA turnover were observed within the hypothalamus. Running increased the ratio of (DOPAC + HVA)/DA in the hypothalamus compared to sedentary mice, independent of ethanol access ($F_{(1,32)} = 4.68$, $p = 0.0386$; see **Figure 4D**). Moreover, ethanol access increased hypothalamic DOPAC concentrations independent of physical activity status ($F_{(1,32)} = 4.75$, $p = 0.0367$; see **Figure 4E**).

Brainstem Area

The brainstem area also had five statistically significant changes in monoamine-related neurochemicals. Most notably, ethanol ingestion increased the ratios of both (DOPAC + HVA)/DA ($F_{(1,32)} = 7.92$, $p = 0.0096$; see **Figure 5A**) and 5-HIAA/5-HT ($F_{(1,32)} = 5.79$, $p = 0.0220$; see **Figure 5B**), which may suggest that alcohol drinking augments turnover of these neurotransmitters systems at their areas of cell body origin (Pickel et al., 1975; Steinbusch et al., 1979). With regards to specific neurochemical changes, L-DOPA concentrations were also mildly increased in mice that had access to ethanol ($F_{(1,32)} = 4.47$, $p = 0.0423$; see **Figure 5C**). Physical activity status ($F_{(1,32)} = 7.82$, $p = 0.0087$) and ethanol access ($F_{(1,32)} = 6.27$, $p = 0.0176$) influenced 5-HIAA levels in brainstem regions, whereby sedentary mice with access to ethanol had higher concentrations of 5-HIAA than sedentary mice with access to water ($p = 0.0280$) and running mice with access to ethanol ($p = 0.0442$) or water ($p = 0.0007$; see **Figure 5D**). Finally, exercise lowered 5-HT concentrations in the brainstem regions, independent of ethanol access during the DID period ($F_{(1,32)} = 13.25$, $p = 0.0010$; see **Figure 5E**).

Caudal Cortical Area

The remaining cortical area (i.e., with the prefrontal cortex removed) had the next highest number of statistically significant monoaminergic-related changes at four. Of particular interest, access to ethanol during the DID period increased the (DOPAC + HVA)/DA ratio ($F_{(1,32)} = 6.31$, $p = 0.0173$; see **Figure 6A**). This finding was likely due to the capacity of ethanol access to increase both DA metabolites DOPAC ($F_{(1,32)} = 6.99$, $p = 0.0126$) and HVA ($F_{(1,32)} = 11.75$, $p = 0.0017$), without resulting in changes to DA concentrations (see **Figures 6B,C**). Moreover, physical activity status and access to alcohol interacted to influence NE concentrations ($F_{(1,32)} = 7.78$, $p = 0.0088$), whereby alcohol access eliminated exercise-induced decreases of NE content (see **Figure 6D**). Indeed, running mice with access to water during DID had lower NE concentrations than sedentary mice with access to water ($p = 0.0451$) and running mice with access to alcohol ($p = 0.0113$).

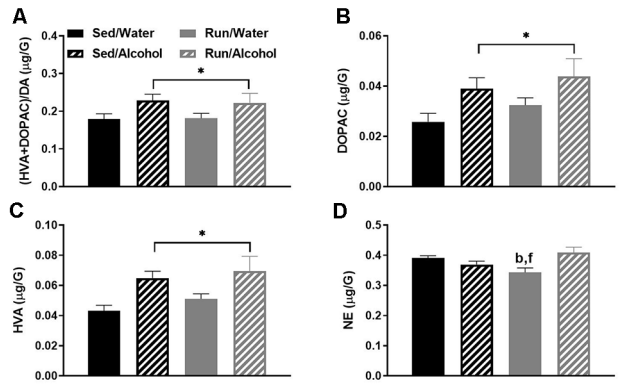

FIGURE 6 | Neurochemical levels in the mouse caudal cortical area immediately following the final DID session. Ratio of (HVA + DOPAC)/DA **(A)**, DOPAC concentrations **(B)**, HVA concentrations **(C)**, and NE concentrations **(D)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

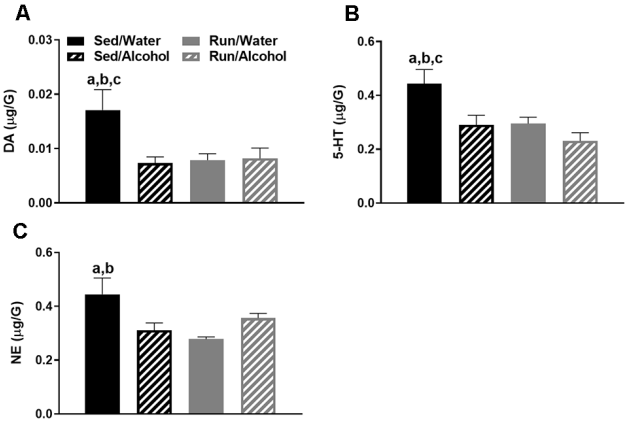

FIGURE 7 | Neurochemical levels in the mouse cerebellum area immediately following the final DID session. DA concentrations **(A)**, 5-HT concentrations **(B)**, and NE concentrations **(C)**. Data represent means +SEM. $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{c}P < 0.05$ Sed/Water from Run/Alcohol.

Cerebellum

The cerebellum also had four statistically significant changes. Overall, physical activity status and alcohol access interacted to influence DA, 5-HT, and NE related neurochemicals in the cerebellum. Foremost, a statistically significant interaction was found between exercise condition and access to alcohol for DA concentrations ($F_{(1,32)} = 4.69$, $p = 0.0379$; see **Figure 7A**). Sedentary mice that had access to water had greater amounts of DA compared to their ethanol available counterparts ($p = 0.0049$), as well as running mice that had access to water ($p = 0.0069$) and ethanol ($p = 0.0076$). For cerebellar 5-HT concentrations, statistically significant main effects were observed for both physical activity status ($F_{(1,32)} = 5.90$, $p = 0.0209$) and alcohol access ($F_{(1,32)} = 6.22$, $p = 0.0180$; see **Figure 7B**). Sedentary mice that had access to water had greater concentrations of 5-HT compared to their ethanol available counterparts ($p = 0.0076$), as well as running mice that had access to water ($p = 0.0086$) and ethanol ($p = 0.0015$). Finally, physical activity status and alcohol access interacted to affect cerebellar NE content ($F_{(1,32)} = 9.49$, $p = 0.0042$; see **Figure 7C**). Sedentary mice that had access to water had greater concentrations of NE compared to their running ($p = 0.0017$) and ethanol drinking counterparts ($p = 0.0097$).

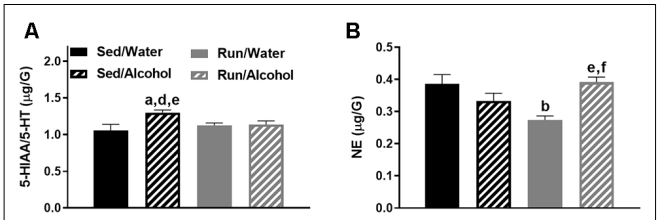

FIGURE 8 | Neurochemical levels in the mouse hippocampus area immediately following the final DID session. The ratio of 5-HIAA/5-HT **(A)**, and NE concentrations **(B)**. Data represent means +SEM. $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

Hippocampal Area

In the hippocampus, the interaction between physical activity status and access to ethanol affected the ratio of 5-HIAA/5-HT ($F_{(1,32)} = 4.51$, $p = 0.0414$) and NE content ($F_{(1,32)} = 16.69$, $p = 0.0003$; see **Figures 8A,B**). Exercise eliminated the increased turnover of hippocampal 5-HT resulting from ethanol access, as sedentary mice with access to ethanol had a greater 5-HIAA/5-HT ratio than running mice with access to ethanol ($p = 0.0436$) or water ($p = 0.0305$), and sedentary mice with access to water ($p = 0.0034$). Finally, access to alcohol eliminated the exercise-induced reductions of hippocampal NE content, as running mice with access to water had lower concentrations of NE than their sedentary ($p = 0.0008$) and alcohol drinking ($p = 0.0003$) counterparts. Running mice with access to ethanol also had marginally greater NE concentrations than their sedentary counterparts did ($p = 0.0467$).

Prefrontal Cortex

Alcohol access and running interacted to augment NE concentrations in the prefrontal cortex ($F_{(1,32)} = 6.06$, $p = 0.0194$), as running mice with access to ethanol had greater NE levels than both sedentary ($p = 0.0244$) and running mice ($p = 0.0005$) that had access to water (see **Figure 9A**). Moreover, access to ethanol increased concentrations of 5-HIAA in the prefrontal cortex ($F_{(1,32)} = 8.21$, $p = 0.0073$; see **Figure 9B**).

DISCUSSION

This study examined the impact of moderate physical activity on monoamine-related neurochemical responses to binge-like ethanol drinking across mouse brain areas that comprise reward, limbic, and motor systems. The results provided several key findings with regards to the impact of physical activity status and alcohol ingestion on monoamine levels throughout the brain.

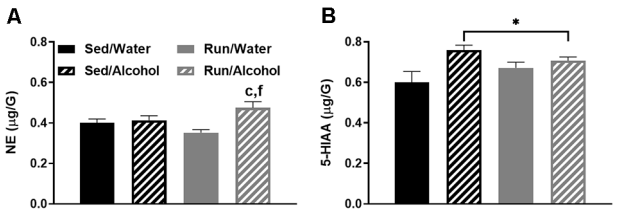

FIGURE 9 | Neurochemical levels in the mouse prefrontal cortex area immediately following the final DID session. NE concentrations **(A)**, and 5-HIAA concentrations **(B)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{c}P < 0.05$ Sed/Water from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

First, physical activity status did not influence drinking behavior during DID and only mildly affected monoamine-related neurochemical concentrations following alcohol ingestion. In particular, NE levels were slightly elevated in the prefrontal cortex and decreased in the striatum of physically active mice following alcohol access (see **Figures 9A**, **3G**). Moreover, wheel access spared alcohol-related increases of 5-HT turnover markers in the hippocampus (see **Figure 8A**). Second, alcohol ingestion eliminated some of the exercise-induced changes to monoamines and metabolite concentrations, including reduced NE content in the hypothalamus, hippocampus, and caudal cortical areas, as well as reduced hypothalamic concentrations of 5-HT and 5-HIAA (see **Figures 4C**, **8B**, **6D**). Finally, the DID paradigm replicated several key monoaminergic concentration changes, yet also yielded some departures from past work that employed commonly used methods of forced ethanol exposure (e.g., i.p. injection, gavage, vapor chamber, etc.). Some of the parallels between the DID model and previous work included augmented correlates of DA-related activity in the striatal and brainstem areas, as well as greater markers of 5-HT turnover in striatal, hippocampal, and brainstem regions (Fadda et al., 1980; Kiianmaa and Tabakoff, 1983; Hellevuo and Kiianmaa, 1988; see **Figures 3**, **5**, **8**). However, alcohol access during DID had a relatively limited influence on neurochemical levels in the prefrontal cortex and in NE concentrations across brain areas, which contrasts some findings of previous work using models of acute ethanol exposure (Pohorecky and Jaffe, 1975; Murphy et al., 1988; Milio and Hadfield, 1992; Lanteri et al., 2008). Overall, these results provide some insight into the potential utility of moderate physical activity to influence neurochemical levels related to the motivation to ingest high volumes of ethanol.

Perhaps the most unexpected outcome of this study was that physical activity status failed to robustly influence monoamine-related neurochemical responses to ethanol ingestion. This outcome was surprising because running can potently modulate monoaminergic activity and receptor plasticity throughout limbic and reward structures, which in turn can alter monoamine responses during distinct events, like exposure to stress or some drugs of abuse (Foley and Fleshner, 2008; Lin and Kuo, 2013; Clark et al., 2015; Arnold et al., 2020; Katsidoni et al., 2020). One possibility is that the relatively mild influence that alcohol ingestion had on monoamine and metabolite concentrations could have limited the ability to detect neurochemical changes that were further influenced by physical activity in this study. Moreover, the possibility remains that despite reaching a daily running distance of nearly 2 km, which was sufficient to elevate markers of muscle adaptation related to training (see **Figure 1C**), restricting the running period to 2.5 h may not have been enough to maximally stimulate brain adaptations that influence monoaminergic activity in response to binge-like ethanol drinking (Drummond et al., 2009). It should be noted that p70S6K1 protein augmentation related to wheel running was reduced in mice with ethanol access (see **Figure 1C**), which is consistent with previous work suggesting impaired muscle growth following periods of high-volume alcohol exposure (Lang et al., 2000; Steiner and Lang, 2014, 2015). However, providing longer periods of exercise, just prior to alcohol access may be problematic, as wheel running is rewarding to rodents and the stress associated with suddenly restricting wheel access may result in greater ethanol ingestion (Greenwood et al., 2011, 2012; Nishijima et al., 2013; Herrera et al., 2016). The methods of the current study were designed to minimize alcohol-seeking due to potential stress from restricted wheel access, by providing the same period running every single day in a different environment from the mouse home cages. Moreover, limiting daily wheel access to a couple of hours may more closely model human patterns of engagement in physical activity than continuous wheel access paradigms, where C57BL/6J mice commonly run over the entirety of 12-h periods and can reach distances of 4–9 km per night (Lightfoot et al., 2004; Clark et al., 2011). Therefore, shorter periods of wheel access may be particularly relevant for modeling the potential influence of physical activity status on human physiological responses to alcohol abuse. Taken together, the results of the current study add to a growing, but conflicting body of literature questioning the utility of exercise as a therapeutic approach to mitigate the severity of alcohol abuse disorders.

Nonetheless, mice with daily access to running wheels displayed some differences in neurochemical concentrations in response to alcohol ingestion compared to their sedentary counterparts that could have implications for motivation to misuse alcohol. Daily running mildly increased NE concentrations in the prefrontal cortex, while lowering NE concentrations in the striatum, in response to ethanol access (see **Figures 3**, **9**). Access to running wheels also prevented the elevated markers of 5-HT turnover in the hippocampus that were related to ethanol drinking (see **Figure 8**). Growing evidence suggests that increases or decreases of brain region-specific noradrenergic signaling are common responses to drugs of abuse, including alcohol (Fitzgerald, 2013; Koob and Volkow, 2016). However, mixed results have been found concerning NE concentrations following acute alcohol exposure in rodent models (Vazey et al., 2018). Moreover, research into the potential alcohol-induced changes to NE in the prefrontal cortex or striatum that have been linked to behavioral or neurophysiological outcomes is limited (Vazey et al., 2018). Some evidence suggests that alcohol's rewarding properties

may depend on heightened NE levels in the prefrontal cortex (Ventura et al., 2006). Therefore, elevated NE levels in the prefrontal cortex could contribute to the urge to drink alcohol that has been reported to follow bouts of exercise (Manthou et al., 2016). However, the current results did not demonstrate an increase in ethanol ingestion in running mice with respect to their sedentary counterparts. Compared to the prefrontal cortex and limbic regions, even less work has been done examining striatal NE levels following acute exposure to ethanol. However, it is worth noting, that a prior history of stress exposure may sensitize striatal NE responses to ethanol exposure (Karkhanis et al., 2014). On the other hand, regularly engaging in physical activity promotes stress-resistance and may (Dishman et al., 1997; Greenwood et al., 2013), desensitize striatal NE activity in response to ethanol. However, the influence of lower striatal NE concentrations on motivation to ingest alcohol remains unresolved. Therefore, more research is required to determine the behavioral or cognitive effects of possible exercise-induced changes to NE levels in the prefrontal cortex and the striatum in response to ethanol ingestion.

Exercise also eliminated the augmented turnover of 5-HT in the hippocampus of mice that had access to ethanol (see **Figure 8A**). This finding is particularly interesting because augmented 5-HT activity in the hippocampus following exposure to large amounts of ethanol has been linked to impaired hippocampal function that may result from an increased inhibition of principal neurons (McBride et al., 1990; Lovinger, 1997; Bare et al., 1998; Belmer et al., 2018). The capacity of moderate physical activity to attenuate alcohol-increased 5-HT turnover in the hippocampus suggests that regularly engaging in physical activity might protect against some hippocampal dysfunction that has been reported with acute episodes of binge drinking. Extensive research has shown robust improvements to hippocampal function following long periods of regular engagement in physical activity (Van Praag et al., 1999; Christie et al., 2008; Clark et al., 2008; Marlatt et al., 2012; Merritt and Rhodes, 2015). Moreover, regularly participating in exercise can facilitate neuroplasticity (e.g., synapse makers and neurogenesis) in models of chronic alcohol-induced hippocampal deterioration (Redila et al., 2006; Helfer et al., 2009; Hamilton et al., 2012; Maynard and Leasure, 2013). Therefore, the benefits of exercise on hippocampal function may also extend to mitigate short-term or spatial memory deficits during acute alcohol challenges. However, to the best of our knowledge, no studies have investigated the influence of physical activity status on hippocampal function during episodes of binge-like alcohol drinking.

Access to alcohol during the DID period eliminated some of the exercise-induced changes to monoaminergic-related neurochemical concentrations. This was particularly pronounced in the hypothalamic area of wheel running mice, where ethanol ingestion eliminated the reduced concentrations of NE, 5-HT, and 5-HIAA (see **Figures 4A–C**), but also to a lesser extent in the hippocampal and caudal cortical areas for NE content (see **Figures 8B**, **6D**). A popular view remains in the scientific community that the anxiolytic or antidepressant properties of exercise may depend on elevated 5-HT, DA, and NE concentrations across brain areas comprising reward and limbic circuits (Meeusen et al., 2001). This assertion appears to be primarily based on data measuring monoamine levels during exhaustive exercise when brain monoaminergic activity is high. However, this is less clear when measured during post-exercise periods or rest, as some evidence suggests that 5-HT and NE concentrations may be lower in trained rodent brain areas including the cortex, hypothalamus, and hippocampus, when compared to non-trained conditions (Barchas and Freedman, 1963; Dey et al., 1992; Hoffmann et al., 1994; Gerin and Privat, 1998; Lambert and Jonsdottir, 1998; Gomez-Merino et al., 2001). The capacity of moderate exercise to potentially lower NE- and 5-HT-related neurochemical levels during post-exercise periods in brain regions involved in mediating mood and stress responses, albeit negated by alcohol ingestion in the current study, might still have significant implications for therapeutic approaches targeted at alcohol dependence. Indeed, increased levels of NE in the brainstem, amygdala, and hypothalamus reported during alcohol withdrawal can induce stress-related negative feelings (e.g., anxiety), which may encourage further alcohol-seeking (Trzaskowska and Kostowski, 1983; Walker et al., 2008; Lee et al., 2011; Koob, 2014). The capacity of post-exercise periods to lower basal NE concentrations in brain regions that mediate responses to stress (see **Figures 4C**, **8B**) might suggest regular exercise could be a useful approach to counteract alcohol craving during withdrawal. Indeed, recent work indicates that mice with access to running wheels display attenuated anxiety-like behavior during periods of alcohol withdrawal (Kolb et al., 2013; Motaghinejad et al., 2015; Lynch et al., 2019). The contribution of potential post-exercise attenuated NE or 5-HT concentrations to the management of alcohol craving during withdrawal could be topics for future investigations.

The DID paradigm has many advantages as a model of binge-like ethanol ingestion. Possibly foremost, the DID procedure capitalizes on rodent's self-motivation to ingest high amounts of ethanol in relatively short periods of time. This is a departure from decades of previous work into the acute impact of alcohol abuse on monoamine levels using rodents, which commonly employed methods of forced high-volume ethanol exposure, involving unconventional stressful routes of alcohol administration (e.g., gavage, i.p. injection, vapor chambers, etc.; Sprow and Thiele, 2012). Factors like stress and alternative routes of administration may promote patterns of neurochemical expression that are distinct from alcohol ingestion alone. Therefore, it is not surprising that the results of this study yielded both similarities and differences from past work into brain monoaminergic responses to high levels of ethanol administration. Similar to previous work, ethanol access during DID increased measures reflecting striatal and brainstem area dopaminergic and serotonergic activity (see **Figures 3**, **5**), which contain the mesolimbic DA system that is suggested to play a key role in the rewarding properties of alcohol (Koob, 2014; You et al., 2019). However, in contrast to past work, ethanol access produced relatively few neurochemical changes in the prefrontal cortex (see **Figure 9**), a brain region that has received some attention for monoaminergic responses following ethanol exposure (Fitzgerald, 2013). Moreover, alcohol

access during DID had a relatively subtle influence on NE concentrations across brain areas. This is interesting because NE has been another focal point of past work investigating the acute and chronic effects of alcohol abuse (Koob and Kreek, 2007; Fitzgerald, 2013; Vazey et al., 2018). The reasons for distinctions between the current and past findings are not entirely clear. However, both the noradrenergic system and prefrontal cortex are key components of the stress response, which is more likely to be evoked during forced ethanol exposure (Koob, 2014; Vazey et al., 2018). Moreover, while mice ingested ethanol at levels comparable to the National Institute on Alcohol Abuse and Alcoholism standard for binge drinking (see **Figure 2**), studies employing techniques like gavage or i.p. injection more rapidly deliver greater volumes of ethanol (Rhodes et al., 2005, 2007; Thiele and Navarro, 2014). Therefore, perhaps exposure to even greater volumes of ethanol at faster rates or longer periods of alcohol exposure are required to evoke NE systems or monoamine responses in the prefrontal cortex similar to past work. Finally, the possibility remains that the estrus cycle of female mice in our study could have created variability in neurochemical concentrations, thereby masking the true magnitude of the effect sizes for the dependent measures. Such an outcome could contribute to deviations from the findings of past work. Despite possible limitations, these data underscore the utility of the DID model in adding a more comprehensive understanding of neurophysiological responses to acute alcohol abuse.

In conclusion, the results of the current study suggest that regularly engaging in moderate physical activity does not influence voluntary binge-patterned ethanol drinking in female mice, and may only mildly influence monoamine-related neurochemical responses to alcohol across brain areas that comprise motor, limbic, and reward circuits. Indeed, alcohol-related changes to monoaminergic levels in these brain areas, observed in the current study and others, have been suggested to contribute to the rewarding properties of alcohol, as well as cognitive dysfunction following episodes of alcohol abuse (Koob and Volkow, 2010). In summary, this work adds to a growing body of research that questions the utility of exercise as a therapeutic approach to curb incidences of alcohol misuse (Giesen et al., 2015; Leasure et al., 2015; Manthou et al., 2016), despite mounting evidence suggesting that regularly engaging in exercise may be more effective at reducing the risk for abusing other illicit substances (Goodwin, 2003; Smith and Lynch, 2012; Lynch et al., 2013).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Iowa State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TB contributed to conceptualizing the study, performing experiments, and writing the manuscript. CR contributed to performing experiments and writing the manuscript. AS and EB contributed to performing experiments and data collection. RV contributed to performing experiments, training co-authors, and data collection. PC contributed to conceptualizing the study, training coauthors, writing the manuscript, and funding the entire study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Behavioral Neuroscience

CORRECTION
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Corrigendum: The Influence of Moderate Physical Activity on Brain Monoaminergic Responses to Binge-Patterned Alcohol Ingestion in Female Mice

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Keywords: alcohol abuse, drinking in the dark, exercise, physical activity, monoamine, rodent models, binge drinking, voluntary ethanol consumption

A Corrigendum on

The Influence of Moderate Physical Activity on Brain Monoaminergic Responses to Binge-Patterned Alcohol Ingestion in Female Mice

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In the original article, there was a mistake in the legend for Figure 5 as published. The legend for Figure 5 does not contain the appropriate key to denote statistically significant differences detected by pairwise comparisons to match the text that corresponds to Figure 5. In: 'dP < 0.05 Sed/Alcohol from Run/Water', the superscript should be "d" rather than "b". The correct legend appears below.

"**Figure 5**. Neurochemical levels in the mouse brainstem area immediately following the final DID session. The ratio of (HVA + DOPAC)/DA **(A)**, the ratio of 5-HIAA/5-HT **(B)**, L-DOPA concentrations **(C)**, 5-HIAA concentrations **(D)**, and 5-HT concentrations **(E)**. Data represent means +SEM. *P < 0.05 main effect alcohol access, $^{\wedge}P < 0.05$ main effect physical activity status, $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol."

In the original article, there was also a mistake in the legend for Figure 9 as published. The legend for Figure 9 does not contain the appropriate key to denote a statistically significant effect of alcohol access and differences detected by pairwise comparisons to match the text that corresponds to Figure 9. The correct legend appears below.

"**Figure 9.** Neurochemical levels in the mouse prefrontal cortex area immediately following the final DID session. NE concentrations **(A)**, and 5-HIAA concentrations **(B)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{c}P < 0.05$ Sed/Water from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol."

The following figures also required corrections and have been replaced.

In the original article, there were mistakes in Figures 2, 5, 7, 8 and 9 as published.

In Figure 2B, the g/kg Ethanol that was reported represents only the last 2.5 h of the drinking in the dark paradigm, and not the total 4 h as stated in the legend. The amounts should have been reported as the total 4 h of ethanol access. The corrected Figure 2 appears below.

In Figure 5C, pairwise comparisons were labeled when the label to denote a significant main effect should have been used. This is correctly reported in the results section as a main effect but is incorrectly labeled in the corresponding Figure 5C. In Figure 5D, the "b" denoting a significant pairwise comparison should be changed to a "d" to accurately reflect the compared groups. This is correctly reported in the results section, just not the corresponding figure. The corrected Figure 5 appears below.

In Figure 7, the Figure key was not present. The pairwise comparison label, "c" in Figure 7C should be removed, as this group comparison was not statistically different. This was correctly described in the results section, but incorrectly labeled in the corresponding Figure 7C. The corrected Figure 7 appears below.

In Figure 8B, the significant pairwise comparison labels, "d" and "f" should be removed from above the "Run/Water" group as these were incorrectly included here but were correctly reported in the corresponding text of the results section. The corrected Figure 8 appears below.

In Figure 9A, the pairwise comparison label, "d" should be removed as this group comparison was not statistically different. This was indicated in the text correctly, but incorrectly labeled in the corresponding Figure 9A. In Figure 9B, pairwise comparisons were labeled when notation to denote a significant main effect should have been used. This is correctly reported in the results section as a main effect but is incorrectly labeled in the Figure 9B. The corrected Figure 9 appears below.

Lastly, in the original article, there was an error in the "Drinking Data" paragraph of the Results section. The amount of g/kg ethanol consumption that was reported only reflected the last 2.5 h of the drinking in the dark paradigm rather than the entire 4-h period.

A correction has been made to *Results*, *Drinking Data*. The corrected paragraph is shown below.

"Physical activity status did not influence the amount of fluid ingested relative to bodyweight for either mice that had access to water or alcohol ($F_{(1,32)} = 0.00$, $p = 0.94$; see **Figure 2A**). However, mice with access to ethanol consumed greater volumes of fluid compared to those with access to water during DID ($F_{(3,32)} = 7.293$, $p < 0.0007$). Mice consumed almost double the volume of ethanol or water on day 5 during the 4-h DID session, when compared to the first 4 days that consisted of 2-h DID sessions ($F_{(4,123)} = 32.26$, $p < 0.0001$; see **Figure 2A**). The amount of ethanol ingested on day 5 of DID was 8.82 g/kg ($\pm$SEM = 0.8) for sedentary mice and 9.36 g/kg ($\pm$SEM = 0.4) for mice with access to running wheels (see **Figure 2B**). Finally, wheel access did not influence the amounts of fluids ingested independent of fluid type ($F_{(1,32)} = 2.87$, $p = 0.11$), or while considering day and fluid type ($F_{(4,123)} = 0.52$, $p = 0.72$)."

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

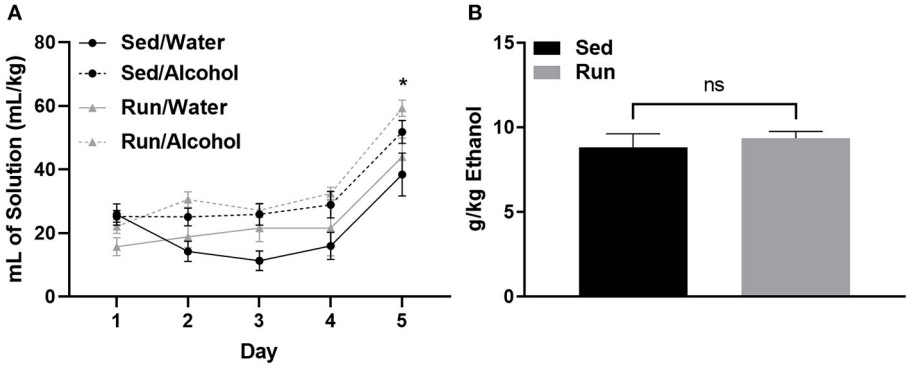

FIGURE 2 | Ethanol consumption during the drinking in the dark protocol. Comparison between the average amount of fluid ingested (±SEM) over the 5-day DID procedure (relative to body mass) for mice that had access to alcohol and water **(A)** and total ethanol intake (relative to body mass) during the final DID session **(B)**. *$P < 0.05$ Main effect of day, $^{ns}P > 0.05$ between Run/Alcohol and Sed/Alcohol. Data represent means ±SEM.

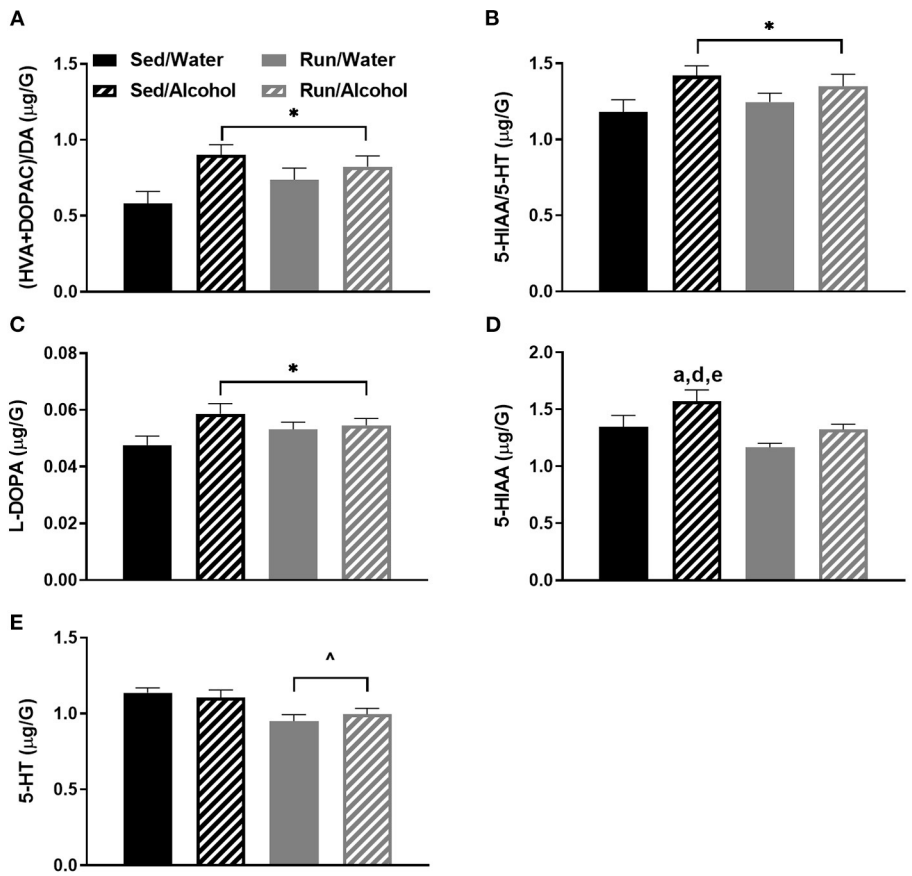

FIGURE 5 | Neurochemical levels in the mouse brainstem area immediately following the final DID session. The ratio of (HVA + DOPAC)/DA **(A)**, the ratio of 5-HIAA/5-HT **(B)**, L-DOPA concentrations **(C)**, 5-HIAA concentrations **(D)**, and 5-HT concentrations **(E)**. Data represent means +SEM. *$P < 0.05$ main effect alcohol access, ^$P < 0.05$ main effect physical activity status, $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol.

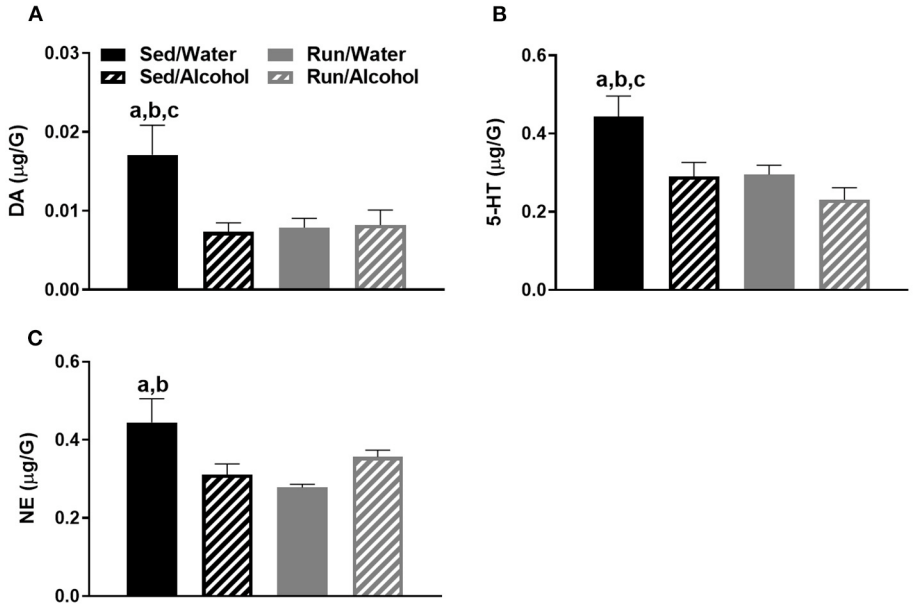

FIGURE 7 | Neurochemical levels in the mouse cerebellum area immediately following the final DID session. DA concentrations **(A)**, 5-HT concentrations **(B)**, and NE concentrations **(C)**. Data represent means +SEM. $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{c}P < 0.05$ Sed/Water from Run/Alcohol.

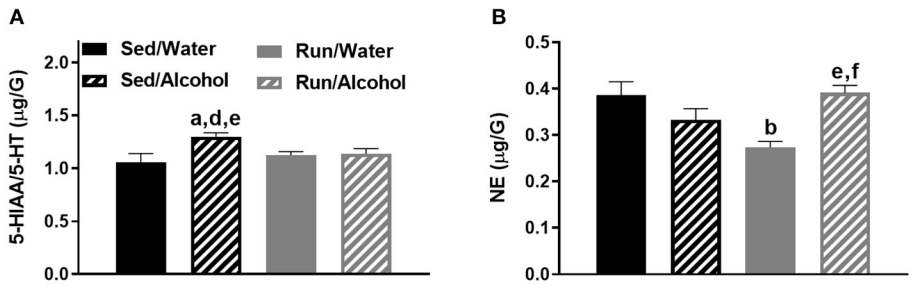

FIGURE 8 | Neurochemical levels in the mouse hippocampus area immediately following the final DID session. The ratio of 5-HIAA/5-HT **(A)**, and NE concentrations **(B)**. Data represent means +SEM. $^{a}P < 0.05$ Sed/Water from Sed/Alcohol, $^{b}P < 0.05$ Sed/Water from Run/Water, $^{d}P < 0.05$ Sed/Alcohol from Run/Water, $^{e}P < 0.05$ Sed/Alcohol from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

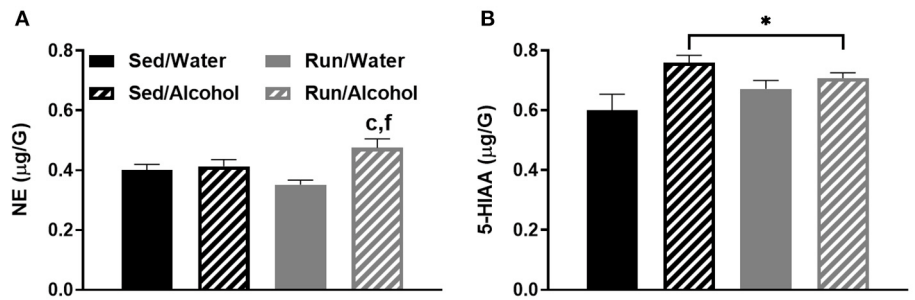

FIGURE 9 | Neurochemical levels in the mouse prefrontal cortex area immediately following the final DID session. NE concentrations **(A)**, and 5-HIAA concentrations **(B)**. Data represent means +SEM. $^{*}P < 0.05$ main effect alcohol access, $^{c}P < 0.05$ Sed/Water from Run/Alcohol, $^{f}P < 0.05$ Run/Water from Run/Alcohol.

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ORIGINAL RESEARCH
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Stress Alters the Effect of Alcohol on Catecholamine Dynamics in the Basolateral Amygdala

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The current rodent study applied *in vivo* fast-scan cyclic voltammetry (FSCV), paired with a pharmacological approach, to measure the release of the catecholamines (CA) dopamine (DA) and norepinephrine (NE) in the basolateral amygdala (BLA) following locus coeruleus (LC) stimulation. The primary goal was to determine if exposure to either social (social defeat) or non-social (forced swim) stress altered LC-evoked catecholamine release dynamics in the BLA. We used idazoxan (α2 adrenergic receptor antagonist) and raclopride (D_2 dopamine receptor antagonist) to confirm the presence of NE and DA, respectively, in the measured CA signal. In non-stressed rats, injection of idazoxan, but not raclopride, resulted in a significant increase in the detected CA signal, indicating the presence of NE but not DA. Following exposure to either stress paradigm, the measured CA release was significantly greater after injection of either drug, suggesting the presence of both NE and DA in the LC-induced CA signal after social or non-social stress. Furthermore, acute administration of alcohol significantly decreased the CA signal in stressed rats, while it did not have an effect in naïve animals. Together, these data reveal that, while LC stimulation primarily elicits NE release in the BLA of control animals, both social and non-social stress unmask a novel dopaminergic component of LC catecholamine signaling. Future studies will be needed to identify the specific neural mechanism(s) responsible for these plastic changes in LC-BLA catecholamine signaling and to assess the possible contribution of these changes to the maladaptive behavioral phenotypes that develop following exposure to these stressors.

Keywords: norepinephrine, dopamine, FSCV, fast-scan cyclic voltammetry, locus coeruleus (LC), social defeat stress, forced swim test (FST)

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INTRODUCTION

Mood and anxiety disorders are highly comorbid with alcohol use disorder (AUD; Grant et al., 2004). For example, those diagnosed with post-traumatic stress disorder are three times more likely to develop an AUD (Kessler et al., 1997). Even in individuals without a formal diagnosis, alcohol is a common tool for those with self-reported social anxiety to cope with stressful situations. These people are more likely to consume alcohol to feel more comfortable

socially (Thomas et al., 2003). Despite the strong relationship between stress and alcohol, much remains unknown about how one can alter the neurobiological consequences of the other.

The locus coeruleus (LC) is a key brainstem nucleus involved in stress and arousal (Szabadi, 2013; Naegeli et al., 2018; Daviu et al., 2019). This pontine structure is the primary source of noradrenergic projections in the central nervous system which target many regions throughout the brain, such as the amygdala, thalamus, hypothalamus, and prefrontal cortex, associating the LC with myriad behavioral, motor, and sensory processes (Samuels and Szabadi, 2008; Deal et al., 2019). In particular, the projection from the LC to the basolateral amygdala (BLA) has been proposed as an important pathway in anxiety-related processes and behaviors. Recent work has shown that optogenetic activation of this pathway produced anxiety-like behavior in rats as demonstrated by a reduction in time spent in the center of an open field test and a reduction in time spent in the open portions of an elevated zero maze (McCall et al., 2017). Moreover, it was recently revealed that the LC-norepinephrine (NE) circuitry can bidirectionally control alcohol-drinking behaviors through different patterns of optoactivation (Deal A. L. et al., 2020).

On other hand, stress has been shown to be a key influencer of catecholamine (CA) activity and dynamics throughout the brain (Tanaka et al., 1991; Ferry et al., 1999; Inglis and Moghaddam, 1999; Anstrom et al., 2009; Karkhanis et al., 2015; Rajbhandari et al., 2015; Holly and Miczek, 2016; Deal et al., 2018; Giustino et al., 2020). For example, studies using *ex vivo* fast-scan cyclic voltammetry (FSCV) have reported that adolescent social isolation stress (Yorgason et al., 2013, 2016) as well as social defeat stress (Deal et al., 2018) resulted in increased dopamine (DA) release and its consequent reuptake in striatal subregions. Furthermore, social isolation stress during adolescence was found to elicit lasting changes to DA and DA transporters in the BLA, specifically a decrease in DA levels as measured by microdialysis and an increase in DA transporters in adulthood (Karkhanis et al., 2015). These findings are further reinforced by *in vivo* voltammetric experiments demonstrating an increased uptake rate and decreased release of NE in the bed nucleus of the stria terminals in rats who experienced social isolation (Fox et al., 2015). Notably, no studies have examined post-stress catecholamine dynamics within the LC-BLA circuitry or whether stress-related alterations in this pathway alter the effects of alcohol on DA and NE transmission.

To explore these questions, we used *in vivo* FSCV, a methodology that permits the real-time detection of extracellular DA and NE concentrations at terminals following electrical stimulation of cell body regions (Budygin et al., 2000, 2007, 2017; Mateo et al., 2004; Oleson et al., 2009; Park et al., 2011, 2012; Fox et al., 2015, 2016). This approach allowed us to examine the effects of two different stressors, repeated social defeat stress (RSDS) and forced swim test (FST), on LC-evoked CA release in the BLA and the sensitivity of the CA signal to acute alcohol. These stress paradigms have been shown to significantly elevate serum corticosterone levels in rats, a key marker of stress circuitry activity (Báez and Volosin, 1994; Miczek et al., 1999; Covington and Miczek, 2005; Barnum et al., 2007; Hueston et al., 2011; Moravcova et al., 2021). After first confirming the CA signal, we pharmacologically identified the components of the CA signal in stressed and non-stressed subjects as NE or DA by injecting either idazoxan (α_2 adrenergic receptor antagonist) or raclopride [dopamine (DA) D_2 receptor antagonist], respectively, and determined whether the evoked CA signal was altered by acute alcohol.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (300–350 g) were used as intruder (stressed) subjects and retired breeder male Long Evans rats (>450 g) served as resident (aggressor) rats (Wood et al., 2010, 2013; Arora et al., 2019). The utilization of different strains resulted in a more robust stress environment due to the aggressive nature of the Long Evans rat (Henry et al., 1993; Snyder et al., 2018). A total of 28 Sprague–Dawley and 6 Long Evans rats were used during this study. Animals were kept on a 12-h light/dark cycle with food and water available *ad libitum*. Animal handling and all procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee (protocol A19-190).

Repeated Social Defeat Stress

Intruder rats were exposed to five 45-min social defeat sessions prior to use for voltammetric recordings (or remained in their home cage to serve as a control). For five consecutive days, intruders were acclimated for 1 h to the testing room and then placed in the cage of a larger, aggressive resident male (see **Figure 1** for experimental design). The rats were allowed to physically interact for 15 min. If blood was observed following a physical bout, the injury was assessed by an observing experimenter for severity. Minor injury resulted in immediate transferal to a protective mesh cage within the resident cage for the remainder of the session. Major injuries, those requiring suturing or other invasive interventions, would have resulted in removal of the intruder rat from the study (no major injuries occurred and thus no rats were excluded from the experiment). Typical sessions included several aggressive physical interactions that resulted in the intruder rat exhibiting subordinate behavior through assuming a supine position. At the conclusion of the physical interaction time, the remainder of the session (30 min) took place while the intruder was protected within a wire mesh cage inside of the resident cage. Visual, auditory, and olfactory signal exposure was still possible during this time.

Forced Swim Test

Rats were forced to swim as described previously (Porsolt et al., 1978). Briefly, animals were allowed to acclimate to the testing room for 1 h and then placed in a transparent acrylic cylinder

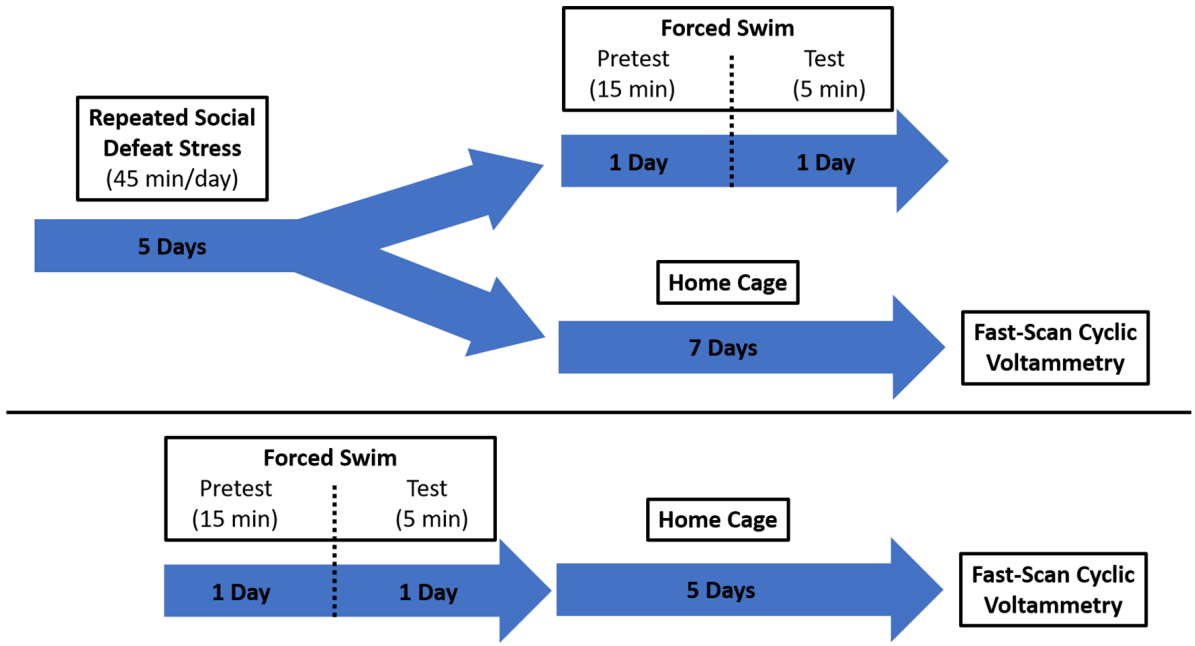

FIGURE 1 | Schematic showing the experimental design. Two groups of rats were exposed to different stress paradigms, social defeat and forced swim. They were then evaluated for protracted effects of stress on catecholamine dynamics using fast-scan cyclic voltammetry (FSCV).

(30 × 50 cm) filled with 40 cm of water (25 ± 1°C) for 15 min (Linthorst et al., 2008; Bogdanova et al., 2013). One day later, the rats were placed in the water-filled cylinder again for 5 min. The rats were allowed to dry off in their home cage, which was placed on a heating pad, for at least 1 h before the cage was returned to the colony room. The cylinder was cleaned with soap and water after each rat.

A separate group of RSDS rats underwent the FST procedure immediately following the last day of social defeat exposure to behaviorally verify a stress-like phenotype. These rats were not included in subsequent voltammetry experiments. Video recordings were made for each rat on both days of FST exposure. Day 2 (test day) recordings were analyzed by an experimenter blinded to the condition and scored for bouts of immobility. The experimenter evaluated behavior every 5 s for the duration of the test (5 min) as either swimming or immobile (Deal A. W. et al., 2020).

Fast-Scan Cyclic Voltammetry

Rats were taken 1 week after the final defeat session or 5 days after the second forced swim session, anesthetized *via* urethane injection (1.35 g/kg, i.p.), and secured in a stereotaxic frame. Holes were drilled to allow for the placement of a carbon fiber recording electrode in the basolateral amygdala (from bregma: AP −2.9; ML + 4.6; DV −8.0), a bipolar stimulating electrode in the ipsilateral locus coeruleus (AP −9.8; ML + 1.3; DV −7.2), and a Ag/AgCl reference electrode in the contralateral hemisphere. The reference electrode was connected to a voltammetric amplifier (UNC Electronics Design Facility, Chapel Hill, NC, USA). The carbon fiber microelectrode (exposed fiber length: 75–100 μm; diameter: 6 μm) was connected to the voltammetric amplifier and secured to the stereotaxic frame arm. Stimulations were made every 10 min by an electrical pulse. Extracellular CA was recorded at the carbon fiber electrode every 100 ms for 15 s by applying a triangular waveform (−0.4 V to +1.3 V and back to −0.4 V vs Ag/AgCl, 400 V/s). The catecholamine signal was identified by observing background-subtracted cyclic voltammograms characterized by oxidation and reduction peaks occurring at +0.6 and −0.2 V, respectively. A stable baseline signal was established (three consecutive recordings within 10% variability) and an average baseline value was calculated. Immediately after establishing a baseline, an injection (i.p.) of saline, raclopride (dopamine D_2 receptor antagonist; 2 mg/kg), idazoxan (α_2 adrenergic receptor antagonist; 5 mg/kg), or alcohol (2 g/kg ethanol) was administered. These idazoxan and raclopride doses effectively increase an electrically-evoked catecholamine efflux clarifying whether DA (raclopride) or NE (idazoxan) is detected in the rat brain with FSCV (Park et al., 2015; Fox et al., 2016; Mikhailova et al., 2019; Deal et al., 2018; Deal A. L. et al., 2020). Some rats received more than one drug during the FSCV experiment. In such cases, the CA signal was allowed to completely return to pre-injection levels before a new baseline was established. Additionally, the order of injections was balanced to prevent any confounding effects of drug interactions. Voltammetric recordings were taken for 60 min after injection. Data were digitized (National Instruments, Austin, TX, USA) and stored on a computer. Each carbon fiber electrode was calibrated after use in an experiment in a flow injection analysis system. Calibrations were performed

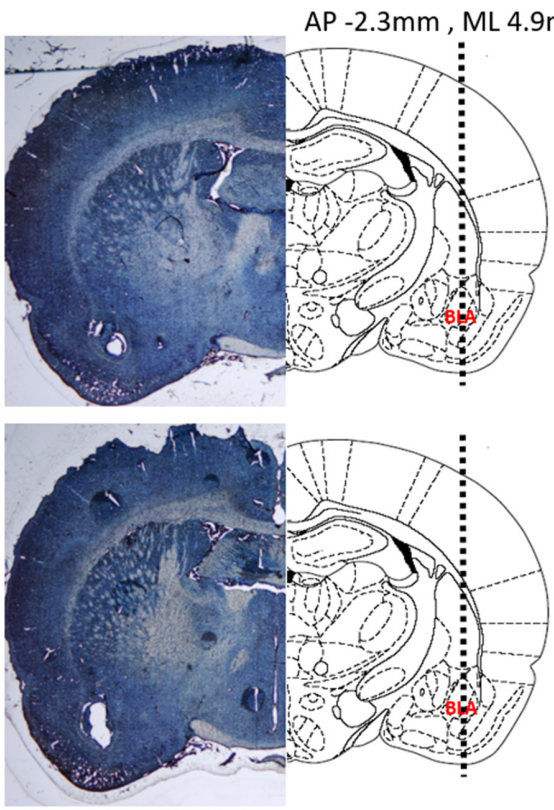

FIGURE 2 | Representative coronal sections demonstrating the location of the recording electrode in two different rat brains. (Left) Thionine stained brain slices showing a lesion at the recording site in the basolateral amygdala (BLA). (Right) Atlas diagrams with the BLA labeled and a dashed line showing the insertion trajectory of the recording electrode. Coordinates provided are relative to Bregma. Diagram adapted from Paxinos and Watson (2004).

in triplicate using a known concentration (10 μM) of DA (Sigma–Aldrich, St. Louis, MO, USA) and calibrated again using a known concentration (10 μM) of NE (Sigma–Aldrich, St. Louis, MO, USA). The voltammetric current was measured at the peak potential.

Histology

Verification of electrode placements was performed as previously described (Park et al., 2010; Budygin et al., 2012). Briefly, a constant current (20 μA, 10 s) was applied to the carbon fiber electrode to create a lesion at the site of recording. Brains were removed and submerged in 10% formaldehyde for a minimum of 3 days. After adequate fixation, the brains were coronally sliced at a thickness of 40–50 μm using a cryostat. Once the slices were mounted on slides, they were stained with 0.2% thionine and cover slipped before imaging was performed using a light microscope. Representative slices from two different rats demonstrating lesions at the site of the recording electrode are shown with accompanying atlas diagrams (**Figure 2**).

Data Analysis

Data were analyzed using GraphPad Prism (GraphPad Software version 7.04, San Diego, CA, USA). Unpaired *t*-test, repeated measures two-way ANOVAs, and Tukey's multiple comparisons tests were used to analyze data where appropriate. Data are presented as mean ± SEM and the criterion for significance was set at $p < 0.05$.

RESULTS

Electrical Stimulation of LC Elicits NE Release in the BLA

We have previously shown that optogenetic activation of the LC results in a robust noradrenergic signal in the BLA, as detected by FSCV (Deal A. L. et al., 2020). Here, we evoked release by electrical LC stimulation that resulted in a consistent, robust, frequency-dependent signal in the BLA which matched the characteristics of CA release (**Figure 3**). Next, in order to identify the constitutive components of this signal, a pharmacological approach was used. More specifically, doses of idazoxan ($n = 5$) or raclopride ($n = 5$) were administered to determine the presence of norepinephrine or dopamine, respectively, compared to a saline control ($n = 7$; **Figure 4**). A repeated measure two-way ANOVA found the main effect of drug ($F_{(2, 14)} = 20.28$; $p < 0.0001$), time ($F_{(8, 112)} = 9.354$; $p < 0.0001$), and a significant interaction ($F_{(16, 112)} = 9.101$; $p < 0.0001$). A follow-up *post hoc* Tukey's multiple comparison test found that idazoxan significantly increased the measured CA signal compared to saline ($p < 0.0001$) and raclopride ($p < 0.001$) and there was no significant difference after raclopride injection ($p = 0.718$). These results demonstrate that NE is the predominant CA evoked in the BLA following LC electrical stimulation.

Electrical LC Stimulation in Socially Stressed Subjects Elicits NE and DA Release in BLA

To determine the effect of stress on evoked CA transients in the BLA, intruder rats were used for *in vivo* FSCV for 1 week following the last 5 days of repeated social defeat stress. In a separate group of rats, we verified that the social defeat procedure resulted in stress by exposing RSDS rats to a forced swim test. A one-tailed unpaired *t*-test found a significantly higher average number of bouts of immobility exhibited by rats exposed to RSDS (11.1 ± 1.65; $n = 5$) compared to non-stressed controls (7.21 ± 1.32; $n = 7$; $t_{(10)} = 1.854$; $p < 0.05$) suggesting a stress-like phenotype.

Similar to the naïve group, idazoxan ($n = 5$) and raclopride ($n = 5$) were administered to determine the catecholamines (CA) present in the detected signal in rats exposed to RSDS and compared to a saline control ($n = 7$; **Figure 5**). A repeated measure two-way ANOVA found the main effect of drug ($F_{(2, 14)} = 22.77$; $p < 0.0001$), time ($F_{(8, 112)} = 20.02$; $p < 0.0001$), and a significant interaction ($F_{(16, 112)} = 9.855$; $p < 0.0001$). A *post hoc* Tukey's multiple comparisons test showed that injection of either idazoxan or raclopride significantly increased the evoked CA signal compared to saline ($p < 0.0001$ and $p = 0.0164$, respectively) and idazoxan increased the signal significantly more than raclopride ($p = 0.0145$). These data show that electrical

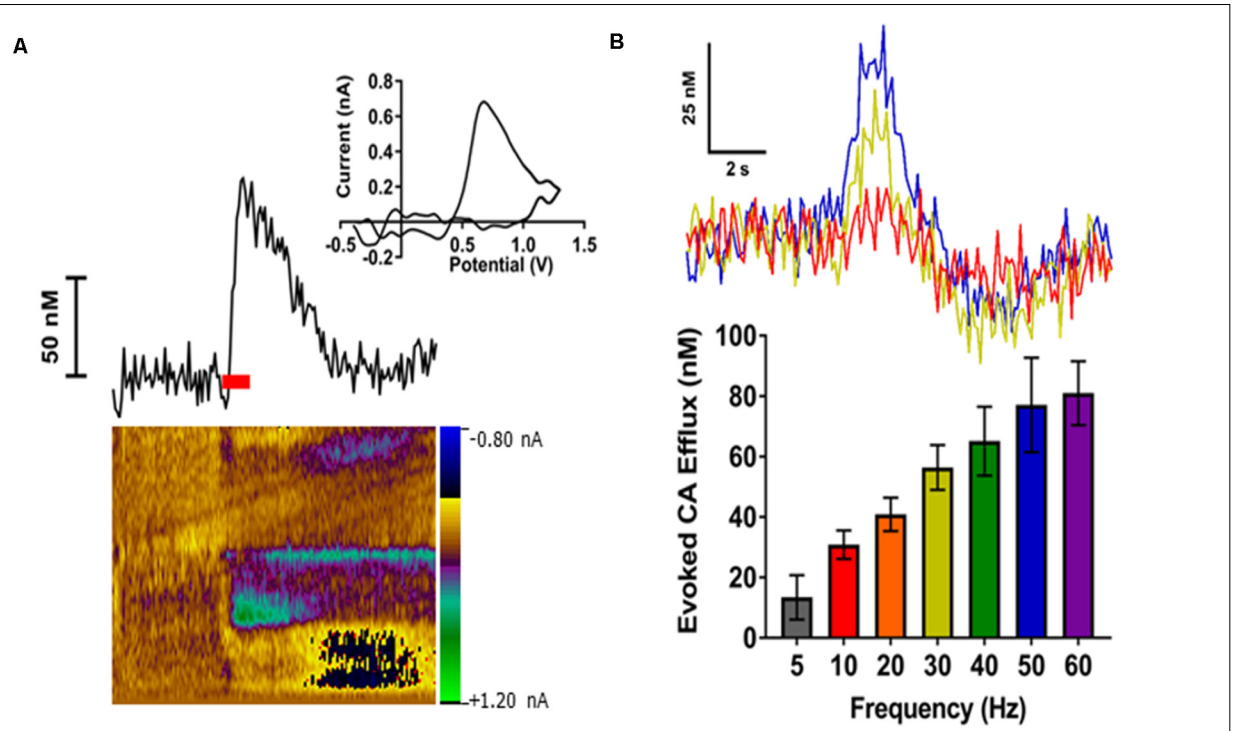

FIGURE 3 | Electrical stimulation of the locus coeruleus (LC) reliably evoked catecholamines (CA) release in the BLA in a frequency-dependent manner. **(A)** A representative trace showing changes in CA concentration over time (top left). The respective cyclic voltammogram (top right) and color plot (bottom) are also shown. The red bar indicates duration of electrical stimulus. **(B)** Representative concentration-time traces of CA release (top) in the BLA of an individual rat demonstrate an increased response as stimulation frequency increases (Red = 10 Hz; Yellow = 30 Hz; Blue = 50 Hz). Averaged CA responses (bottom) show higher frequency stimulations resulted in increased efflux concentrations compared to lower frequency stimulations. Data are presented as mean ± SEM.

stimulation of the LC evokes both NE and DA release in the BLA of subjects exposed to repeated social defeat stress.

Exposure to Forced Swim Stress Results in NE and DA Release in the BLA Following LC Stimulation

In order to determine if this effect of social stress on CA release was unique or generalizable to other stressors, a separate cohort of naïve rats (not exposed to RSDS) was exposed to FST and then used for *in vivo* FSCV (**Figure 6**). A repeated measures two-way ANOVA calculated the main effect of drug ($F_{(2, 13)} = 10.3$; $p < 0.01$), time ($F_{(8, 104)} = 21.26$; $p < 0.0001$), and a significant interaction ($F_{(16, 104)} = 7.489$; $p < 0.0001$). A *post hoc* Tukey's multiple comparisons test found that both idazoxan ($n = 5$) and raclopride ($n = 5$) increased the measured CA release compared to saline controls ($n = 6$; $p = 0.0458$ and $p < 0.01$, respectively). Taken together these experiments suggest that stress, either social and non-social, results in the release of DA, in addition to NE, in the BLA following LC electrical stimulation.

Interestingly, when compared across stress paradigms, raclopride administration resulted in a significantly higher efflux of dopamine following FST compared to either RSDS or non-stressed, naïve controls ($n = 5$ for all groups; two-way RM ANOVA: drug, $F_{(3, 18)} = 12.26$, $p < 0.0001$; time, $F_{(8, 144)} = 14.36$, $p < 0.0001$; interaction, $F_{(24, 144)} = 7.814$, $p < 0.0001$; Tukey's multiple comparisons test: $p < 0.05$). In a similar comparison, injection of idazoxan did not result in a significant difference between the stress and control groups ($n = 5$ for all groups; two-way RM ANOVA: drug, $F_{(3, 18)} = 13.32$, $p < 0.0001$; time, $F_{(8, 144)} = 41.71$, $p < 0.0001$; interaction, $F_{(24, 144)} = 6.848$, $p < 0.0001$; Tukey's multiple comparisons test: $p > 0.05$) This suggests that there are possible stressor-specific effects on dopamine release which should be considered when using these different paradigms.

Stress Exposure Does Not Alter LC-Evoked NE in the BLA

Additional analysis of the signal prior to injection of saline or drug was conducted to determine if stress exposure altered NE release. A one-way ANOVA found that there was no difference in NE release between the non-stressed ($n = 5$; 251.2 ± 62.6), RSDS ($n = 8$; 214.7 ± 30.2), and FST subjects ($n = 6$; 184.9 ± 29.6; $F_{(2, 16)} = 0.617$; $p = 0.552$). However, though not significant, some tendency towards reduced electrically-evoked NE release following forced swim test exposure was evident. A comparison of DA release was not possible because raclopride did not significantly alter the CA signal in non-stressed subjects thus suggesting DA was not present (**Figure 4**).

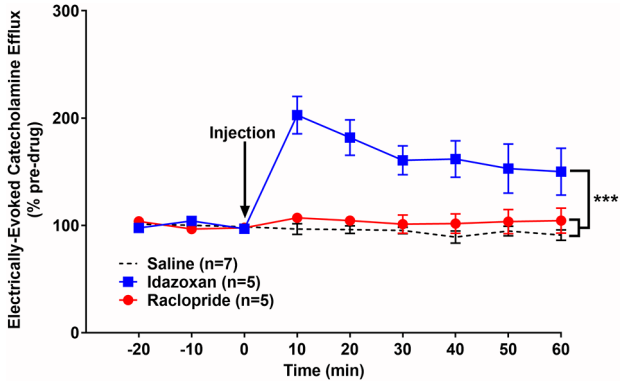

FIGURE 4 | Idazoxan significantly increased norepinephrine (NE) in the basolateral amygdala (BLA) following locus coeruleus (LC) electrical stimulation in non-stressed rats. Anesthetized rats were used to quantify LC-evoked CA release in the BLA, as measured by fast-scan cyclic voltammetry. After a stable baseline was established, saline, idazoxan, or raclopride was administered (black arrow). Idazoxan resulted in a significant increase in the measured signal compared to the saline control. There was no change in the CA signal following injection of raclopride compared to the saline control. Efflux values are presented as mean (± SEM) percent of a pre-injection baseline average. Saline: $n = 7$; Idazoxan: $n = 5$; Raclopride: $n = 5$. ***$p < 0.001$.

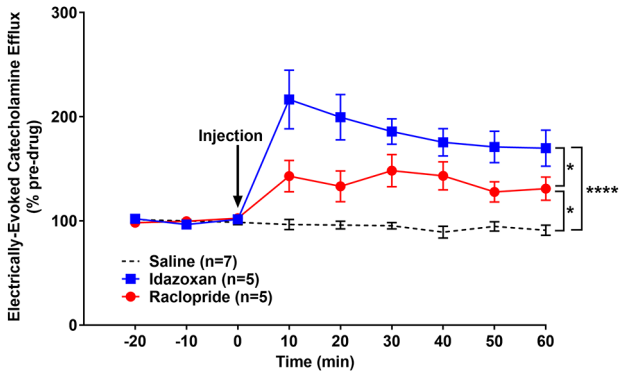

FIGURE 5 | Idazoxan and raclopride significantly increased norepinephrine and dopamine (DA), respectively, in the basolateral amygdala (BLA) following locus coeruleus (LC) electrical stimulation in rats exposed to repeated social defeat stress (RSDS). Rats exposed to 5 days of social defeat stress were anesthetized and used to quantify LC-evoked CA release in the BLA, as measured by fast-scan cyclic voltammetry. After a stable baseline was established, saline, idazoxan, or raclopride was administered (black arrow). Idazoxan and raclopride resulted in a significant increase in the measured signal compared to the saline control. Efflux values are presented as mean (± SEM) percent of a pre-injection baseline average. Saline: $n = 7$; Idazoxan: $n = 5$; Raclopride: $n = 5$. *$p < 0.05$; ****$p < 0.0001$.

Alcohol Decreases the Evoked CA Signal in the BLA by LC Stimulation in Stressed Subjects

In order to explore whether stress is capable of altering the effect of alcohol on CA dynamics, ethanol (2 g/kg, i.p.) was acutely administered to anesthetized subjects previously exposed to RSDS ($n = 5$) or FST ($n = 4$; **Figure 7**). A repeated measures,

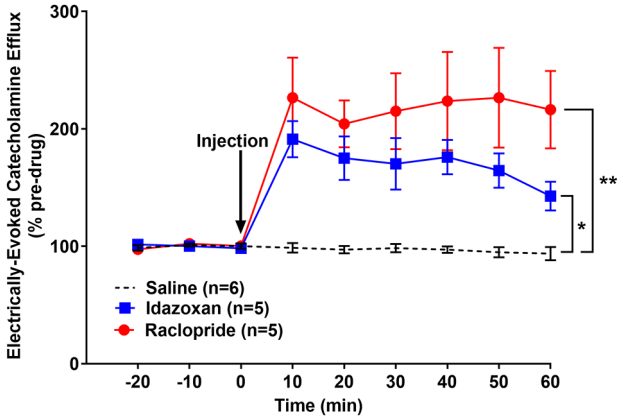

FIGURE 6 | Idazoxan and raclopride significantly increased norepinephrine and dopamine, respectively, in the basolateral amygdala (BLA) following locus coeruleus (LC) electrical stimulation in rats exposed to a forced swim test (FST). Rats exposed to a forced swim test were anesthetized and used to quantify LC-evoked CA release in the BLA, as measured by fast-scan cyclic voltammetry. After a stable baseline was established, saline, idazoxan, or raclopride was administered (black arrow). Idazoxan and raclopride resulted in a significant increase in the measured signal compared to the saline control. Efflux values are presented as mean (± SEM) percent of a pre-injection baseline average. Saline: $n = 6$; Idazoxan: $n = 5$; Raclopride: $n = 5$. *$p < 0.05$; **$p < 0.01$.

two-way ANOVA was conducted and found a main effect of condition ($F_{(3, 16)} = 8.408$; $p < 0.001$), time ($F_{(8, 128)} = 11.79$; $p < 0.0001$), and a significant interaction ($F_{(24, 128)} = 4.026$; $p < 0.0001$). A *post hoc* Tukey's multiple comparisons test found that alcohol attenuated CA release in both RSDS and FST groups compared to saline ($p < 0.01$ and $p = 0.0309$, respectively) and non-stressed controls ($p < 0.01$ and $p = 0.0262$, respectively). In addition, there was no significant change following alcohol injection in control animals compared to saline ($p > 0.05$). These data show that alcohol decreases LC-evoked BLA CA release in subjects exposed to stress but does not have an effect in non-stressed controls.

DISCUSSION

These experiments demonstrate robust stress-dependent alterations in LC-evoked CA release in the BLA following LC stimulation. In control subjects, LC electrical stimulation elicits a strong, predominantly noradrenergic signal in the BLA whose efflux is not altered by acute alcohol. However, in animals that have been exposed to stress, through either social defeat or forced swim, electrical stimulation of the LC results in a CA efflux that is mediated by both noradrenergic as well as dopaminergic components and this response is attenuated following administration of alcohol. Taken together, these findings suggest a role for stress in altering catecholamine dynamics in the BLA and a change in the sensitivity of evoked CA to alcohol.

Although NE release evoked by LC stimulation was previously detected with *in vivo* FSCV in the prefrontal cortex and bed nucleus of the stria terminals (Park et al., 2011, 2012, 2013;

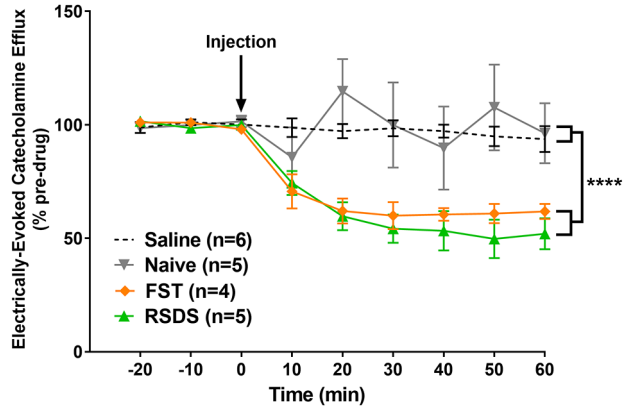

FIGURE 7 | Alcohol significantly reduced the measured catecholamine (CA) efflux in the basolateral amygdala (BLA) following locus coeruleus (LC) electrical stimulation of stressed subjects. Rats were exposed to either repeated social defeat stress or the forced swim test prior to being used to quantify LC-evoked CA release in the BLA as measured by fast-scan cyclic voltammetry. After a stable baseline was established, alcohol was injected (black arrow). In stressed subjects, those exposed to either repeated social defeat stress (RSDS) or forced swim test (FST), alcohol resulted in a significant decrease in the measured CA signal compared to non-stressed, naïve controls. Efflux values are presented as mean (± SEM) percent of a pre-injection baseline average. Saline: $n = 6$; Naïve: $n = 5$; FST: $n = 4$; RSDS: $n = 5$. ****$p < 0.0001$.

Fox et al., 2016; Deal et al., 2019), our results for the first time demonstrate the possibility to measure real-time NE efflux in the BLA. Based on pharmacological characterizations, we ruled out the involvement of DA efflux in the signal measured in naïve rats as previously reported for the prefrontal cortex and bed nucleus of the stria terminals (Park et al., 2011; Fox et al., 2016; Deal et al., 2019). As expected, the electrically-evoked NE release was frequency-dependent, while the average maximum concentrations were ∼2–3 fold lower than concentrations in the prefrontal cortex (Deal et al., 2019). An intriguing finding was the raclopride-induced increase in the detected CA signal in subjects following exposure to both stress paradigms. These data clearly indicate the appearance of a DA component that was not detected in control animals. The origins of the evoked DA signal are unclear and therefore should be further explored. However, some speculations can be offered.

A major dopaminergic source in the brain is the ventral tegmental area (VTA), which has connections with the LC and BLA (Shelkar et al., 2017; Breton et al., 2019). Indeed, social defeat stress has been shown to increase phasic DA release in the nucleus accumbens (NA), a region with strong dopaminergic input from the VTA (Anstrom et al., 2009). Further, chronic stress exposure can lead to morphological changes in VTA DA neurons and enhanced excitability of this neuronal population (Douma and de Kloet, 2020). Perhaps a stress-promoted increase in the excitability of VTA dopaminergic neurons could result in the triggering of DA release in the BLA in response to LC stimulation. Therefore, one hypothesis to address the current findings is that the stress-triggered DA portion of the evoked CA signal reflects a persistent increase in the excitability of VTA projections.

Stress has also been shown to alter the electrophysiological characteristics of LC neurons (Jedema and Grace, 2003; Borodovitsyna et al., 2018). Previous studies have reported that exposure of adolescent rats to restraint and predator odor stressors resulted in increased excitability and spontaneous discharge of LC neurons, which were evident 1 week after stress exposure (Borodovitsyna et al., 2018). Thus, it is possible that in the present study, forced swim or repeated social defeat stress induced a similar increase in LC neuron excitability, altering the activation dynamics of LC inputs to VTA DA neurons, resulting in amygdalar DA release. Therefore, another possible origin for the DA component in the CA signal of stressed subjects is from changes to the NE neurons of the LC that innervate the VTA. In fact, a third possibility can combine both mentioned above scenarios.

Finally, multiple studies have found that various stressors result in an increased expression of tyrosine hydroxylase (TH) mRNA in LC neurons (Mamalaki et al., 1992; Rusnák et al., 2001). While these studies did not confirm the lasting effects of stress on TH mRNA, such an increase in this rate-limiting enzyme of DA synthesis could result in elevated levels of DA in LC NE neurons. Supporting this possibility, another study found that chronic social defeat stress resulted in an upregulation of dopamine β-hydroxylase (DBH) mRNA and protein in the LC as well as increased DBH protein levels in terminal regions of the LC, such as the amygdala, hippocampus, and frontal cortex (Fan et al., 2013). The increase in the production of this ezyme that converts DA to NE can be induced by accumulation of DA inside of NE neurons. Consequently, LC stimulation of stressed rats could result in the efflux of both DA and NE from noradrenergic projections to the BLA due to elevated levels of DA production within the LC NE neurons. Though not induced by stress, there is evidence to support the release of DA from noradrenaline neurons in some brain areas, including prefrontal cortex and hippocampus (Devoto et al., 2003, 2019; Kempadoo et al., 2016).

The finding that consequences of social and non-social stress on CA dynamics are not different is somewhat surprising. However, it is important to highlight that our experiments focused on evoked NE and DA releases which are not necessarily correlated with basal activity of neurotransmitters. Therefore, this question requires further exploration with complimentary techniques which can evalutate changes in other modes of CA neurotransmission.

Another stress-induced change in the CA signal was observed following the administration of alcohol. In non-stressed animals, alcohol did not significantly alter the measured CA efflux. However, following stress exposure, alcohol attenuated the evoked CA signal in the BLA. Unfortunately, due to the presence of both NE and DA in the CA signal, as evidenced by idazoxan and raclopride increasing the measured efflux, it is not currently possible to disentangle the effects of alcohol on NE or DA in the stressed subjects. However, it is notable that when only NE was evoked in

the BLA following LC stimulation, alcohol did not affect the measured signal. These findings may suggest that CA efflux decreased after stress because alcohol only influenced the DA portion. In fact, divergent effects of alcohol on NE and DA transmission under normal conditions have been observed in some brain areas. For example, it was found using FSCV that LC-induced NE in the mPFC is not affected by acute alcohol, while VTA-induced DA in the same brain region is decreased by alcohol (Shnitko et al., 2014; Deal et al., 2019). Moreover, a microdialysis study revealed that alcohol (2 g/kg) increased extracellular DA but not NE in the BLA of group housed rats (Karkhanis et al., 2015). As discussed previously (Jones et al., 2006), an increase in DA levels, as measured by microdialysis, would be consistent with a decrease in the amount of electrically-evoked DA detected with FSCV *in vivo*. This may be simply due to autoreceptor feedback induced by increased cell firing rates and accumulating DA concentrations in terminals. Further, the effect of acute alcohol on extracellular DA concentrations can be sensitized in the BLA following stress through adolescent social isolation (Karkhanis et al., 2015). Therefore, there are multiple studies which provide indirect evidence to support the involvment of DA in the effect of alcohol on evoked CA release in the BLA.

However, we cannot exclude the role of NE in this effect. Indeed, while extracellular NE concentrations were unchanged by alcohol under normal conditions, social isolation stress resulted in a significant increase in this measure (Karkhanis et al., 2015). Strikingly, alcohol-induced increases in both NE and DA levels measured by microdialysis exhibited a similar time course to the decrease in electrically-evoked CA release mesured by voltammetry. Taken together, the current and previous findings support the hypothesis that alcohol may be differently targeting DA and NE release in the BLA following stress exposure.

In conclusion, we have shown that stress, whether social or non-social, can lead to lasting alterations in CA activity that, in turn, affect how alcohol influences CA release in the BLA. Stress changes the LC-induced CA landscape of the BLA, introducing a locus of effect for alcohol that is not present in non-stressed subjects.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EB and JW designed the electrochemical and behavioral experiments. AD performed the electrochemical and behavioral tests and analyzed the data. JP conducted the histology. All authors contributed to the article and approved the submitted version.

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Influence of Age and Genetic Background on Ethanol Intake and Behavioral Response Following Ethanol Consumption and During Abstinence in a Model of Alcohol Abuse

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Genetic background and age at first exposure have been identified as critical variables that contribute to individual vulnerability to drug addiction. Evidence shows that genetic factors may account for 40–70% of the variance in liability to addiction. Alcohol consumption by young people, especially in the form of binge-drinking, is becoming an alarming phenomenon predictive of future problems with drinking. Thus, the literature indicates the need to better understand the influence of age and genetic background on the development of alcohol dependence. To this aim, the inbred rat strains Lewis (LEW, addiction prone) and Fischer 344 (F344, addiction resistant) were used as a model of genetic vulnerability to addiction and compared with the outbred strain Sprague-Dawley (SD) in a two-bottle choice paradigm as a model of alcohol abuse. During a 9-week period, adolescent and adult male rats of the three strains were intermittently exposed to ethanol (20%) and water during three 24-h sessions/week. Adult and adolescent SD and LEW rats escalated their alcohol intake over time reaching at stable levels, while F344 rats did not escalate their intake, regardless of age at drinking onset. Among adolescents, only F344 rats consumed a higher total amount of ethanol than adults, although only SD and LEW rats escalated their intake. Adult LEW rats, albeit having a lower ethanol consumption as compared to SD rats but greater than F344, showed a more compulsive intake, consuming higher amounts of ethanol during the first hour of exposure, reaching a higher degree of ethanol preference when start drinking as adolescents. Behavioral analysis during the first hour of ethanol consumption revealed significant strain differences, among which noticeable the lack of sedative effect in the LEW strain, at variance with F344 and SD strains, and highest indices of withdrawal (most notable jumping) in LEW rats during the first hour of abstinence days. The present results underscore the importance of individual genetic background and early onset of alcohol use in the progression toward abuse and development of alcohol addiction.

Keywords: adolescence, alcohol use disorders, genetic vulnerability, Fischer 344 rats, Lewis rats

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INTRODUCTION

The transition from use to abuse of licit and illicit substances can be due to several interacting factors. Among these, genetic background and age at the time of first exposure to the substance have been shown to contribute significantly. Evidence from twin studies indicates that genetic background accounts for 50–60% of the variance in liability to alcohol dependence in humans (Heath and Martin, 1994; True et al., 1999; Heath et al., 2001; Agrawal and Lynskey, 2008; Kendler et al., 2008; Grant et al., 2009; Agrawal et al., 2012). Moreover, early onset of alcohol use, especially in the form of binge-drinking, is concerning given that it has been correlated with alcohol abuse and dependence and other disorders later in life (Grant and Dawson, 1997; DeWit et al., 2000; Dawson et al., 2008; Sartor et al., 2009; Spear, 2011; Liang and Chikritzhs, 2013; Yuen et al., 2020).

Although environmental and genetic factors contribute to the etiology of Alcohol Use Disorder (AUD), a more thorough understanding as to why some individuals become addicted while others do not presumably requires the detailed study of differential actions of ethanol on brain structure/function. The old theory on ethanol pharmacology as an unspecific pharmacological agent has been replaced by detailed pharmacological studies that show several specific molecular targets for ethanol (Abrahao et al., 2017). Among the different effects of alcohol, at multiple molecular targets, the effects on dopamine (DA), endocannabinoid, and opioid systems appear of critical importance for the rewarding and reinforcing properties of alcohol (Spanagel, 2009; Abrahao et al., 2017). Clearly, understanding the etiology of AUD has to keep into account likely different adaptive changes occurring in the above systems following repeated exposure to ethanol. Due to both technical and ethical reasons, this is difficult to study in humans, and therefore, we decided, more than a decade ago, to investigate differences in DA transmission functionality in an animal model of genetic vulnerability to addiction, the inbred rat strains Fischer 344 (F344) and inbred Lewis (LEW). Studies by several groups suggest that the genetic vulnerability of LEW strain to addiction, as compared with the F344 strain, is the result of differences in several neurotransmitter systems in basal conditions but, more importantly, following exposure to drugs of abuse and stress (see Cadoni (2016) for a review). We have previously shown that the greater sensitivity of LEW strain to drugs of abuse, compared with F344 one, might be the result of its greater mesolimbic DA transmission responsiveness to these drugs. Indeed LEW rats, as compared with F344 strain, display higher DA release in the nucleus accumbens shell and core in response to morphine, nicotine, cocaine, and Δ^9-tetrahydrocannabinol (Cadoni and Di Chiara, 2007; Cadoni et al., 2009, 2015). More notably, LEW strain shows different adaptive changes following repeated exposure to drugs of abuse, retaining (if exposed at adulthood) or even increasing (if exposed at adolescence) their DA response in the nucleus accumbens shell to drug challenge (Cadoni et al., 2015; Cadoni et al., 2020; Lecca et al., 2020). These differences might contribute to the proneness of LEW strain rats to develop higher drug intakes as seen in self-administration paradigms (Picetti et al., 2010; Picetti et al., 2012; Lecca et al., 2020). In addition to being more sensitive to several drugs of abuse, LEW rats appear to be high alcohol preferring, reaching higher rates of intake when compared with F344 rats (Li and Lumeng, 1984; Suzuki et al., 1988; Wilson et al., 1997), and showing neurochemical characteristics (firing modality of dopaminergic neurons, D2 receptors density, etc.) similar to other rat strain lines selected in the world to model alcohol abuse (Minabe et al., 1995; Flores et al., 1998).

Thus, in order to further investigate the influence of genetic background and age of first exposure on the development of alcohol dependence, we compared the intakes of LEW and F344 rats, as an animal model of genetic vulnerability to drug addiction, given intermittent access to high ethanol concentration (20% V/V) in a two-bottle choice test. This protocol has been gaining popularity for animal modeling of alcohol abuse, because, given the repeated cycles of abstinence from alcohol, it leads to escalation of voluntary ethanol intake and preference, thus, mimicking the human condition (Wise, 1973; Simms et al., 2008; Carnicella et al., 2014). To further compare the above genotypes with a group representative of a variable genetic background, we used the outbred rat strain Sprague-Dawley (SD). Analysis of alcohol intake (as daily, weekly, and cumulative intake in 9 weeks) has been expanded to a careful analysis of behavior following ethanol intake and during withdrawal days. Indeed, behavioral analysis following ethanol intake, such as behavioral activation or sedation, and, more importantly, following abstinence days, might provide useful information about the rewarding and motivational value of alcohol helpful for further investigations. Thus, the first aim of the study was to evaluate the influence of the genetic background on ethanol intake and thus, likely, on the development of alcohol abuse and second to clarify the influence of age at first exposure depending on the genetic background. Indeed, even if human studies suggest that age of onset of alcohol use is associated with the risk to develop AUD later in life, recent systematic reviews suggest that this association is less consistent and might be driven by confounding factors, such as the history of alcohol problems in the family, preceding mental health problems, socioeconomic status, or genetic background (Maimaris and McCambridge, 2014; Marshall, 2014; Kuntsche et al., 2016). Even animal studies on this issue are not always consistent (Labots et al., 2018; Spear, 2018; Towner and Varlinskaya, 2020; Mugantseva et al., 2021) and therefore, further investigation is needed to help in clarifying the role of causality between early alcohol onset and increased risk of later AUD.

MATERIALS AND METHODS

Animals

Male outbred SD, LEW, and F344 rats (Charles River, Calco, Italy) of 5 weeks (30–35 postnatal day, PND) or 9 weeks (58–63 PND) of age at arrival were group housed and left to acclimatize to the new housing conditions for 1 week, under standard conditions of temperature (23°C) and humidity (60%) and a 12 h light-dark cycle (light on 08:00 a.m.) with food and water *ad libitum*. Thereafter, they were single housed in polycarbonate cages

(480 mm × 265 mm × 210 mm, mod. 2154F, Tecniplast S.p.A., Buguggiate, Varese, Italy) with matching type wire lids. Thus, at the beginning of ethanol exposure, rats were mid-adolescents (6 weeks of age, 35–42 PND) or adults (10 weeks of age, 65–70 PND). This age for adolescent rats has been intentionally selected because our previous study indicates this age as the most sensitive to the DA releasing effects by drugs of abuse (Corongiu et al., 2020). A total of 101 rats were used, 50 adults and 51 adolescents. They were randomly assigned to the ethanol or water control group. All experimental procedures have been carried out in accordance with the European Council directive (2010/63/UE L 276 20/10/2010) and with the guidelines approved by the Ethical Committee of the University of Cagliari (OPBA).

Intermittent Alcohol Access Procedure

Adult or adolescent rats, individually housed, were exposed to a two-bottle choice regimen (water vs. ethanol 20% V/V) with an intermittent alcohol access for three 24-h sessions per week (Monday-Wednesday–Friday) for a total of 9 weeks. Access to alcohol started at the beginning of the light cycle (08:00) and ended after 24 h. When alcohol was not available, both bottles were filled with water and the amount consumed was recorded for each side. The left and right positions for alcohol and water bottles were switched between sessions to avoid any side bias effect on intake. Both water and alcohol were made available through graduated 190 ml capacity polycarbonate bottles (ACBT0152) equipped with stainless steel caps (ACCP0111) (Tecniplast S.p.A., Buguggiate, Varese, Italy). The bottles were refilled with fresh solutions at every session. On the day of alcohol availability, after having recorded water consumption, animals were weighed and returned to their home cages and soon after the two bottles (water and alcohol) were placed in the cage. Alcohol and water intake in 24 h was monitored by weighing the bottles (accuracy 0.1 g) and then intake referred to each animal as g/kg of body weight. In addition, at sessions 1, 9, 12, and 19, alcohol intake after the first hour of exposure (T1) was recorded as an index of the animal motivation to drink at the beginning and following the abstinence day. Possible fluid spillage was calculated by using multiple bottles filled with water and 20% ethanol, positioned in empty cages. The mean of this passive leakage was subtracted from the weight change of individual fluid bottles of experimental subjects at each analysis point. Control animals were kept in the same conditions but both bottles contained water. This group served as a control for water intake.

Behavioral Recording During First Hour of Ethanol Exposure or Withdrawal

During the first hour of alcohol exposure and the first hour of withdrawal day of the sessions indicated below, the animal behavior was recorded and then analyzed and scored as the percentage of time spent in each behavioral category in 60 min (after ethanol exposure: sedation, locomotion, licking, sniffing, and rearing; after ethanol withdrawal: jumping, paw treading, locomotion, head burying, gnawing). Behavioral effects were recorded during the first week of exposure (1st and 3rd sessions), third week (9th session), fifth week (13th session), and seventh week (19th session). Withdrawal score was recorded the day after the 8th and 19th sessions but since there were no substantial differences between sessions scores have been pooled.

Statistics

All data were expressed as mean ± SEM. Statistical analysis was carried out by Statistica for Windows (Version 7.0 Statsoft, Tulsa, OK, United States). Daily and weekly ethanol intake data (g/kg) were analyzed by three-way and two-way ANOVA for repeated measures to unveil significant differences, with age and strain as between factors and sessions or weeks as within measure. One way and two-way ANOVAs were applied to cumulative ethanol and water intake, respectively, with strain, age, and ethanol exposure as independent factors. Differences in ethanol preference were analyzed within each age group by two-way ANOVA for repeated measures with strain as between factor and weeks as within factor. Three-way ANOVA was applied to ethanol intake at T1 with strain and age as between factors and sessions as within factors. Behavioral scores were analyzed by one-way ANOVA for each behavioral item with strain as between factor. Results showing significant effects following ANOVA were subjected to Tukey's *post hoc* test. The significance level was set at $p < 0.05$.

RESULTS

Alcohol Consumption in Adult and Adolescent Rats

Figure 1 shows the daily consumption of alcohol and water in the three strains of rats together with daily water intake and body weight gain curve during the alcohol exposure regimen. Three-way ANOVA for repeated measure was applied to ethanol intake that revealed significant main effects of strain, age, and time ($F_{strain}(2,47) = 19.58$, $p < 0.00001$; $F_{age}(1,47) = 9.04$, $p < 0.01$; $F_{time}(26,1222) = 13,16$, $p < 0.000001$) and a significant interaction of time × strain ($F_{52,1222} = 3.21$ $p < 0.00001$) and time × age ($F_{26,1222} = 3.32$, $p < 0.00001$) but not for strain × age and time × strain × age ($p > 0.05$). *Post hoc* analysis revealed that overall adolescents drink more ethanol than adults ($p < 0.01$), SD drink more than LEW and F344 ($p < 0.01$, $p < 0.001$), and LEW more than F344 ($p < 0.01$).To better highlight differences over time between strains, we performed a two-way ANOVA for repeated measures within each age group which showed significant strain differences in ethanol intake [*adults*: $F_{strain}(2,26) = 13.71$, $p < 0.0001$; $F_{time}(26,676) = 6.55$, $p < 0.0001$; $F_{strain\ x\ time}(52,676) = 2.73$, $p < 0.0001$; *adolescents*: $F_{strain}(2,21) = 6.86$, $p < 0.01$; $F_{time}(26,546) = 13.55$, $p < 0.0001$; $F_{strain\ x\ time}(52,546) = 1.52$, $p < 0.05$]. Tukey's *post hoc* analysis showed that SD and LEW rats, both adult and adolescent, increase their alcohol intake over time while F344 rats do not, no matter if adults or adolescents. Adolescent rats start drinking greater amounts of alcohol compared with adults (SD: 2.45 ± 0.5 vs. 1.1 ± 0.32 g/kg/day; LEW: 1.79 ± 0.31 vs. 1.1 ± 0.30; F344: 1.64 ± 0.26 vs. 0.48 ± 0.13), although there were no significant

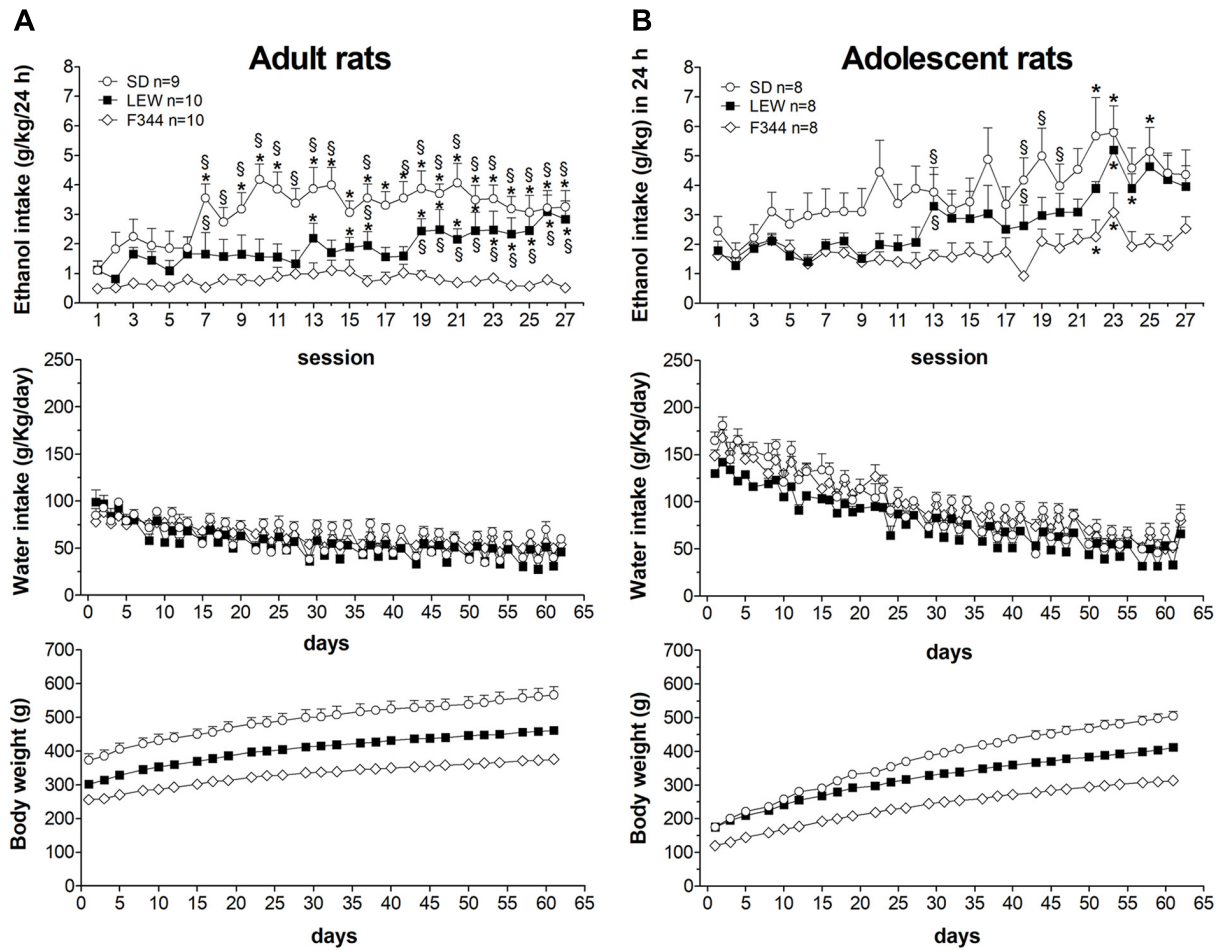

FIGURE 1 | Daily pattern of alcohol intake. Alcohol intake (g/kg/day) by adults **(A)** and adolescents **(B)** onset rats of Sprague-Dawley (SD), Lewis (LEW), and Fischer 344 (F344) strains and daily water intake (g/kg/day) during ethanol regimen. Lower panels show the body weight curves for the three strains during the entire experimental period. Results are expressed as means ± SEM. *$p < 0.05$ vs. first session within the same strain and §$p < 0.05$ vs. the corresponding value in the F344 strain by two-way ANOVA for repeated measures followed by Tukey's *post hoc* test.

differences between strains at least in the first 3 weeks of exposure (from 1st to 12th session, **Figure 1B** upper panel).

The same analysis revealed that SD rats consume greater alcohol amounts than LEW and F344 and LEW greater than F344 strain when adult (**Figure 1A** upper panel), but these differences fail to emerge when they were adolescent at the onset of ethanol exposure, at least in the first 3 weeks of exposure (**Figure 1B** upper panel). Analyzing alcohol consumption as weekly intake by two-way ANOVA for repeated measures (**Figure 2**) highlights better the escalation in ethanol intake in SD and LEW strains, but not in F344 one, in both age groups. Water intake decreased over time in the three strains in both age groups [*adults*: $F_{time}(8,208) = 70.10$, $p < 0.0001$; *adolescents*: $F_{time}(8,168) = 335,23$, $p < 0.00001$] but to a greater extent in LEW rats [*adults*: $F_{strain}(2,26) = 2.3$, $p = 0.11$; $F_{strain\ x\ time}(16,208) = 2.59$, $p < 0.01$, *post hoc* $p < 0.05$, see **Figure 2A** for details; *adolescents*: $F_{strain}(2,21) = 15.54$, $p < 0.0001$; $F_{strain\ x\ time}(16,168) = 1.021$, $p = 0.4$], and more in adolescent than adult rats [$F_{agextime}(8,128) = 16.13$, $p < 0.0001$] given that adolescent rats drink more water than adults (**Figures 2A,B**).

One-way ANOVA within each strain applied to cumulative alcohol intake in the 9-week period did not reveal any significant difference between adults and adolescents in SD and LEW rats ($F_{1,15} = 0.94$, $p = 0.34$; $F_{1,16} = 2,72$, $p = 0.11$, respectively), although generally daily ethanol intake in adolescent individuals was higher than adult ones (mean intake g/kg/day during last week SD: 4.64 ± 0.25 vs. 3.18 ± 0.06; LEW: 4.26 ± 0.20 vs. 2.79 ± 0.19; F344: 2.19 ± 0.18 vs. 0.62 ± 0.08), while adolescent F344 rats show the greater total amount of alcohol consumed compared with their adult counterparts, as clearly apparent in **Figure 3A** ($F_{1,16} = 15.68$, $p < 0.01$). Analysis of cumulative ethanol intake between strains in adults shows that SD ethanol intake was greater than LEW and F344 ($F_{2,25} = 12.09$, $p < 0.001$; *post hoc* $p < 0.05$ and $p < 0.001$) and LEW intake greater than F344 ($p < 0.05$), while in adolescent groups, only SD ethanol intake was greater than that one of F344 rats ($F_{2,21} = 6.99$, $p < 0.01$; *post hoc* $p < 0.01$).

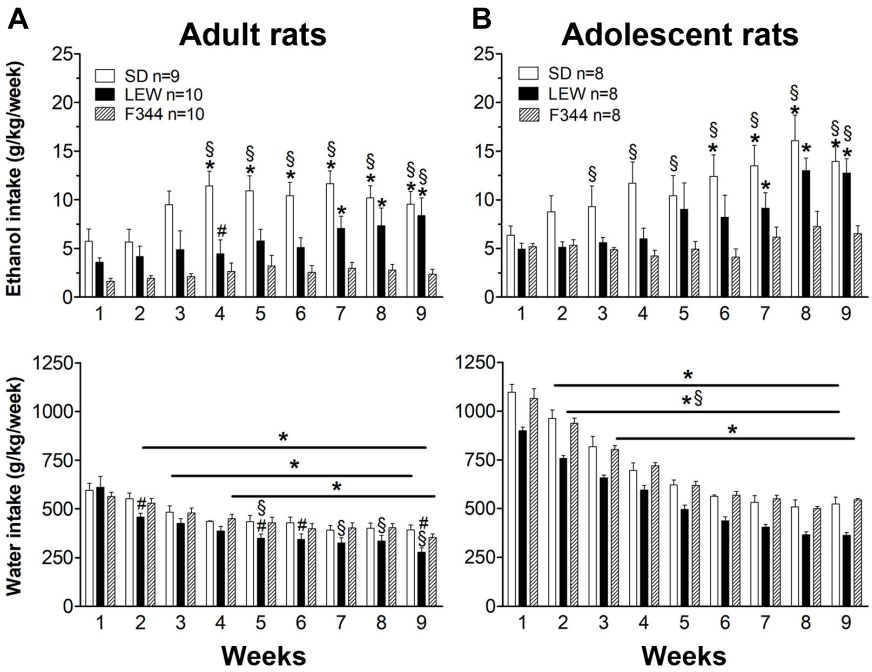

FIGURE 2 | Weekly pattern of ethanol and water intake. Weekly ethanol and water intake (g/kg/week) in the two age groups **(A)** adults and **(B)** adolescents of the three strains. Results are expressed as means ± SEM. *$p < 0.05$ vs. first week within each strain, #$p < 0.05$ vs. Sprague-Dawley (SD) strain, §$p < 0.05$ vs. Fischer 344 (F344) strain by two-way ANOVA for repeated measures followed by Tukey's *post hoc* test.

Comparison of cumulative water intake between controls and ethanol exposed group of each strain and age group (**Figure 3B**) revealed no difference between ethanol and control groups in the total amount of water intake (adults: SD $F_{1,14} = 0.11$, $p = 0.74$; LEW: $F_{1,15} = 0.08$, $p = 0.77$; F344: $F_{1,15} = 0.57$, $p = 0.46$; adolescents: SD $F_{1,15} = 0.7$, $p = 0.4$; LEW: $F_{1,15} = 0.3$, $p = 0.5$; F344: $F_{1,15} = 1.7$, $p = 0.2$), indicating that even if ethanol exposed groups reduce their water intake during alcohol sessions they drink more water during days in which alcohol was not available.

Two-way ANOVA applied to cumulative water intake revealed that among adult controls, LEW rats drink less than SD and F344 rats ($F_{2,18} = 5.79$, $p < 0.01$), while among adolescents, ethanol exposed LEW rats drink significantly less water than the other two strains ($F_{2,21} = 15.54$, $p < 0.0001$, *post hoc* $p < 0.001$), with no difference between control groups ($F_{2,24} = 3.2$, $p = 0.05$), at variance with what observed in adult rats. These results are consistent with the higher ethanol preference observed in the LEW strain (**Figure 4**, lower panel).

The analysis of alcohol preference, calculated as a percentage of ethanol consumption on the total amount of liquid drunk by each rat, revealed that ethanol preference in LEW and SD strain increases over time [$F_{\text{strain x time}}$ (16,208) = 3.04, $p < 0.001$, see **Figure 4** legend for *post hoc* details] reaching the same amount of ethanol preference and being higher than that one of F344 rats in the adult group (**Figure 4**, upper panel). A similar increase of ethanol preference was observed in adolescents, but a significant increase of preference was observed also in F344 strain, while, at the end of alcohol exposure, only LEW strain showed an ethanol preference higher than F344 strain [$F_{\text{strain x time}}$(16,168) = 2.95, $p < 0.001$, see **Figure 4** legend for *post hoc* details), reaching almost 50% of ethanol preference.

Figure 5 shows the percentage of alcohol intake during the first hour of exposure at different time points of ethanol exposure (1st, 9th, 12th, and 19th sessions). Three-way ANOVA for repeated measures revealed that adult LEW rats consume in general a greater percentage of their daily intake than F344 strain during the first hour of exposure, while this difference fades in adolescent groups [$F_{\text{strain x age x session}}$ (6,141) = 3.49, $p < 0.01$, see **Figure 5** legend for *post hoc* comparisons between strains and ages].

Behavioral Effects After Ethanol Exposure and During Withdrawal

Behavioral observations made during the first hour of ethanol exposure revealed different patterns of effects depending on the strain and onset age (**Figures 6**, **7**).

In adult rats, following the first 3 sessions of alcohol exposure, there was a significant difference between strains. While SD and F344 rats manifested a profound sedation following ethanol intake ($F_{2,26} = 36{,}72$, $p < 0.0001$; *post hoc* $p < 0.001$), LEW rats showed a behavioral activation characterized by locomotion, sniffing, and hedonic reactions, such as genitals licking (and associated penile erection) and licking of the cage walls (*locomotion*: $F_{2,26} = 10.09$, $p < 0.001$; *licking*: $F_{2,26} = 8.11$, $p < 0.01$; *sniffing*: $F_{2,26} = 3.41$, $p < 0.05$), this last item being completely absent in the other two strains (**Figure 6A**, see figure legend for *post hoc* significance). Following multiple ethanol

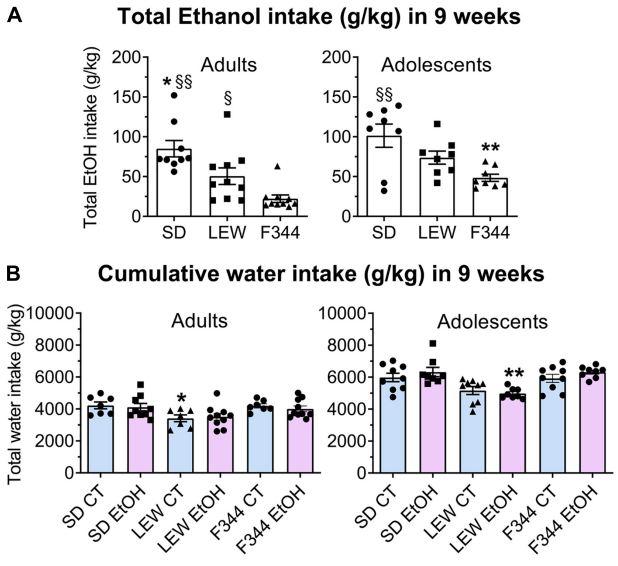

FIGURE 3 | Cumulative ethanol and water intake in 9 weeks. **(A)** Total ethanol intake (g/kg/9 weeks) in adult and adolescent groups of the three strains. Data are expressed as means ± SEM. *$p < 0.05$ Sprague-Dawley (SD) vs. Lewis (LEW), §§$p < 0.001$ SD vs. Fischer 344 (F344), §$p < 0.05$ LEW vs. F344, **$p < 0.01$ F344 adolescents vs. adults by one-way ANOVA followed by Tukey's *post hoc* test. **(B)** Cumulative water intake (g/kg/9 weeks) in adult and adolescent groups of the three strains. Water intake by control rats (CT) is reported for comparison with ethanol-exposed rats (EtOH). *$p < 0.05$ LEW CT vs. SD and F344 CT; **$p < 0.001$ LEW EtOH vs. SD and F344 EtOH by two-way ANOVA followed by Tukey's *post hoc* test.

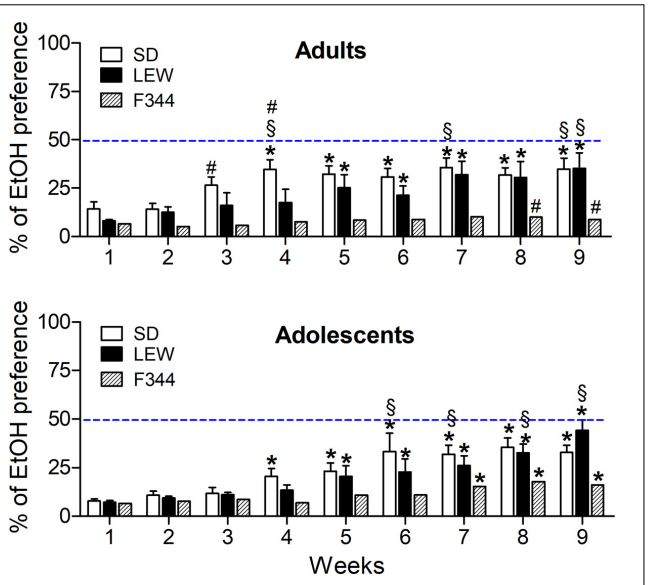

FIGURE 4 | Alcohol preference expressed as percentage of total fluid intake in adult and adolescent groups of the three strains. Data are expressed as means ± SEM. *$p < 0.05$ vs. preference in the first week within each strain; §$p < 0.05$ vs. corresponding value in the Fischer 344 (F344) strain; #$p < 0.05$ vs. the corresponding value in the adolescent group of the same strain by two-way ANOVA for repeated measures followed by Tukey's *post hoc* test.

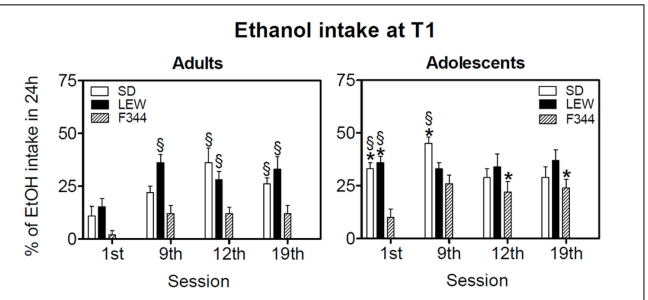

FIGURE 5 | Ethanol intake during the first hour of the drinking session (T1). Data are expressed as means ± SEM. *$p < 0.05$ vs. the corresponding value in adult rats, §$p < 0.05$ vs. corresponding value of Fischer 344 (F344) rats by three-way ANOVA for repeated measures followed by Tukey's *post hoc* test.

exposures, a locomotor sensitization appeared in SD strain ($F_{3,51} = 3.08$, $p < 0.05$, *post hoc* $p < 0.05$ seventh week vs. first, third, and fifth weeks) accompanied by a total disappearance of sedation, which was still present in F344 strain even during the seventh week ($F_{2,26} = 149$, $p < 0.0001$, see **Figure 6** legend for *post hoc* comparisons). Repeated exposure to ethanol induced the appearance of hedonic reactions in SD rats (licking), still lacking in F344 strain (**Figures 6B–D**). In LEW rats, there was not an increase in locomotor activity but the appearance of confined sniffing and licking still higher when compared with the other two strains (third week: $F_{2,26} = 15.53$, $p < 0.0001$; fifth week: $F_{2,26} = 10.78$, $p < 0.001$; seventh week: $F_{2,26} = 15.78$, $p < 0.0001$, see **Figure 6** legend for *post hoc* comparisons).

Behavioral effects in adolescent rats were characterized by an almost absent sedation in SD rats ($F_{1,15} = 8.87$, $p < 0.01$, *post hoc* $p < 0.05$), but still present in the F344 strain, although there was a trend toward reduction when compared with the adult counterpart ($F_{3,48} = 2.7$, $p = 0.05$). Adolescent LEW rats displayed a reduction in locomotion when compared with the adult counterpart ($F_{1,16} = 6.15$, $p < 0.05$; *post hoc* $p < 0.05$) but showed the appearance of stereotypies (confined sniffing and gnawing), **Figure 7**.

Analysis of behavior during the first hour of withdrawal showed a profound difference between strains (**Figure 8**). In the adult group, LEW strain manifested significant "craving like" reactions (mainly jumping, locomotion, and gnawing) when compared with the other two strains, notably vs. F344 strain. Time spent in jumping by the LEW strain was higher than that one by the other two strains ($F_{2,26} = 10.02$, $p < 0.001$) as well as locomotion, and gnawing time spent was higher than that one by the F344 strain (*locomotion*: $F_{2,26} = 7.98$, $p < 0.01$; *gnawing*: $F_{2,26} = 3.75$, $p < 0.05$). In the adolescent group, differences between strains were similar to those observed in the adult group, with the only difference of a reduced locomotion in SD rats ($F_{1,15} = 5.14$, $p < 0.05$; *post hoc* $p < 0.05$) when compared with the adult counterpart (**Figure 8**).

It is worth noting, in regard to withdrawal reactions, that animals manifesting greater "craving like" reactions drank greater amounts of water from the bottle placed on the same side where, the previous day, the alcohol bottle was placed (data not shown).

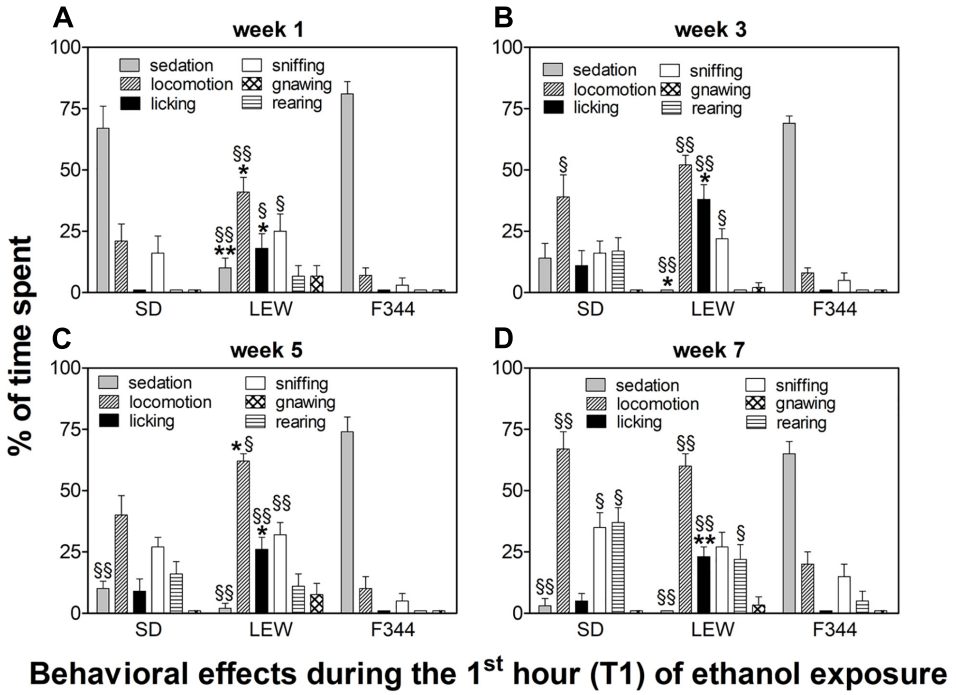

FIGURE 6 | Behavioral effects in adult onset rats during the first hour of the drinking session. Data are expressed as means ± SEM. **(A)** first week: data are referred to the first and third sessions of exposure. **(B)** third week: data are from the 9th session. **(C)** fifth week: data are from the 13th session; and **(D)** seventh week: data are from the 19th session. *$p < 0.05$ and **$p < 0.001$ Lewis (LEW) vs. Sprague-Dawley (SD), §$p < 0.05$ and §§$p < 0.001$ vs. corresponding value in Fischer 344 (F344) strain by one-way ANOVA followed by Tukey's *post hoc* test.

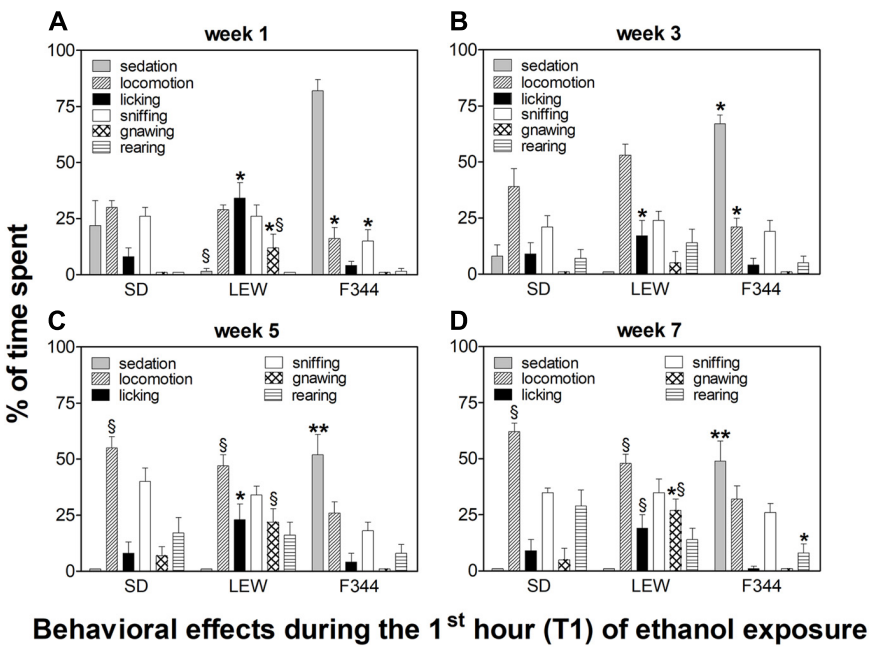

FIGURE 7 | Behavioral effects in adolescent onset rats during the first hour of the drinking session. Data are expressed as means ± SEM. **(A)** first week: data are referred to the first and third session of exposure; **(B)** third week: data are from the 9th session; **(C)** fifth week: data are from the 13th session; and **(D)** seventh week: data are from the 19th session. *$p < 0.05$ Lewis (LEW) vs. Sprague-Dawley (SD) and Fischer 344 (F344) strain, **$p < 0.001$ F344 vs. LEW and SD, §$p < 0.05$ SD and LEW vs. F344 strain by one-way ANOVA followed by Tukey's *post hoc* test.

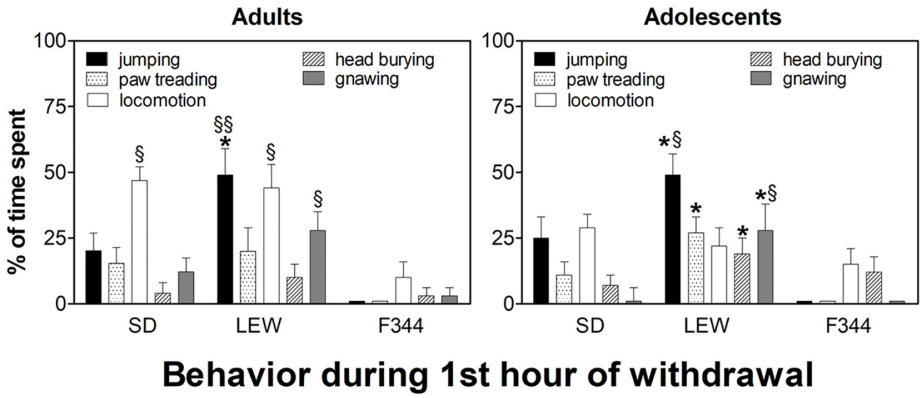

FIGURE 8 | Behavioral reactions during the first hour of withdrawal in the three strains in adult and adolescent onset rats. Data are expressed as means ± SEM. Results from the day after the 8th and 19th drinking sessions were pooled since no significant difference was observed. *$p < 0.05$ Lewis (LEW) vs. Sprague-Dawley (SD), §$p < 0.05$ LEW and SD vs. Fischer 344 (F344), §§$p < 0.001$ LEW vs. F344 by one-way ANOVA followed by Tukey's *post hoc* test.

DISCUSSION

The results presented highlight differences in alcohol intake and behavioral effects induced by repeated and intermittent alcohol consumption, and alcohol withdrawal, due to age of first exposure and genetic background of the individual. The intermittent alcohol access paradigm utilized in this study shows how genetic background may affect drinking pattern. While SD and LEW strains display an escalation of alcohol intake, F344 strain does not, thus, confirming previous reports for the SD strain (Li et al., 2010; Bito-Onon et al., 2011; Li et al., 2011), and adding new evidence for the inbred rat strains LEW and F344. Comparison of the age groups revealed that while in the adult onset group, differences between strains emerge after the first 2 weeks of exposure, in the adolescent onset group, differences due to genetic background emerge later on, when the rats become adults. This pattern of results is consistent with what is observed in humans. Individual differences in alcohol (but also in other substances of abuse) initiation and patterns of use are reported to be strongly influenced by social and familial environmental factors, while later levels of use appear to be strongly influenced by genetic factors (Kendler et al., 2008; van Beek et al., 2012). Although adolescent rats start drinking more than adults, there were no statistical differences between adult and adolescent SD and LEW rats in the total amount of alcohol intake during the entire period (9 weeks) of exposure. However, F344 strain, considered an addiction resistant strain, on the basis of several previous findings (Cadoni, 2016), although not escalating alcohol intake over time (at least in a period of 9 weeks as in the present study) displays a significantly higher total ethanol intake if starting to drink as adolescent, shows an increase in ethanol preference in the last 2 weeks of exposure, and an increased ethanol intake at T1. This would suggest that even individuals resistant to develop AUD might be at risk if they start to drink as an adolescents.

One of the reasons why the adolescent onset of drinking seems to affect more the F344 strain (addiction resistant) than LEW (addiction prone) and SD ones might be the different impact of ethanol on developing brain of these strains. It is well known that during adolescence, there is a rearrangement of several neuronal circuitries, with changes in number and subunits composition of different neurotransmitter receptors in different brain areas (Spear, 2000; Brenhouse and Andersen, 2011). Indeed, there are several fundamental differences between strains, in particular, between F344 and LEW rats (Cadoni, 2016), in neurotransmitter systems targeted by alcohol (gabaergic, dopaminergic, serotonergic, cannabinoidergic) which might play a role in the impact of ethanol on adolescent brain of each strain. For examples, adolescent THC (Δ^9-tetrahydrocannabinol) exposure affects differently DA transmission in SD, LEW, and F344 rats, respectively, by decreasing, leaving unaffected, and increasing DA transmission responsiveness in the nucleus accumbens shell of these strains (Cadoni et al., 2008; Cadoni et al., 2015). Similar results were obtained after adolescent nicotine exposure, and different adaptive changes in DA transmission have been observed depending on adolescent or adult drug exposure (Cadoni et al., 2020). It is conceivable that if similar adaptive changes occur following repeated ethanol exposure too, they could affect differently drinking pattern in each strain. Another point to be considered in this regard is the fact that SD strain is an outbred strain, at variance with F344 and LEW, and therefore, the greater variability in the SD strain might have masked a likely effect of early onset in some individuals but not in others, while this variability is almost absent in the inbred strains. Even if there were not differences between adolescent and adult rats of the SD and LEW strains, analysis of ethanol preference in the two age groups of the three strains revealed that LEW adolescent rats display the highest ethanol preference the last week of exposure, reaching on average almost 50% of ethanol preference, with some animals drinking nearly exclusively alcohol.

The fact that SD rats, both adults and adolescents, drink more than LEW rats (considered an addiction-prone strain) might be explained by a greater sensitivity of the LEW strain to ethanol rewarding effect and/or different ethanol pharmacokinetics in the two strains. Indeed, previous findings in SD, LEW, and F344

rats have shown LEW rats having a slower alcohol metabolism, thus, leading to higher blood ethanol concentrations (BEC) at differing time points following alcohol exposure when compared with SD (Bito-Onon et al., 2011), but also with F344 rats (Suzuki et al., 1988; Roma et al., 2007; Roma et al., 2008). Moreover, on the basis of our previous studies (Cadoni and Di Chiara, 2007; Cadoni et al., 2015; Cadoni et al., 2020) showing a higher sensitivity of the LEW strain to the DA releasing properties of different drugs of abuse and different adaptive changes in mesolimbic DA transmission following repeated drug exposure, it can be hypothesized that similar pattern of response might occur following ethanol too. Both of these reasons might explain why LEW rats drink little less than SD ones.

In the present study, we compared also cumulative water intake between controls and ethanol group of each strain and age group. This analysis revealed a difference between adult and adolescent groups with adolescent rats drinking a larger amount of water as compared to adults. This is a general feature in human infants/adolescents, and presumably in rodents too, and is due to high requirements for water of infants/adolescents to maintain an adequate body composition (Iglesia et al., 2015). This high requirement can be in part explained by a proportionally higher body water content than adults. Although we did not observe differences within each age between the water and ethanol group of the three strains, we detected a reduced water intake in control adult rats of the LEW strain and a reduced water intake in adolescent ethanol-exposed rats of the same strain. This would indicate that the reduced water intake in adult control LEW rats cannot be explained only by body mass, given that F344 rats although having a lower body weight consume the same amount of water as SD strain, but other factors might be involved. On the other hand, we observed a reduced water intake in adolescent LEW ethanol-exposed rats, which would be consistent with an increased ethanol preference in this strain of rats.

Ethanol intake during the first hour of alcohol availability after the day of abstinence (expressed as a percentage of 24 h intake) is somehow a measure of individual compulsiveness to consume alcohol, and it is related to the state of withdrawal experienced by the animal during the sessions following the first one. In general, adolescent rats consume more alcohol than adults during the first session, and this is likely correlated to a different motivation (high novelty seeking behavior in adolescents), but also to alcohol effects experienced, given that the first exposure usually produces sedative effects in adults while producing activating effects in adolescents, as it has been reported in the result section (see also **Figures 6**, **7**). Although, as described before, SD showed the highest rates of alcohol consumption, they are not always compulsive as the LEW strain, consuming most of their daily intake during the dark phase.

Analysis of behavior during the first hour following ethanol consumption revealed significant differences between strains and age groups. In the adult group, while SD and F344 strains showed a prevalent sedative effect at the first week, LEW rats displayed a significant behavioral activation characterized by locomotion, sniffing, and licking. A low sedative response following alcohol intake is considered a risk factor to develop AUD later in life in humans and in laboratory animals (Crews et al., 2016). Moreover, behavioral effects observed following ethanol intake would suggest a greater DA transmission stimulation (Wise and Bozarth, 1987) and a greater hedonic effect (as shown by the presence of licking). Following repeated exposures to ethanol, SD rats developed behavioral sensitization, manifested as tolerance to the sedative effect of alcohol, and increased time spent in locomotor activity as previously observed in this strain (Wilson et al., 1997). These striking differences between strains in sedative effects might be due to differences between strains in gamma-aminobutyric acid (GABA) levels, GABA-A receptors distribution and/or functionality (Cadoni, 2016), and different adaptive changes in DA transmission in striatal areas (ventral and dorsal striatum). Indeed, previous studies have shown that LEW and F344 strains differ not only in basal levels of GABA and Glutamate in NAc but also in response to acute ethanol (Selim and Bradberry, 1996) and cocaine reinstatement in a self-administration paradigm (Miguéns et al., 2011).

The behavior observed in the adolescent group following alcohol consumption revealed a marked reduction of sedative effect in SD strain (almost absent) and a modest reduction in the F344 strain. The lower sedative effect observed in adolescent rats following ethanol consumption is a general hallmark of adolescence, both in humans and rodents (Little et al., 1996; Moy et al., 1998; Novier et al., 2015; Spear, 2018), and it appears correlated to developmental changes of GABA-A receptors, and other receptor types too (N-methyl-D-aspartate [NMDA], DA) (Crews et al., 2007; Novier et al., 2015). Therefore, sedative or activation effects do not appear to be correlated to the amount of ethanol intake in this study, as previously observed in other studies showing that adolescent rats recover the righting response following a sedative dose of ethanol in spite of higher brain alcohol levels than adults (Silveri and Spear, 1998). F344 rats displayed also a behavioral activation (locomotion, sniffing, and rearing), almost absent in adult counterparts, indicating a different impact on neurotransmitter systems mediating alcohol effects in the adolescent brain (Spear, 2018). Adolescent LEW rats, on the contrary, do not display any sedative effects as previously observed in adult counterpart but, at variance with SD strain, show a reduced locomotor activity compared with adults, and appearance of more stereotypies, such as confined sniffing and gnawing, this last one being significantly higher than SD and F344 rats. The appearance of stereotyped activity is usually correlated to an intense DA receptors activation, especially in striatal areas (Kelly et al., 1975; Bordi and Meller, 1989; Delfs and Kelley, 1990). This difference might be due to a different impact of alcohol on DA transmission between adults and adolescents and/or to different adaptive changes in DA transmission following alcohol exposure during the critical period of adolescence. Indeed, our previous studies on adolescent rats have shown a higher sensitivity, in terms of DA released, to several drugs included opiates (Corongiu et al., 2020) and different adaptive changes in mesolimbic DA transmission following adolescent exposure to cannabinoids, opiates, and nicotine in LEW, F344, and SD rats, as discussed above.

It is worthy of note in this regard that LEW rats are, among several rat lines, the most sensitive to the rewarding

and activating effects of nicotine (Cadoni et al., 2009; Chen et al., 2012) and that rats selectively bred for high alcohol consumption/responsivity are also more likely to self-administer nicotine (Deehan et al., 2018), in agreement with human studies, suggesting a common genetic basis for alcohol and nicotine addiction (Cross et al., 2017).

The analysis of behavior during ethanol withdrawal indicates the LEW strain as the most affected and this might be a factor influencing the establishment of alcohol dependence. Indeed, withdrawal syndrome has been implicated as one of the mechanisms for the progression from impulsive to compulsive substance use. In this regard, jumping behavior in rodents, highly expressed by LEW rats in this study during withdrawal, is widely considered as an index of opiates withdrawal intensity and is commonly used to test opiate dependence (Ritzmann, 1981; Kest et al., 2001; Dunn et al., 2019). Reasons for this heightened vulnerability of LEW strain might be searched in the mechanism of action of alcohol and LEW characteristics in DA and opioid systems (Cadoni, 2016). In fact, the reinforcing and rewarding effects of alcohol are primarily mediated by the DA mesolimbic and opioid systems (Spanagel, 2009). In addition to stimulate mesolimbic DA transmission, ethanol releases endorphins into the nucleus accumbens (Olive et al., 2001). Therefore, it is likely that repeated alcohol consumption might have induced adaptive changes in opioid receptors similar to those observed after repeated morphine or heroin. It is worthy of note that LEW rats show, in comparison with F344 strain, opposite changes in endogenous opioids following chronic morphine (Cadoni, 2016) and an increased binding to mu-opioid receptors (Sánchez-Cardoso et al., 2007) consistent with a higher score of "craving like" reactions observed in this strain following repeated exposure to heroin (Cadoni et al., 2015), and a similar correlation has been reported in human alcoholics showing higher rates of craving during abstinence (Heinz et al., 2005; Hansson et al., 2019).

A limitation of this study might be due to the experimental condition utilized. The single housed condition of the animals, especially during adolescence, might have affected the results obtained. This choice has been, however, imposed by the need to evaluate individual alcohol intake, but also by the need to perform other biochemical recordings in another group of animals on the same ethanol regimen (microdialysis experiments actually still running). It is well known that isolation is a stressful condition for adolescent individuals (Burke et al., 2017; Walker et al., 2019) which can lead to increased ethanol intake in two-bottle choice paradigms (McCool and Chappell, 2009; Karkhanis et al., 2015), but not in operant self-administration paradigms (Noori et al., 2014). Moreover, stress effects on alcohol consumption might be affected by genetic background (Spanagel et al., 2014) and therefore social isolation might have affected differently the three strains. However, given that we preserved social interaction at least in early adolescence, by group housing animals, and considered that the most critical period for social isolation is between PND 21 and 41, during which rats show most social play behavior (Lesscher et al., 2015; Burke et al., 2017), it could be hypothesized a limited effect on our animals, since most of them were isolated at PND 38–40.

Moreover, it should be emphasized the lack of testing in female rats in the present study. Indeed, it is well recognized that sex/gender may affect substance abuse in general (Becker and Chartoff, 2019; Cornish and Prasad, 2021) and alcohol-induced outcomes, such as drinking pattern, sensitivity to ethanol, anxiety, and neuroinflammation (Robinson et al., 2021). Therefore, further investigation will need to extend the results of the present study to female rats.

Although conclusive remarks will need additional research, by using operant self-administration procedures, to better evaluate the reinforcing and motivational value of alcohol in these strains, nonetheless, the present results highlight the influence of age and genetic background on vulnerability to AUD. On the basis of alcohol consumption (escalation of alcohol intake), it appears that both SD and LEW rats are the most vulnerable to develop alcohol dependence. However, on the basis of behavioral reactions, in particular, during withdrawal days, and alcohol preference, the LEW strain seems the more prone to develop alcohol dependence. On the other hand, although the F344 strain seems to be the less vulnerable strain to develop alcohol dependence, since it does not escalate ethanol intake and does not manifest "craving like" reactions, comparisons between adult and adolescent groups indicate that adolescent F344 rats, increasing their ethanol preference over time, might be at risk to develop alcohol dependence.

In conclusions, the results presented show that the influence of genetic background on alcohol consumption emerges more at adulthood than during adolescence and that adolescent onset of drinking might increase the risk to develop AUD even in genetically less vulnerable individuals.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee at the University of Cagliari.

AUTHOR CONTRIBUTIONS

CC and SF were responsible for the study concept and design. SC, CD, AuP, EE, AnP, and CC contributed to the acquisition of animal data. CC, DL analyzed the data. CC wrote the manuscript. All authors critically reviewed content and approved the final version of the manuscript.

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Reinforcement History Dependent Effects of Low Dose Ethanol on Reward Motivation in Male and Female Mice

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Alcohol use disorders (AUDs) are more prevalent in men than in women, though AUD diagnoses in women are growing rapidly, making an understanding of sex differences in alcohol-related behaviors increasingly important. The development of AUDs involves the transition from casual, low levels of alcohol drinking to higher, maladaptive levels. The ability of low dose alcohol to drive reward and drug seeking may differ in males and females, and this could underlie differences in susceptibility to AUD. In this study we sought to determine whether a history of chronic, low dose ethanol exposure (0.5 g/kg; i.p.) could drive sucrose reward seeking and motivation, and whether this differed between male and female mice. Adult mice were trained to lever press for a liquid sucrose reward on two reinforcement schedules: a random interval (RI) schedule and a variable ratio (VR) schedule. After training, mice were tested on each of these levers for reward motivation using a progressive ratio test. We found that a history of low dose ethanol exposure increased sucrose reward motivation in male mice, but only on the RI lever and only when exposure occurred proximal to learning. Female mice were more motivated for sucrose on the RI lever than the VR lever regardless of ethanol exposure condition. These findings indicate that training on different reinforcement schedules affects reward motivation. Further, we show that males are more susceptible to the effects of low dose ethanol on sucrose reward motivation than females. These data broaden our understanding of sex differences in reward seeking as a result of ethanol exposure.

Keywords: sex, reward, motivation, microstructure, ethanol, schedule

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INTRODUCTION

While alcohol use disorders (AUDs) present a significant societal and economic burden, the majority of people who drink alcohol do so at low, casual levels that do not reach criteria for an AUD (SAMHSA, 2019). However, these chronic, lower drinking levels can produce behavioral and neurobiological changes that may promote the transition from casual drinking to heavy drinking

seen in the development of AUD. A greater understanding of how low levels of alcohol drinking or alcohol exposure impact inflexible behavior can increase understanding of how susceptibility to AUD is conveyed.

The physiological effects of acute ethanol are distinct at low doses vs. high doses. Lower doses (0–0.75 g/kg) produce stimulatory effects, while higher doses (1 g/kg +) produce sedative effects (Cui and Koob, 2017). Low dose ethanol has also been shown to be more neuroprotective, as it reduces inflammation and increases production of neurotrophic factors (Gahring et al., 1999; Tizabi et al., 2018). However, low dose ethanol effects on reward-seeking behavior are neither well known nor well studied. Clinical and preclinical studies have shown that the impacts of ethanol exposure on memory and behavior depend on the timing of exposure in relation to learning (Tyson and Schirmuly, 1994). It has also been shown that acute ethanol exposure, even at low doses, up to 2 h after a learning event can promote memory recall (Alkana and Parker, 1979; Hewitt et al., 1996). Further supporting the importance of exposure timing, conflicting results have been found when chronic ethanol exposure occurs proximal to learning vs. distal (Corbit et al., 2012; Barker et al., 2020).

Men are currently more likely to be diagnosed with an AUD than women, but the gap has been narrowing in recent years (Keyes et al., 2008; White, 2020). It is especially important to understand how sex may impact low dose alcohol effects as women escalate from casual use to addiction more rapidly than men and may therefore be differentially susceptible to low dose ethanol effects (Becker and Koob, 2016). Further, women suffer greater negative health outcomes with more rapid onset and from lower doses of alcohol than men (Foster et al., 2018), so it is crucial to investigate outcomes of low dose ethanol in both sexes to understand the course of alterations.

As research into sex differences progresses, it is increasingly clear that male and female rodents exhibit differing patterns of reward seeking. Generally, female rodents have been found to be more motivated than males to work for highly palatable foods, like sucrose (Seaman et al., 2008; Sherrill et al., 2011; Sinclair et al., 2017). Females also develop sucrose-seeking habits faster than males (Quinn et al., 2007). There appear to be no striking sex differences in the attribution of incentive salience to food cues (Pitchers et al., 2015), suggesting that increased motivation observed in females is not driven by a greater sensitivity to food-related cues. In addition to these baseline differences, exposure to drugs and alcohol may have sex-specific or sex-determined outcomes. For example, it has been shown that the effects of higher doses of ethanol on behavior can depend on sex and, in some cases, even reinforcement schedule (Chaudhri et al., 2005; Barker et al., 2017a; Giacometti et al., 2020). Despite this, there is a lack of research into sex differences in the outcomes of lower doses of ethanol.

This study thus investigated the effects of low dose ethanol exposure on male and female mice to determine whether repeated low dose ethanol exposure impacted sucrose reward seeking and motivation. Further, we investigated how timing of low dose ethanol exposure and training history impacted outcomes.

MATERIALS AND METHODS

Subjects

Adult male and female C57BL/6J mice (9 weeks of age; 42 males, 30 females) from The Jackson Laboratory were used in these studies in accordance with the Drexel University Institutional Animal Care and Use Committee guidelines. The mice were housed in a vivarium with a standard 12:12 h light/dark cycle and were given 1 week to acclimate to the facility before beginning any experiments. Some mice (24 males and 18 females) underwent stereotaxic surgery with a pAAV-hSyn-EGFP retrograde adeno-associated virus (Addgene plasmid # 50465, RRID: Addgene_50465) targeting the nucleus accumbens shell (AP + 1.5 mm ML + 0.6 mm DV −4.7 mm) prior to beginning behavioral experiments. These mice were not used for any other experiments. Following recovery from surgery or following the acclimation period, mice were restricted to approximately 90% of their *ad libitum* weight and were then maintained at that weight for the length of the experiments. All mice were group housed for the duration of the study.

Operant Set-Up

All operant training occurred in standard Med-Associates operant boxes for mice, housed within sound attenuating chambers that included a fan for ventilation and white noise. The left wall of the box was curved and featured five nose poke holes with lights that were not activated or used for any of these studies. The right wall of the chamber had two retractable levers on either side of a reward magazine that had slots for pellet and/or liquid reinforcer. A house light was fitted above the magazine. The back wall, door, and ceiling of the box were made with Plexiglas. The floor was made with standard metal bars and was raised above a removable tray. Besides the house light, which turned on at the start of the session and remained on for the length of the session, there were no discrete cues presented during any of the behavioral sessions.

Instrumental Training

Prior to starting instrumental training, mice were habituated to the operant box and reward delivery magazine. For these sessions, the mice were placed in the operant box and the 10% liquid sucrose reward (20 ul, in tap water) was delivered into the magazine every 60 s for a total of 15 min. Mice only had one magazine training session per day. After 2 days of magazine training, mice were trained to lever press for sucrose on two separate levers. Only one lever was accessible at a time, and the levers were presented consecutively during the session. Thus, for the first half of the session the mice had access to one lever, then that lever would retract, and they would have access to the other lever for the rest of the session. Each lever was accessible for 15 min, and the whole session lasted 30 min. The order of which lever was accessible first alternated each day for each mouse and was counterbalanced across all groups and conditions.

Initially, both levers delivered reinforcer on a fixed ratio 1 (FR1) schedule where each lever press resulted in reward delivery. Mice were trained on the FR1 schedule until they reached stable

responding (at least 15 lever presses on each lever, maintained for 3 days). Mice that did not reach stable responding on both levers were excluded (seven males, one female). The schedules of reinforcement for each lever then diverged, such that the left lever began reinforcing on a random interval (RI) schedule and the right lever began reinforcing on a variable ratio (VR) schedule. On a RI schedule, the first press after a randomly determined interval (averaging 30 s for RI30, and 60 s for RI60) has elapsed was reinforced. On a VR schedule, the first lever press after a variable number of presses was reinforced (averaging 5 presses for VR5, and 8 presses for VR8). It has been shown that RI schedules promote inflexible behavior whereas VR schedules maintain flexible behavior (Adams and Dickinson, 1981; Dickinson et al., 1983; Gremel and Costa, 2013; Barker et al., 2017a). Mice were trained for 3 days on the RI30/VR5 schedule and then for 3 days on the RI60/VR8. After training, a subset of mice was tested for inflexible behavior on a contingency degradation and outcome devaluation test before beginning testing on the progressive ratio (PR) schedule. Data from those additional tests are being excluded for the purposes of this manuscript.

Ethanol Exposure

Previous studies have shown that the effects of ethanol on learning can depend on exposure timing in relation to behavior, especially if exposure occurs within protein synthesis dependent memory consolidation (e.g., 1–3 h after learning) (Bourtchouladze et al., 1998; Hernandez and Abel, 2008). This study tested and controlled for exposure timing dependent effects by injecting saline or low dose ethanol (0.5 g/kg; i.p.) daily either 1 h (during this window) or 4 h (outside of this window) after behavior. No differences were observed between saline mice that received injections at 1 vs. 4 h, so saline mice were collapsed across groups. Mice were exposed to ethanol starting on the first day of FR1 training through the last day of RI60/VR8 training. There was no further ethanol exposure after the last day of training, therefore there was no ethanol exposure proximal to the PR testing. PR testing took place 1–3 weeks after the final training day (i.e., after last ethanol exposure). The time between the final ethanol exposure and PR testing was determined by training and acquisition length, and there were no differences across groups and sexes.

Progressive Ratio Testing

As PR testing began 1–3 weeks after the final RI60/VR8 training session, mice were given two additional days of RI60/VR8 retraining before beginning testing on the PR. There was no additional ethanol exposure on these retraining days. Mice were tested for sucrose reward motivation on the PR test, which measures how much an animal is willing to lever press for a particular reward. The PR schedule used here was an arithmetic schedule, where the number of lever presses required for reinforcer delivery increased by 4 every time reward was delivered. An arithmetic schedule was chosen as this has been shown to be sufficient for measuring appetitive motivation in rodents previously (Gourley et al., 2008). The test session ended either after 5 min had passed without a lever press (e.g., the "breakpoint") or when the maximum session length was reached. The maximum session length was 4 h for the first cohort of mice (n = 20 males) but was changed to 8 h for all subsequent cohorts when four mice hit the maximum session length without reaching their breakpoint. Most mice reached their breakpoints within the confines of the session length, regardless of whether it was 4 or 8 h. For the RI lever, four males and one female did not reach their breakpoint; for the VR lever two males did not reach their breakpoint. Mice that did not reach their breakpoints were still included in the analysis using the maximum breakpoint reached at session termination. There were no differences between mice that had the 4 h capped sessions vs. the 8 h capped sessions. Each mouse was only tested once for each lever on each day, and the order of which lever was tested first was counterbalanced across all groups and conditions.

It is important to understand not just the whole behavioral output, but also the differences that exist as part of a "behavioral microstructure." Investigating this microstructure can reveal latent differences in behavioral strategy that are not otherwise clear using traditional measures (Robinson and McCool, 2015; Fuchs et al., 2019; Yamada and Kanemura, 2020). For example, differences may exist in how often a mouse checks the reward magazine for reward delivery, which may reflect reward tracking or attention to reinforcement schedule. Differences in these strategies between mice and between groups may reflect different mechanisms, even if the overall behavioral output or phenotype is the same. Thus, one measure of interest for this study was magazine checking after a lever press, as differences in reward delivery tracking, as measured by what percentage of lever presses were followed by a magazine entry, could relate to reward evaluation. Alternatively, magazine checking could relate to sensitivity to the PR, as a mouse that checks the magazine more often for reward may also be more sensitive to progressively increasing lever press requirements on the PR and would stop responding sooner.

Statistical Analyses

GraphPad PRISM was used for all statistical analyses. A repeated measures ANOVA (rmANOVA) or mixed effects analysis (when there were missing values) was used for all training and testing data. Sidak's, Tukey's, and Dunnett's corrections were used for *post-hoc* analyses as appropriate. Correlational analyses were performed using linear regression.

RESULTS

Effects of Low Dose Ethanol and Schedule on Sucrose Reward Seeking in Males

To determine whether low dose ethanol impacted reward seeking behavior, adult male and female mice were trained to lever press for sucrose on two levers with differing reinforcement schedules (**Figure 1A**). In males, a main effect of day was observed on the RI lever (**Figure 1B**) [rmANOVA, $F_{(2.787,89.20)} = 13.45$, $p < 0.0001$], with *post-hoc* analyses revealing a significant escalation in responding on the last day of training as compared to the

first (Sidak's, $p < 0.0001$). No main effect of ethanol exposure [$F_{(2,32)} = 0.6281$, $p = 0.5401$] nor interaction [day × exposure, $F_{(16,256)} = 0.7632$, $p = 0.7264$] was observed on the RI lever. A main effect of day was also observed on the VR lever (**Figure 1C**) [rmANOVA, $F_{(1.796,55.69)} = 13.50$, $p < 0.0001$], with *post-hoc* analyses revealing a significant escalation in responding on the last day of training as compared to the first (Sidak's, $p = 0.0020$). Similar to the RI lever, no main effect of ethanol exposure [$F_{(2,31)} = 0.6349$, $p = 0.5368$] or interaction [day × exposure, $F_{(16,248)} = 1.099$, $p = 0.3562$] was observed on the VR lever. These findings show that responding escalated on both RI and VR schedules, and that post-training, low dose ethanol exposure did not impact basal reward seeking on these schedules in males.

Differences in the number of reinforcers delivered across training were also examined in males. On the RI lever (**Figure 1D**), a main effect of day was observed [rmANOVA, $F_{(3.530,112.9)} = 47.81$, $p < 0.0001$]. *Post-hoc* analyses revealed that the number of reinforcers delivered on the last day of training was significantly reduced as compared with the first day (Sidak's, $p < 0.0001$), consistent with the leaner reinforcement schedule. A main effect of day was also observed for reinforcer delivery on the VR lever (**Figure 1E**) [rmANOVA, $F_{(4.343,134.6)} = 71.49$, $p < 0.0001$] with *post-hoc* analyses revealing that the number of reinforcers delivered on the last day of training was significantly reduced as compared with the first day (Sidak's, $p < 0.0001$).

As there were no effects of ethanol exposure on lever pressing on either lever, male mice were collapsed across exposure conditions to compare responding on the two levers directly (**Figure 1F**). A main effect of training day [Mixed effects analysis, $F_{(1.734,58.95)} = 21.71$, $p < 0.0001$] and a day × lever interaction [$F_{(2.568,84.43)} = 3.533$, $p = 0.0236$] were observed; Sidak's *post-hoc* analysis showed that lever pressing was significantly higher on the second day of FR1 training on the RI lever as compared to the VR lever in males ($p = 0.0284$). Male reinforcer delivery data were collapsed across exposure conditions to compare overall reinforcer delivery on the RI vs. VR lever (**Figure 1G**). A main effect of training day [rmANOVA, $F_{(4.518,149.1)} = 134.9$, $p < 0.0001$] and a day × lever interaction [$F_{(3.926,129.6)} = 3.180$, $p = 0.0164$] were observed; Sidak's *post-hoc* analysis showed that male mice received significantly more sucrose reinforcers on the RI lever as compared to the VR lever on the 3rd day of RI30/VR5 training (day 6 overall; $p = 0.0248$).

Effects of Low Dose Ethanol and Schedule on Sucrose Reward Seeking in Females

In females, when comparing lever presses across exposure conditions on the RI lever (**Figure 2A**) a main effect of day was observed [rmANOVA, $F_{(5.035,129.0)} = 10.42$, $p < 0.0001$], with *post-hoc* analyses revealing a significant escalation in responding on the last day of training as compared to the first day (Sidak's, $p < 0.0001$). No main effect of ethanol exposure [$F_{(2,26)} = 0.3853$, $p = 0.6841$] nor interaction [day × exposure, $F_{(16,205)} = 1.446$, $p = 0.1234$] were observed on the RI lever. On the VR lever (**Figure 2B**), a main effect of day was also observed [rmANOVA, $F_{(5.629,144.2)} = 9.456$, $p < 0.0001$], with *post-hoc* analyses revealing a significant escalation in responding on the last day of training as compared to the first (Sidak's, $p = 0.0034$). No main effect of ethanol exposure [$F_{(2,26)} = 0.4340$, $p = 0.6525$] or interaction [day × exposure, $F_{(16,205)} = 0.8284$, $p = 0.6524$] were observed on the VR lever. Similar to data from the males, these findings show a significant escalation of responding on RI and VR schedules, but no effect of post-training, low dose ethanol on basal reward seeking in females.

Reinforcer delivery across training was also analyzed in females. On the RI lever (**Figure 2C**), a main effect of day was observed [rmANOVA, $F_{(3.667,94.88)} = 46.89$, $p < 0.0001$] with *post-hoc* analyses revealing reinforcer delivery was significantly reduced on the final day of training as compared to the first day (Sidak's, $p < 0.0001$). A main effect of day was also observed on the VR lever (**Figure 2D**) [rmANOVA, $F_{(3.764,93.64)} = 122.0$, $p < 0.0001$], with *post-hoc* analyses showing that reinforcer delivery was significantly reduced on the final day of training as compared to the first day (Sidak's, $p < 0.0001$). As in males, these reductions in total reinforcer delivery are consistent with the increasingly lean reinforcement schedules.

As there were no effects of ethanol exposure on lever pressing on either lever, female mice were collapsed across exposure conditions to compare responding on the two levers directly (**Figure 2E**). A main effect of training day [Mixed effects analysis, $F_{(4.768,133.5)} = 17.90$, $p < 0.0001$] and lever [$F_{(1.000,28.00)} = 9.453$, $p = 0.0047$] were observed, with female mice pressing significantly more on the RI lever as compared to the VR lever. Female reinforcer delivery data were also collapsed across exposure conditions to compare overall reinforcer delivery on the RI vs. VR lever (**Figure 2F**). A main effect of training day [Mixed effects analysis, $F_{(5.005,135.1)} = 139.8$, $p < 0.0001$], lever [$F_{(1.000,27.00)} = 28.12$, $p < 0.0001$], and a day × lever interaction [$F_{(2.981,79.74)} = 3.332$, $p = 0.0238$] were observed. Sidak's *post-hoc* analysis showed that reinforcer delivery was significantly higher on the RI lever than the VR lever for all RI30/VR5 and RI60/VR8 training (training days 4–9; day 4 $p = 0.0004$, days 5–9 $p < 0.0001$).

Effects of Low Dose Ethanol on Sucrose Reward Motivation

To determine whether a history of low dose ethanol impacted sucrose reward motivation, male and female mice were tested on a PR schedule on the levers previously reinforced on RI or VR schedules. In males, a significant exposure condition × lever interaction was observed for the maximum ratio reached during the PR session (**Figure 3A**) [rmANOVA, $F_{(2,32)} = 3.492$, $p = 0.0425$]. *Post-hoc* analyses revealed that male mice with a history of exposure occurring proximal to learning (1 h EtOH group) reached significantly higher ratios on the RI lever vs. the VR lever (Sidak's, $p = 0.0241$). Further, 1 h EtOH male mice also reached higher ratios on the RI lever as compared to saline- (Tukey's, $p = 0.0075$) and 4 h EtOH-exposed (Tukey's, $p = 0.0298$) mice. A three-way ANOVA revealed that there were no effects of surgical history on breakpoints [surgery, $F_{(1,29)} = 0.5864$, $p = 0.4500$; ethanol × surgery × lever, $F_{(2,29)} = 1.306$, $p = 0.2863$],

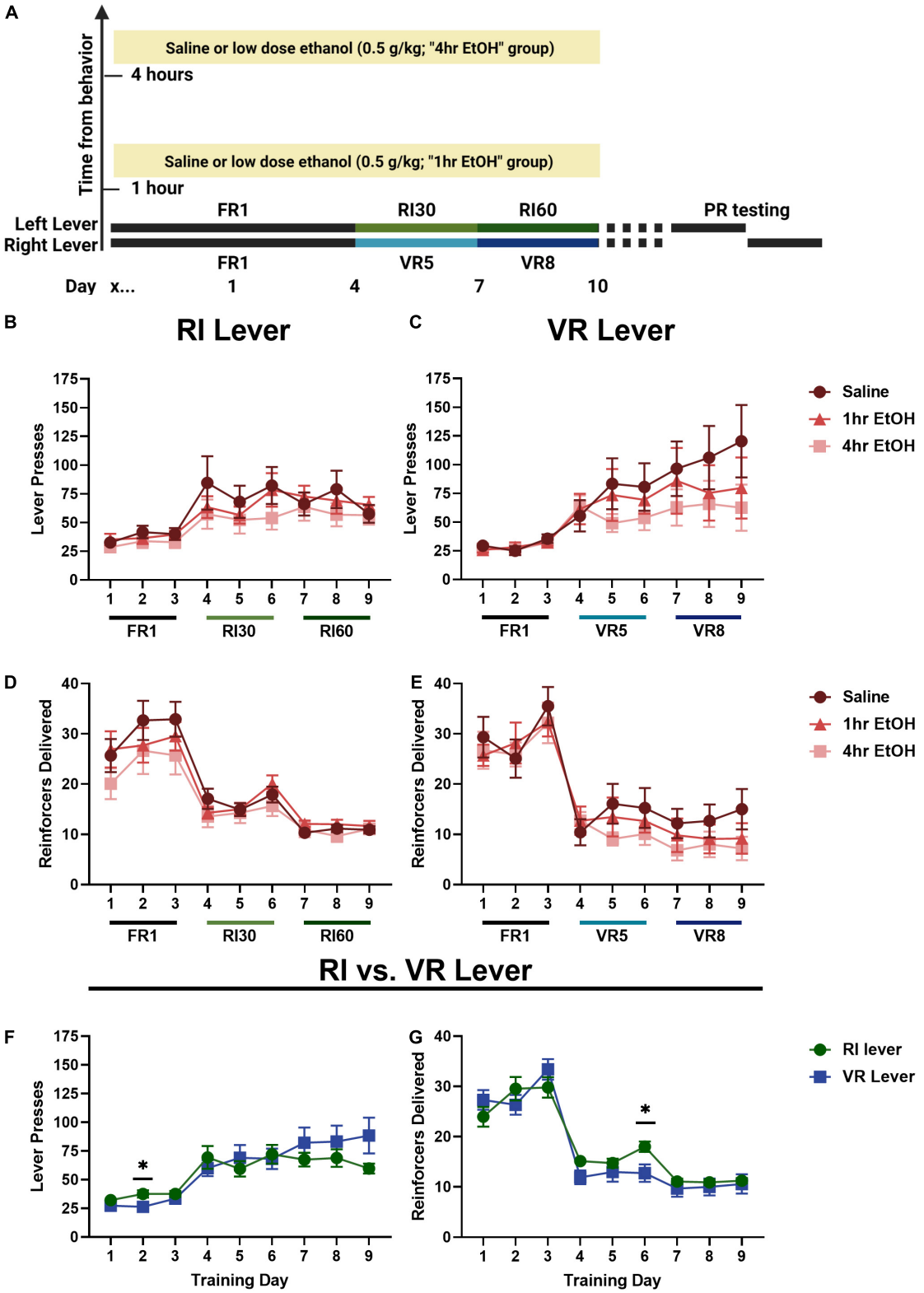

FIGURE 1 | Low dose ethanol exposure does not impact basal reward seeking in males. **(A)** A timeline of the behavioral experiments and ethanol exposure. In males, there is no effect of low dose ethanol exposure on responding during training on the RI **(B)** or VR **(C)** lever. There is also no effect of low dose ethanol exposure on reinforcer delivery during training on the RI **(D)** or VR **(E)** lever. Overall, neither lever pressing **(F)** nor reinforcer delivery **(G)** was different on the RI or VR lever for males. Data shown as mean ± SEM (*$p < 0.05$).

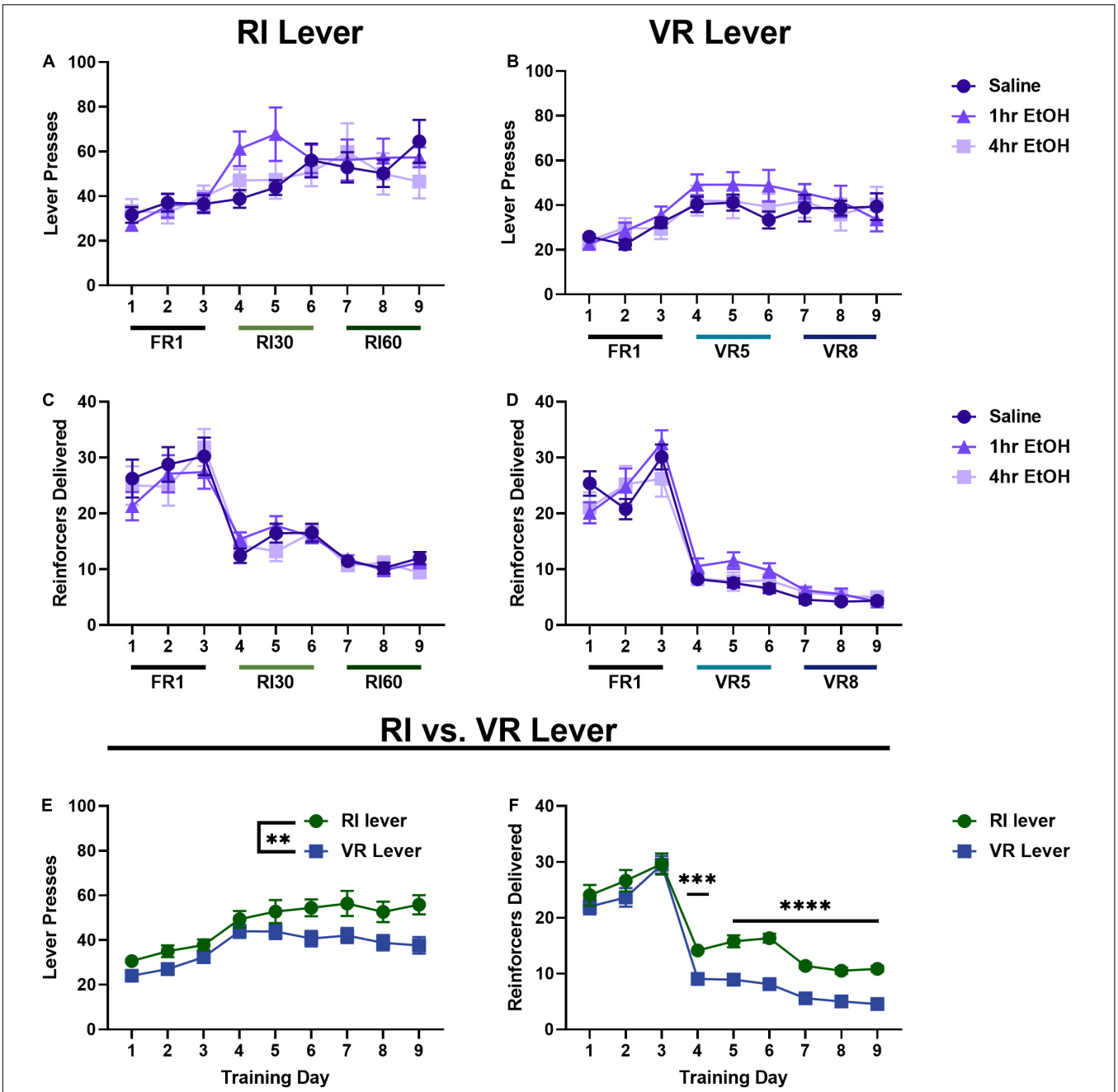

FIGURE 2 | Reinforcer schedule but not ethanol exposure impacts reward seeking in females. In females, there is also no effect of low dose ethanol exposure on responding on the RI **(A)** or VR **(B)** lever. There is also no effect of low dose ethanol exposure on reinforcer delivery on the RI **(C)** or VR **(D)** lever. Overall, females press more **(E)** and receive more reinforcers **(F)** on the RI lever as compared to the VR. Data shown as mean ± SEM (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

and thus animals were collapsed across history of surgery for further analyses. When comparing overall response rates on the PR (**Figure 3B**), a main effect of exposure condition was observed [rmANOVA, $F_{(2,32)} = 4.402$, $p = 0.0205$]. *Post-hoc* analyses indicated that 1 h EtOH male mice had significantly higher response rates as compared to saline- (Dunnett's, $p = 0.0456$) and 4 h EtOH-exposed (Tukey's, $p = 0.0248$) mice. These results suggest that the effects of low dose ethanol on reward motivation are determined by not only exposure timing, but also reinforcement schedule history in male mice.

In female mice, for the maximum ratio reached within the PR session a main effect of lever was observed (**Figure 3C**) [rmANOVA, $F_{(1,26)} = 6.264$, $p = 0.0189$] such that female mice reached significantly higher ratios on the RI lever as compared to the VR lever. There was no main effect of exposure condition [$F_{(2,26)} = 0.3414$, $p = 0.7139$] or exposure condition × lever

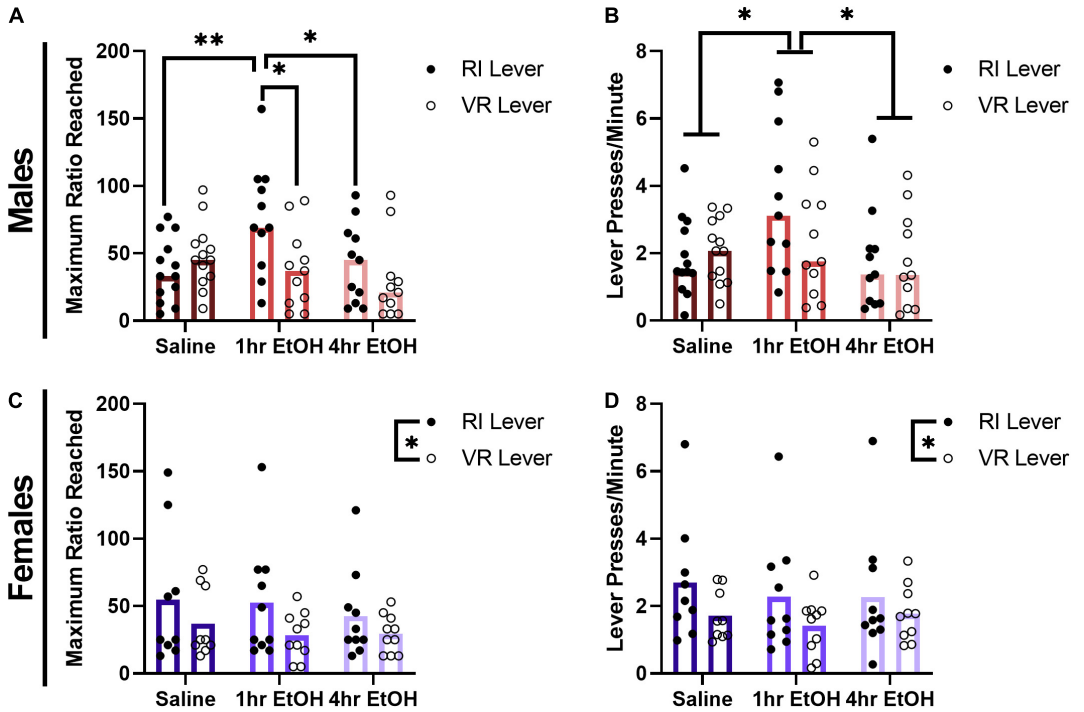

FIGURE 3 | Effect of low dose ethanol on sucrose reward motivation depends on sex and reinforcement schedule. **(A)** Male mice with a history of 1 h EtOH exposure are significantly more motivated for sucrose, but only on the RI lever. **(B)** One hour EtOH male mice also exhibit significantly higher response rates during the PR. **(C)** Female mice are more motivated for sucrose on the RI lever, regardless of exposure history. **(D)** Female mice also exhibit higher response rates on the RI lever during PR testing. Data shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$).

interaction [$F_{(2,26)} = 0.2013$, $p = 0.8189$] observed in females for the maximum ratio reached. A three-way ANOVA revealed that there were no effects of surgical history on breakpoints [surgery, $F_{(1,23)} = 3.064$, $p = 0.0934$; ethanol × surgery × lever, $F_{(2,23)} = 0.1006$, $p = 0.9047$], and thus animals were collapsed across history of surgery for further analyses. A similar pattern was seen when comparing response rates on the PR test in females (**Figure 3D**), where there was again a main effect of lever [rmANOVA, $F_{(1,26)} = 5.877$, $p = 0.0226$]. No main effect of exposure condition [$F_{(2,26)} = 0.2560$, $p = 0.7761$] nor an exposure condition × lever interaction [$F_{(2,26)} = 0.2083$, $p = 0.8133$] were observed. These findings show that female mice are more motivated for sucrose reward on the RI lever, regardless of ethanol exposure condition.

Effects of Ethanol on Progressive Ratio Microstructure

Reward magazine checking behavior was analyzed by comparing the percent of lever presses that were followed by a magazine entry for each PR session. In males (**Figure 4A**), a main effect of condition was observed [rmANOVA, $F_{(2,57)} = 3.742$, $p = 0.0297$], with *post-hoc* analyses revealing that 1 h EtOH mice check the magazine after lever pressing significantly less than saline mice (Dunnett's, $p = 0.0383$). There was no significant difference observed between 4 h EtOH and saline mice, although there was a trend toward reduced magazine checking after a lever press in the 4 h EtOH group vs. saline (Dunnett's, $p = 0.0519$). This was not matched with differences in total magazine entries on the PR, as a rmANOVA analysis showed there were no significant main effects [exposure condition, $F_{(2,32)} = 1.981$, $p = 0.1545$; lever, $F_{(1,32)} = 2.111$, $p = 0.1560$] or interactions [exposure condition × lever, $F_{(2,32)} = 2.311$, $p = 0.1154$] present. In females (**Figure 4B**), there were no significant main effects [rmANOVA, lever, $F_{(1,24)} = 3.090$, $p = 0.0915$] or interactions [lever × exposure condition, $F_{(2,24)} = 0.9416$, $p = 0.4039$] observed for magazine checking after a lever press. Similar to males, there were no differences in total magazine entries during PR testing observed in females [exposure condition, $F_{(2,26)} = 2.466$, $p = 0.1046$; lever, $F_{(1,26)} = 3.104$, $p = 0.0899$; exposure condition × lever, $F_{(2,26)} = 0.2042$, $p = 0.8166$].

To determine whether magazine checking behavior after a lever press during the PR was related to the maximum ratio reached, a linear regression and correlational analysis was performed. No differences were found based on ethanol exposure for either males or females, so data were collapsed across exposure condition for each sex. For male mice (**Figure 4C**), there was a significant negative correlation between the ratio reached and magazine checking on the RI lever [$R^2 = 0.3781$, $F_{(1,32)} = 19.46$, $p = 0.0001$] but not VR lever [$R^2 = 0.0053$, $F_{(1,27)} = 0.1432$, $p = 0.7081$]. The slopes of the regression lines for the RI and VR lever in males were significantly different [$F_{(1,59)} = 8.044$, $p = 0.0062$]. For female mice (**Figure 4D**), there was a significant negative correlation between the ratio reached

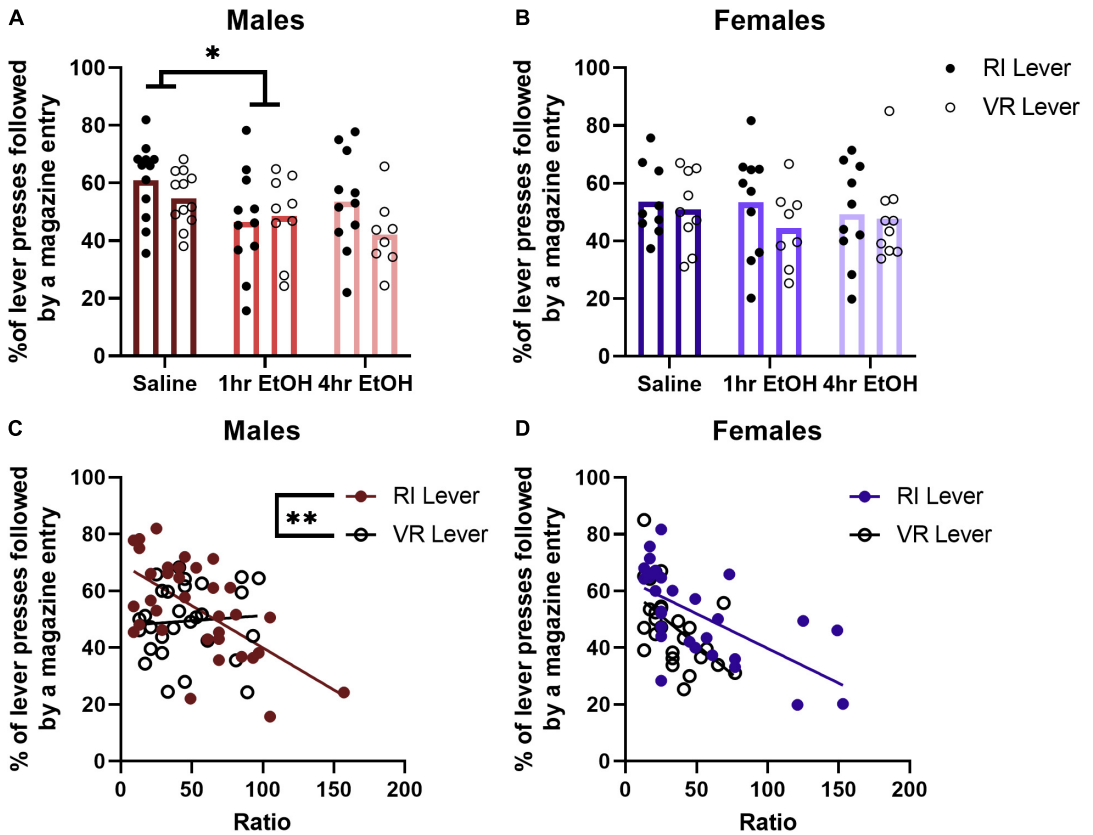

FIGURE 4 | Magazine checking behavior after a lever press is related to performance on the PR. **(A)** One hour EtOH male mice check the magazine after lever pressing significantly less than saline mice. **(B)** There are no differences in magazine checking after lever pressing in females. **(C)** Magazine checking is correlated with performance only on the RI lever in males. **(D)** Whereas in females, magazine checking is correlated with PR performance on both levers. Data shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$).

and magazine checking observed on both the RI [$R^2 = 0.3906$, $F_{(1,27)} = 17.31$, $p = 0.0003$] and VR [$R^2 = 0.2953$, $F_{(1,25)} = 10.48$, $p = 0.0034$] levers. The slopes of these regression lines were not significantly different in females [$F_{(1,52)} = 1.319$, $p = 0.2560$]. These findings suggest that magazine checking behavior after a lever press is related to reward motivation on the PR.

DISCUSSION

Our findings in males show that low dose ethanol exposure can drive sucrose reward motivation as only the males exposed to ethanol proximal to learning (1 h EtOH group), but not distal (4 h group) exhibited increased breakpoints on the PR. Moreover, the fact that this is due to a history of low dose ethanol and not acute exposure surrounding testing highlights a long-lasting outcome of low dose ethanol exposure. This increase in reward motivation in males was matched with reduced checking of the magazine after a lever press, suggesting that low dose ethanol may be shifting reward encoding and behavioral strategy in males. Furthermore, that there was a relationship between checking after a lever press with breakpoints only on the RI lever, not VR lever, suggests that males may be using strategies specific to reinforcement schedule history. In contrast to these findings, low-dose ethanol exposure did not impact sucrose reward motivation in female mice. There was an effect of reinforcement schedule history as females reached higher breakpoints on the RI lever as compared to the VR lever. However, since there was also an effect of schedule on response rate and reinforcers earned during training in females, it is difficult to separate the contributions of reinforcement schedule history alone on breakpoints in females.

Increased sucrose reward motivation was observed on the RI lever as compared to the VR lever in both males and females. RI schedules promote inflexible, habitual behavior that is insensitive to changes in the action-outcome relationship and to changes in reward value (Adams and Dickinson, 1981; Dickinson et al., 1983). It has long been theorized that habit and motivation are two separate processes (Dickinson et al., 2002; Everitt and Robbins, 2016), so the fact that there are schedule-dependent effects on reward motivation was unanticipated. The progressively increasing ratio of responding required to receive reinforcer delivery could be seen as a change in the action-outcome relationship. Insensitivity to this change as a result of overtraining on a RI schedule could then drive lever pressing on the PR. Thus, action-outcome insensitivity may be disguised as higher motivation on this test. Indeed, others have reported that

mice which were insensitive to changes in outcome value also reached higher breakpoints on the PR, further supporting this interpretation (Gourley et al., 2016).

It may alternatively be that behavioral strategy is being shifted, as our male 1 h EtOH mice check the reward magazine less than saline mice regardless of lever. Our findings show that magazine checking behavior after a lever press and motivation as measured by a PR test are clearly related, but whether magazine checking just reflects motivational state or drives it is unknown. Magazine checking may reflect tracking and expectation of reward delivery, so mice that reach higher breakpoints thus exhibit less checking behavior because of high lever press-to-reward delivery ratios. Alternatively, greater magazine checking may reflect greater tracking of the outcome, or a greater attribution of value to the outcome.

This study showed that low dose ethanol can drive sucrose reward motivation in male mice, but whether it can drive ethanol reward motivation as well has yet to be determined. Some studies have shown that the effects of chronic ethanol on reward seeking behavior are similar for non-drug rewards and drug rewards (Kampov-Polevoy and Garbutt, 1997; Krahn et al., 2006; Sjoerds et al., 2013; Giacometti et al., 2020), whereas others find that they are different (Busse et al., 2005; Tryhus et al., 2021). One potential explanation for our findings is that low dose ethanol exposure after learning is shifting the way reward is encoded, thereby enhancing reward value, and increasing motivation. If this is the case, repeated low dose ethanol exposure is likely to increase reward motivation for an ethanol reward similarly to a sucrose reward. On the other hand, low dose ethanol exposure may interfere with sucrose metabolism and enhance reward value through this mechanism. If this is the case, these observed effects of low dose ethanol on reward motivation may not transfer to an ethanol reward as readily.

While there were no differences in responding or reinforcer delivery during training on the RI vs. VR lever in males, there were in females. In recent years, a number of tasks have been developed in which responding is maintained on multiple reinforcement schedules (Gremel and Costa, 2013; Barker et al., 2017b). The current findings indicate that under conditions in which schedules have been calibrated to match response rates in males, female mice discriminate between reinforcement schedules with a preference for responding on the interval schedule over the ratio schedule. The unmatched responding and subsequent reinforcer delivery in the females during training may relate to the schedule difference observed in PR testing. However, the presence of a main effect of reinforcement schedule in the males, where response rates were matched, suggests that these differences do not relate entirely to responding during training. Females respond differently to some stimuli then males, and behavioral measures that have been extensively used in males are not always appropriate, representative, or accurate measures in females (Chen et al., 2021; Shansky and Murphy, 2021). These results suggest that females are less inclined to respond on ratio schedules than interval schedules under conditions that are matched for males, and it will be important to consider this difference when designing experiments and matching schedules of reinforcement in the future. Additionally, it is possible that learning on two levers would yield different outcomes as compared to one lever or schedule, but our data and others do not suggest generalization of response strategies (Gremel and Costa, 2013; Barker et al., 2017b).

There is a paucity of research investigating the long-term impacts of low dose ethanol, especially in the context of reward, and this is particularly pronounced in female subjects. Women display shorter reaction times and greater cognitive performance following low to moderate alcohol consumption than men (Taberner, 1980; Dufouil et al., 1997), and have worse health outcomes at lower doses of ethanol than men (Foster et al., 2018). Thus, low dose ethanol appears to affect females differently than males, but the mechanisms underlying this are unknown. Physiologically, it has been shown that female rats exhibit greater accumbal dopamine levels following low dose ethanol exposure than males (Blanchard and Glick, 2002). So, it is possible that the dose used in these studies (0.5 g/kg) produces different physiological and behavioral effects in female mice than in males, and therefore does not increase sucrose reward motivation as observed in males.

This study was focused on determining the effects of low dose ethanol exposure and reinforcement schedule history on reward seeking in both males and females, but did not directly compare basal differences in task acquisition or progressive ratio responding in these groups (Garcia-Sifuentes and Maney, 2021). Future studies should be designed to investigate and compare different ethanol doses in males and females directly. There have been sex differences observed on the effects of ethanol on locomotor response in rodents, but this appears to depend heavily on the strain and species (Frye and Breese, 1981; Erickson and Kochhar, 1985; Middaugh et al., 1992). Sex differences have also been observed in sucrose reward seeking with higher doses of ethanol (Barker et al., 2017a), so it is possible that the ability of ethanol to modulate appetitive behavior is different between males and females across ranges of ethanol doses.

Multiple brain regions known to be important for the encoding and updating of reward value information are impacted by chronic ethanol (Lescaudron and Verna, 1985; DePoy et al., 2013; Barker et al., 2015; Trantham-Davidson et al., 2017; Ewin et al., 2019), and may be targets for the low dose ethanol effects observed here. In particular, the infralimbic prefrontal cortex, nucleus accumbens shell, and dentate gyrus have been shown to be activated as a result of low dose ethanol using c-Fos and Arc as markers of neuronal activity in rats, and this was not impacted by sex (Randall et al., 2020). Additionally, it has been shown that brain-derived neurotrophic factor (BDNF) is increased in the hippocampus following low to moderate alcohol consumption (Tizabi et al., 2018).

The 1–3 h window after learning encompasses protein synthesis dependent memory consolidation (Bourtchouladze et al., 1998) and is the primary time point where differences are observed in male mice here. Ethanol exposure during this critical period could impact normal protein synthesis associated with consolidation and may thus be enhancing reward motivation by shifting the way reward learning is encoded during training. This is further supported by the absence of an effect of ethanol administration 4 h after

training on motivation, whereas ethanol exposure 1 h after training, during the period where protein synthesis-dependent consolidation/reconsolidation takes place, increased reward motivation in male mice. As the hippocampus is a region critical for memory consolidation and reconsolidation (Fanselow and Dong, 2010), changes in the activity of or protein synthesis in this region as a result of repeated low dose ethanol exposure could also be related to these observed differences, particularly in males.

This study demonstrated that repeated low dose ethanol exposure can enhance appetitive reward motivation, and that these effects were long lasting. These experiments advance our understanding of low dose ethanol exposure impact on maladaptive behavioral patterns that may contribute to aberrant drug and reward seeking, and further how sex may mediate this susceptibility.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Drexel University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KB designed the experiments together with JB. KB performed the experiments, analyzed the data, and wrote the manuscript with edits and input from JB. BS analyzed data for the experiments. All authors contributed to the article and approved the submitted version.

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Adaptation of the 5-choice serial reaction time task to measure engagement and motivation for alcohol in mice

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Alcohol use disorder (AUD) is related to excessive binge alcohol consumption, and there is considerable interest in associated factors that promote intake. AUD has many behavioral facets that enhance inflexibility toward alcohol consumption, including impulsivity, motivation, and attention. Thus, it is important to understand how these factors might promote responding for alcohol and can change after protracted alcohol intake. Previous studies have explored such behavioral factors using responding for sugar in the 5-Choice Serial Reaction Time Task (5-CSRTT), which allows careful separation of impulsivity, attention, and motivation. Importantly, our studies uniquely focus on using alcohol as the reward throughout training and testing sessions, which is critical for beginning to answer central questions relating to behavioral engagement for alcohol. Alcohol preference and consumption in male C57BL/6 mice were determined from the first 9 sessions of 2-h alcohol drinking which were interspersed among 5-CSRTT training. Interestingly, alcohol preference but not consumption level significantly predicted 5-CSRTT responding for alcohol. In contrast, responding for strawberry milk was not related to alcohol preference. Moreover, high-preference (HP) mice made more correct alcohol-directed responses than low-preference (LP) during the first half of each session and had more longer reward latencies in the second half, with no differences when performing for strawberry milk, suggesting that HP motivation for alcohol may reflect "front-loading." Mice were then exposed to an Intermittent Access to alcohol paradigm and retested in 5-CSRTT. While both HP and LP mice increased 5-CSRTT responding for alcohol, but not strawberry milk, LP performance rose to HP levels, with a greater change in correct and premature responding in LP versus HP. Overall, this study provides three significant findings: (1) alcohol was a suitable reward in the 5-CSRTT, allowing dissection of impulsivity, attention, and

motivation in relation to alcohol drinking, (2) alcohol preference was a more sensitive indicator of mouse 5-CSRTT performance than consumption, and (3) intermittent alcohol drinking promoted behavioral engagement with alcohol, especially for individuals with less initial engagement.

KEYWORDS

alcohol, 5-choice serial reaction time task, alcohol preference, intermittent alcohol access, behavioral engagement

Introduction

Excessive alcohol consumption is a prevalent activity that may progress to Alcohol Use Disorder (AUD), and ∼3/4th the ∼$250 billion/year cost of drinking in the US comes from the ∼1/7th of adults who binge (CDC, 2014). Excessive intake can contribute strongly to the substantial harms of alcohol, including enhanced risk of drinking problems (Esser et al., 2014; Grant et al., 2015; Gowin et al., 2017), while reducing excess intake lowers health risks and relapse (Dawson et al., 2005; Moos and Moos, 2006; Rehm et al., 2009). Higher risk for binge drinking has been linked to high trait impulsivity (Poulos et al., 1995; Crews and Boettiger, 2009; Schumann et al., 2011), and non-dependent drinkers with higher self-reported impulsive behavior achieve higher blood alcohol levels during free-access self-administration, and experience greater euphoria from alcohol (Vaughan et al., 2019). Impulsivity is complex construct (Meda et al., 2009; MacKillop et al., 2016; Strickland and Johnson, 2021), with variants related to motor (impulsive action) and cognitive (impulsive choice) functions (see section "Discussion"), and is considered an important risk factor for AUD. As this disorder develops, it is accompanied by significant changes in cognitive behavioral control (Tapert et al., 2004; Wilcox C. E. et al., 2014). The desire for intoxication and the increased tolerance of adverse consequences are examples of motivational changes in people with AUD (Larimer et al., 1999; Lau-Barraco et al., 2017; Radke et al., 2021). Further, an "attentional bias" will typically develop that promotes behavior toward alcohol cues over natural rewards (Fadardi and Cox, 2006; Monem and Fillmore, 2019; Heitmann et al., 2020). Together, impulsivity, motivation, and attention are key aspects of behavioral engagement with alcohol that we seek to investigate, especially changes in such responding after chronic alcohol use.

The 5-choice serial reaction time task (5-CSRTT) is a multifaceted behavioral paradigm that has been thoroughly characterized in rodents to elucidate impulsive, attentional, motivational, and perseverative behavior in the same session (Robbins, 2002; Bari et al., 2008; Semenova, 2012). Thus, the 5-CSRTT is valuable for assessing a broad range of measures of behavioral performance, when compared to many other tasks. Interestingly, a 5-CSRTT version adapted for humans predicts higher alcohol intake in more impulsive individuals, suggesting high translational value (Sanchez-Roige et al., 2014a). While determining clear correlations between behavioral factors in human studies remains challenging, rodent studies give the ability to dissect important contributors to behavioral engagement for alcohol. However, to date, studies examining the relation of alcohol and impulsivity have primarily determined 5-CSRTT responding for sugar in relation to alcohol exposure (Semenova, 2012; Sanchez-Roige et al., 2014a,b; Irimia et al., 2015; Pena-Oliver et al., 2015; Starski et al., 2019, 2020).

Here, we have uniquely adopted the 5-CSRTT to have alcohol as the reward, allowing us greater precision in identifying the nature of behavioral engagement, with the goal of understanding how impulsivity, motivation, and attention for alcohol might relate to preference or consumption. Interestingly, we found that 5-CSRTT performance was significantly related to alcohol preference rather than consumption level. Thus, it is interesting that, in addition to high trait impulsivity, people at risk for binging have higher alcohol preference (Poulos et al., 1995; Crews and Boettiger, 2009; Schumann et al., 2011), and impulsivity can be linked to preferences in rodents (Oberlin and Grahame, 2009; Dick et al., 2010; Lejuez et al., 2010; Adams et al., 2013; Elder et al., 2019; Herman and Duka, 2019) (see section "Discussion"). In addition, after intermittent alcohol consumption, mice overall increased their performance, but this was especially pronounced in initially low-responding mice, suggesting that protracted drinking may be particularly hazardous for individuals with lower initial drive for alcohol. Finally, we also performed several days of 5-CSRTT responding for strawberry milk, with our previously used methods (Starski et al., 2019, 2020). Sweet milk responding was higher than alcohol and with greater accuracy, did not relate to alcohol preference, and had minimal changes with intermittent drinking, suggesting important specificity in the alcohol-engagement relationship. We provide herein a robust and valuable model to help understand inter-relationships between different aspects of engagement for alcohol, and how they could be altered by intermittent drinking, which together promote excessive intake.

Materials and methods

Animals

Forty-eight male C57BL/6J mice from Jackson Laboratories Inc., were individually housed, starting at 8 weeks old, in standard Plexiglass cages with *ad libitum* access to food and water until water restriction. Mice were maintained in a 12 h:12 h reverse light-dark cycle. Animal care and handling procedures were approved by the Indiana University Institutional Animal Care and Use Committee in accordance with NIH guidelines.

5-Choice serial reaction time task

(A) A detailed description of early-stage, late-stage, and strawberry milk (SM) training can be found in **Supplementary methods**. All mice were trained and tested under a 10 s stimulus duration (SD) and 5 s intertrial interval (ITI): this was done to reduce challenge within the task, since this is, to our knowledge, the first investigation using an intoxicant (10% alcohol) as the reward in 5-CSRTT. **Figure 1A** shows the overall timeline of studies, and **Figure 1B** and **Supplementary Figure 1** give visual representations of several typical session events.

(B) 5-Choice serial reaction time task motivators: For alcohol sessions, mice were water restricted because we wanted to heighten their response levels, and, importantly, in the oral modality that is used for alcohol; in other words, water restriction was more ethological as comparison for alcohol versus food restriction. For SM sessions, SM is much more of a nutrient, while alcohol has calories it is consumed more as an intoxicant. For further information see **Supplementary discussion**.

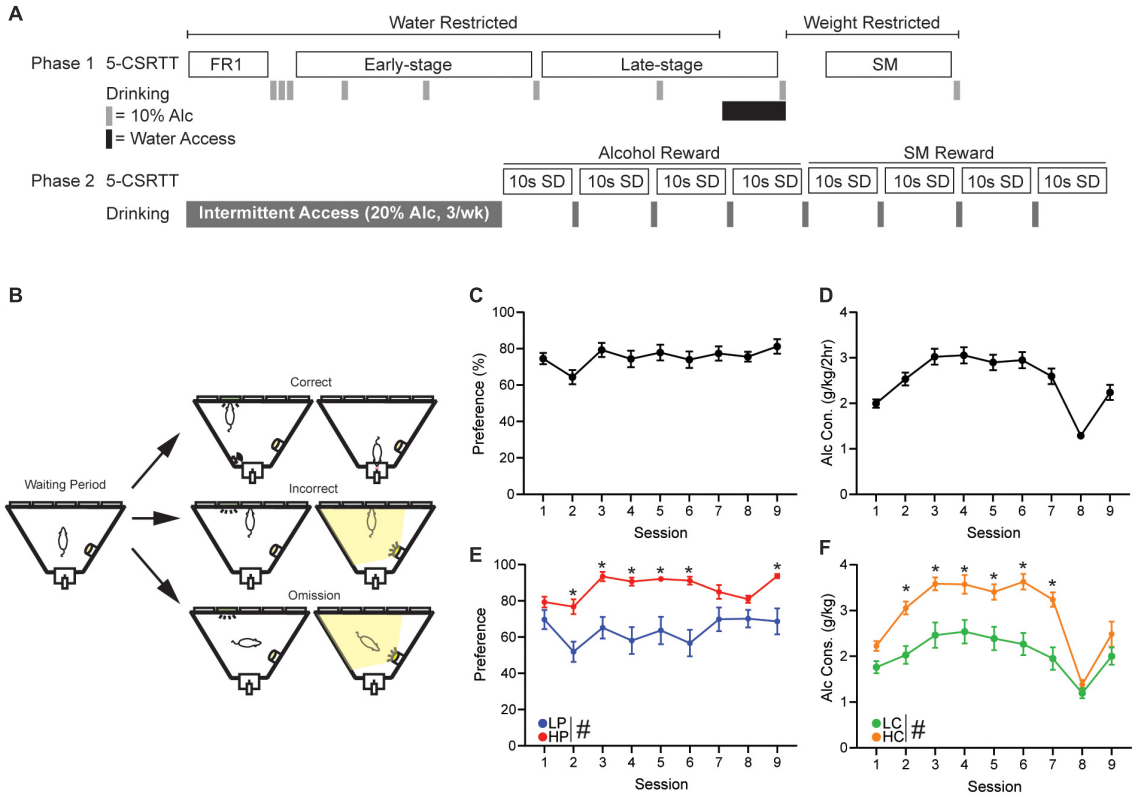

FIGURE 1
5-Choice Serial Reaction Time Task training schedule and alcohol drinking. **(A)** Schedule of 5-CSRTT and drinking behavior. **(B)** Graphic detailing a correct, incorrect, and omission within the 5-CSRTT. Briefly, a head entrance into an illuminated reward tray initiates the waiting period. When the waiting period ends, a light will appear in 1 of 5 ports. A touch in the illuminated port is a correct response and will result in reward delivery. A touch in an unlit port is an incorrect response and will cause a punishing flash of light and no reward. If the lit port extinguishes and the limited hold period elapses, this will result in an omission causing a punishing flash and no reward. Overall **(C)** alcohol preference and **(D)** alcohol consumption of all mice, across the 9 days of 2-h DID two-bottle choice drinking (gray bars). **(E)** Alcohol preference when mice were separated by median split of preference (HPvLP: $F_{1,46} = 82.06$, $p < 0.0001$). *Post hoc* revealed significance on sessions 2–6 and 9. **(F)** Alcohol consumption when mice were separated by median split of consumption ($F_{1,46} = 81.31$, $p < 0.0001$; time: $F_{6{\cdot}11,279{\cdot}4} = 18.30$, $p < 0.0001$; interaction: $F_{8,366} = 2.22$, $p = 0.0253$). *Post hoc* revealed significance on sessions 2–7. Session 8 reflects the time in which the mice were given 1 week of water access before being water restricted again. $^{\#}p < 0.05$ for group effect. $^{*}p < 0.05$ *post hoc* significance. All data are expressed as ± standard error mean.

(C) Post-Intermittent Access Testing (**Figure 1A**, Phase 2). For these studies, mice drank IA2BC interspersed with 5-CSRTT testing (see **Supplementary Figure 9**). Mice were given four sessions for alcohol reward and 4 for a SM reward using a 10 s SD and 5 s ITI duration. Mice continued intermittent access alcohol intake across the testing weeks, resulting in approximately 6 weeks of total intermittent access, and were not weight restricted or continuously water restricted. Importantly, 5-CSRTT testing occurs after a IA2BC session. Briefly, a testing day begins with removal of the 24 h 20% alcohol bottle and no water access until, 5–9 h later, we begin behavioral testing; this timing ensures mice were performing during acute withdrawal (Hwa et al., 2011; Metten et al., 2018). Water bottles were given immediately after behavioral testing was completed for the day and remained until the next IA2BC session (**Supplementary Figure 9**). For analysis we excluded the first day to remove potential burst in behavior from reintroducing the mice to the task.

Drinking in the dark and intermittent access

(A) Throughout 5-CSRTT training, mice were given weekend drinking in the dark (DID) session of 10% alcohol for 2 h to promote response to the reward (**Figure 1A**, Phase 1). Custom-built, low drip sipper tubes were used to reduce dripping from overactive mice that may climb on the cage. These tubes consisted of a Falcon 15 mL conical tube (Fisher Scientific, Hampton, NH, USA) with the bottom cone cut and filed down. A sipper (Ancare Corp., Bellmore, NY, USA) was then placed inside the tube and shrink-wrapped using a heat gun. A rubber stopper (size:0#, StonyLab, Nesconset, NY, USA) was used to plug the opposite end. Bottles were weighed before and after sessions and consumption was calculated using the weekly weight of the mouse. Preference was calculated as the total amount of alcohol consumed divided by total liquid consumed.

(B) Similar to Lei et al. (2019), mice were given 24 h access to 20% alcohol every Sunday, Tuesday, and Thursday starting at 7:00 a.m. (**Figure 1A**, Phase 2). Consumption was calculated using the weekly weight of the mouse. During non-alcohol days, two-bottles filled with water were present to maintain familiarity with the bottles.

Statistical analysis

The 48 mice were categorized as High/Low preference or consumption after the Late-Stage sessions and once the DID behavior was completed. Specifically, alcohol consumption and preference were calculated for each animal based on the overall average of the nine DID sessions. For classifying preference groups, mice were ordered from greatest preference to least preference and a median split was used to divide the 48 mice into two equal-sized groups. For classifying consumption groups, the same mice were instead ordered from greatest consumption to least consumption and a median split was used to divide mice into two equal-sized groups. The same sets of analyses were conducted for preference and consumption. 5-CSRTT and alcohol consumption studies were analyzed by two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test where appropriate. Sphericity was not assumed, and the Geisser–Greenhouse correction was used. For missing data points (spill during drinking, or animal non-responding), a mixed-model analysis of variance was used. All group analyses of pre-IA2BC versus post-IA2BC and performance changes were tested for normal distribution (Shapiro–Wilk test), and then an appropriate test (parametric or non-parametric) was used to measure differences (*t*-test, Mann–Whitney, Paired *t*-test, Wilcoxon). Non-parametric data is reported simply in the main text, with specific values in the **Supplementary material**. All statistical analyses were calculated using Prism 9.0 software (Graphpad Software Inc., San Diego, CA, USA), with significance set at $p < 0.05$.

Results

Mice with high alcohol preference or consumption show higher engagement in early-stage training

To better understand the relationship between alcohol drinking behavior and 5-CSRTT performance, drinking preference and consumption was calculated from the first nine 2 h-DID two-bottle-choice sessions (**Figures 1C,D**). A median split was then performed to compare response patterns in High alcohol Preference (HP, $n = 24$) versus Low Preference (LP) mice ($n = 24$, **Figure 1E**), and a separate median split was performed to compare high consumption (HC, $n = 24$) versus low consumption (LC, $n = 24$) mice (**Figure 1F**). For all subsequent analyses, performance measures were analyzed separately by preference and by consumption.

Each 5-CSRTT session involved unlimited trials in an hour period, where a nosepoke to an illuminated stimulus in one of five ports led to rear reward delivery (detailed further in **Figure 1B** legend). During the first 10 days of training ("early-stage"), HP mice performed significantly more trials than LP mice (**Figure 2A1**, HPvLP: $F_{1,46} = 11.36$, $p = 0.0015$; time: $F_{1.93,88.74} = 9.59$, $p = 0.0002$), with higher accuracy (**Figure 2A2**, HPvLP: $F_{1,46} = 4.84$, $p = 0.0329$; time: $F_{4.81,221.2} = 6.57$, $p < 0.0001$; interaction: $F_{9,414} = 1.96$, $p = 0.0421$) and significantly more correct responses compared to LP mice (**Figure 2A3**, HPvLP: $F_{1,46} = 11.16$, $p = 0.0017$, time: $F_{1.76,80.8} = 8.5$, $p = 0.0008$; interaction: $F_{9,414} = 4.57$,

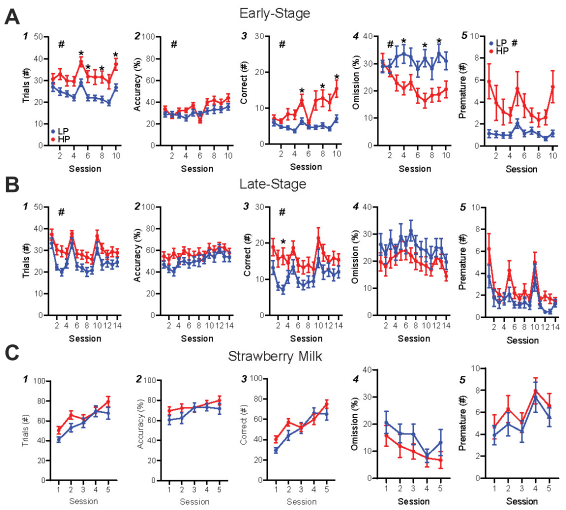

FIGURE 2
HP versus LP early-stage and late-stage alcohol, and SM training in the 5-CSRTT. **(A)** Early-stage performance showed differences in trials, accuracy, correct (*post hoc* significance on session 3), % omission, and premature between HP and LP mice. **(B)** Late-stage performance displayed HP-LP differences in trials and correct, but not accuracy, % omission or premature. **(C)** SM performance showed no differences in any metric. n = 24/group for preference. $^{\#}p < 0.05$ for group effect. $^{*}p < 0.05$ *post hoc* significance. All data are expressed as ± standard error mean.

$p < 0.0001$). In addition, the percent of trials that were omissions in early-stage was lower in HP mice (Figure 2A4, HPvLP: $F_{1,46} = 11.4$, $p = 0.0015$; interaction $F_{9,414} = 2.48$, $p = 0.0091$). However, raw premature responses were significantly higher in HP mice (Figure 2A5, HPvLP: $F_{1,46} = 6.96$, $p = 0.0113$; time: $F_{1.39,64.07} = 4.13$, $p = 0.0334$; interaction: $F_{9,414} = 2.48$, $p = 0.0091$), as were the percentage of premature responses (Supplementary Figure 3G, HPvLP: $F_{1,46} = 7.04$, $p = 0.0109$; time: $F_{2.71,124.6} = 2.93$, $p = 0.0411$; interaction: $F_{9,414} = 3.27$, $p = 0.0007$). Together, these suggest that HP have greater engagement than LP mice early in training, with greater number of trials, correct, and accuracy, and also fewer omissions and greater premature responding (which could reflect greater impulsivity, or simply greater engagement with the task).

Similar to our HP versus LP comparison, we found that mice with HC overall performed more in early training trials than LC mice (Supplementary Figure 2A1, HCvLC: $F_{1,46} = 4.12$, $p = 0.0482$; time: $F_{1.81,83.1} = 9.56$, $p = 0.0003$) and had more correct responses than LC mice (Supplementary Figure 2A3, HCvLC: $F_{1,46} = 4.57$, $p = 0.0380$; time: $F_{1.67,76.91} = 8.11$, $p = 0.0013$; interaction: $F_{9,414} = 2.25$, $p = 0.0185$), although with no *post hoc* differences in any session. In addition, and unlike HP versus LP, HC, and LC mice were not different in accuracy (Supplementary Figure 2A2, HCvLC: $F_{1,46} = 1.34$, $p = 0.2528$; time: $F_{4.55,209.3} = 6.47$, $p < 0.0001$) or percentage of omissions (Supplementary Figure 2A4, HCvLC: $F_{1,46} = 3.44$, $p = 0.0699$). Together, these suggest that differences in performance early in training were more related to alcohol preference differences rather than consumption.

Preference predicts performance, while alcohol consumption does not, during late-stage training

In late-stage training, we continued with a 10 s SD and 5 s ITI, since we wanted to explore potential behavioral differences under simpler task requirements in this first-time assessment of 5-CSRTT with alcohol as the reward. HP and LP mice continued to show significant performance differences in late-stage training, while HC and LC mice did not. HP mice performed more trials (Figure 2B1, HPvLP: $F_{1,46} = 5.93$, $p = 0.0188$; time: $F_{7.74,355.9} = 18.91$, $p < 0.0001$) and correct responses (Figure 2B3, HPvLP: $F_{1,46} = 4.84$, $p = 0.0329$; time: $F_{7.2,331.4} = 9.11$, $p < 0.0001$) than LP mice. However, by late-stage training, there were no differences between preference groups for accuracy (Figure 2B2, HPvLP: $F_{1,46} = 1.92$, $p = 0.1730$; time: $F_{7.52,345.8} = 5.37$, $p < 0.0001$), omissions (Figure 2B4, HPvLP: $F_{1,46} = 1.88$, $p = 0.1772$; time: $F_{6.81,313.1} = 3.01$, $p = 0.0049$), raw premature responses (Figure 2B5, HPvLP: $F_{1,46} = 3.801$, $p = 0.0573$; time: $F_{4.90,225.4} = 10.48$, $p < 0.0001$), or percentage of premature responses (Supplementary Figure 3H, HPvLP: $F_{1,46} = 3.96$, $p = 0.0525$; time: $F_{8.71,400.5} = 5.43$, $p < 0.0001$). In contrast, HC and LC mice did not show differences in trials performed (Supplementary Figure 2B1, HCvLC: $F_{1,46} = 1.71$, $p = 0.1979$; time: $F_{7.8,359} = 19.10$, $p < 0.0001$), accuracy (Supplementary Figure 2B2, HCvLC: $F_{1,46} = 0.119$, $p = 0.7319$; time: $F_{7.56,347.8} = 5.34$, $p < 0.0001$), number of correct responses (Supplementary Figure 2B3,

HCvLC: $F_{1,46}$ = 0.717, p = 0.4016; time: $F_{7.45,342.9}$ = 9.2, $p < 0.0001$; interaction: $F_{13,598}$ = 1.89, p = 0.0286), or omissions (**Supplementary Figure 2B4**, HCvLC: $F_{1,46}$ = 0.139, p = 0.7115; time: $F_{6.74,309.9}$ = 3.03, p = 0.0048). Together, our data suggest, perhaps surprisingly, that preference is a better indicator of established (later-stage) performance under a more basic version of 5-CSRTT than consumption, since HP had more trials and correct responses than LP, while HC and LC were not different.

Estimated intake during sessions was calculated by the 200 μl initial "free reward" and each subsequent correct response that delivers 20 μl. When calculating average intake by preference, there was a trend that HP mice may have higher intake as they get more correct responses compared with LP mice (**Supplementary Figure 4N**, HPvLP: $F_{1,46}$ = 2.842, p = 0.0986; time: $F_{2.644,121.6}$ = 7.669, p = 0.0002). When analyzed by consumption, there were no differences in intake levels (**Supplementary Figure 4O**, HCvLC: $F_{1,46}$ = 0.1393, p = 0.7107; time: $F_{2.656,122.2}$ = 8.002, p = 0.0001).

Unlike alcohol, performance for strawberry milk reward is not related to alcohol preference

After late-stage alcohol testing, mice were switched for five sessions to a strawberry milk (SM) reward in the 5-CSRTT to determine whether preference-related performance for alcohol (**Figures 2A,B**) might be related to more basic differences in motivation for reward learning. Overall, mice had more than twice the number of responses for SM relative to alcohol [**Supplementary Figure 4M**, paired t-test, $t(94)$ = 10.38, $p < 0.0001$]. However, there were no differences in any response measure between HP and LP mice, including in number of trials (**Figure 2C1**, HPvLP: $F_{1,46}$ = 1.571, p = 0.2163; time; $F_{2.76,127.0}$ = 42.46, $p < 0.0001$; interaction: $F_{4,184}$ = 3.017, p = 0.0193), accuracy (**Figure 2C2**, HPvLP: $F_{1,46}$ = 1.278, p = 0.2639; time: $F_{2.81,129.3}$ = 7.62, p = 0.0001), correct responses (**Figure 2C3**, HPvLP: $F_{1,46}$ = 1.242, p = 0.2709; time $F_{2.73,125.5}$ = 43.63, $p < 0.0001$), omissions (**Figure 2C4**, HPvLP: $F_{1,46}$ = 1.119, p = 0.2956; time: $F_{2.67,122.9}$ = 7.03, p = 0.0013), raw premature responses (**Figure 2C5**, HPvLP: $F_{1,46}$ = 0.7796, p = 0.3818; time: $F_{3.41,157.0}$ = 4.471, p = 0.0032), or percentage of premature responses (**Supplementary Figure 3I**, HPvLP: $F_{1,46}$ = 0.6390, p = 0.4282). Responding for SM was also unrelated to higher versus lower consumption (**Supplementary Figures 2**, **3**). Importantly, these findings suggest that HP and LP mice had similar ability to learn and perform for a high-value reward, and thus that reduced alcohol responses in LP mice did not reflect differences in basic reward behavior, but, instead, a difference in engagement in responding for alcohol.

Reward latency is increased during alcohol sessions, but not in strawberry milk, due to occasional longer reward latency trials

Reward latency, the time from giving a correct response to entering the reward tray, is a critical metric thought to identify motivation for the reward, with faster latency taken to indicate higher drive (Asinof and Paine, 2014). However, our initial analyses found that reward latency was not different between HP and LP mice during early-stage (**Figure 3A**, HPvLP: $F_{1,46}$ = 0.9441, p = 0.3363) or late-stage (**Figure 3B**, HPvLP: $F_{1,46}$ = 12.00, p = 0.2790) sessions for alcohol, or during strawberry milk sessions (**Figure 3C**, HPvLP: $F_{1,46}$ = 0.0173, p = 0.8959). Furthermore, when averaging the reward latency of the final five ethanol sessions against the five SM sessions, reward latencies were significantly slower for alcohol compared with strawberry milk: HP mice had longer latencies for alcohol compared to HP responding for strawberry milk [HP-SM, **Figure 3D**, paired t-test, $t(46)$ = 3.706, p = 0.0006]. Similarly, LP mice had longer latencies for alcohol compared to LP-SM [**Figure 3D**, paired t-test, $t(45)$ = 3.719, p = 0.0006]. Also, HP and LP mice had similar reward latencies during SM testing [**Figure 3D**, student's t-test, $t(46)$ = 0.3285, p = 0.7440].

To better understand potential differences in reward latency, we examined the distribution of such latencies. Indeed, we found that alcohol reward latencies could be separated into several time intervals, those more similar to SM, and others that were much longer. Specifically, when comparing average latencies under 2.5 s, latency length was similar and quick when responding for alcohol or SM [**Figure 3E**, paired t-test, $t(90)$ = 0.6917, p = 0.4909]. In contrast, the average length of reward latency above 2.5 s was significantly greater during alcohol testing [**Figure 3F**, paired t-test, $t(90)$ = 2.666, p = 0.0091]. There were also significantly more longer-latency (> 2.5 s) responses during alcohol versus SM sessions [**Figure 3G**, paired t-test, $t(94)$ = 3.794, p = 0.0003]. To better understand these longer latencies across a session, we identified whether they occurred in the first half or second half of a session (relative to number of completed trials). HP mice had significantly more longer latencies during the second half of the session for alcohol (**Figure 3H**, 1st-v-2nd-half: $F_{1,38}$ = 6.318, p = 0.0163), while LP mice did not (**Figure 3I**, 1st-v-2nd-half: $F_{1,32}$ = 0.1731, p = 0.6801). In addition, longer-latency responses for SM were fewer than for alcohol for HP and were equally distributed throughout the session in HP mice (**Figure 3J**, 1st-v-2nd-half: $F_{1,38}$ = 1.332, p = 0.2556) and LP mice (**Figure 3K**, 1st-v-2nd-half: $F_{1,32}$ = 0.243, p = 0.089). Cumulatively, these data suggest that HP mice exhibited a decrease in motivation for alcohol in the second half of the session, perhaps where mice getting more alcohol within the 5-CSRTT task participate less later in the session (addressed further in section "Responses

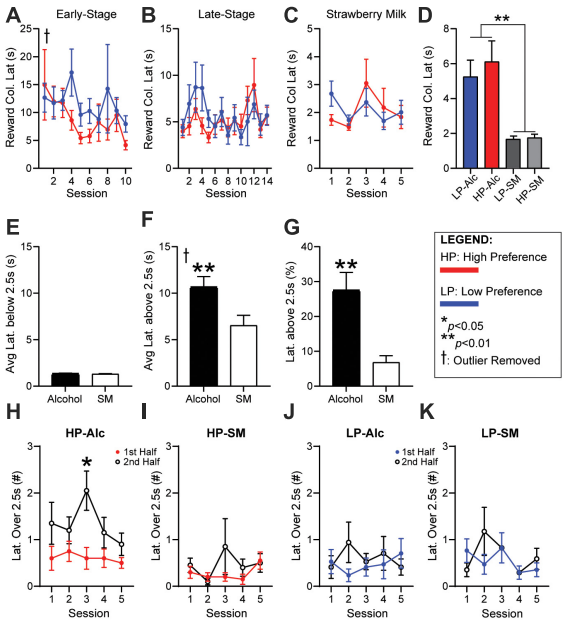

FIGURE 3
Reward latencies are longer during the second half of HP sessions for alcohol, not SM. No differences in **(A)** early-stage, **(B)** late-stage, or **(C)** SM reward latency. **(D)** Average reward latency was greater for alcohol (HP/LP) compared to SM sessions (HP-SM/LP-SM). **(E)** Average latencies below 2.5 s were similar for alcohol and SM. **(F)** Average latency above 2.5 s were significantly greater during alcohol sessions. **(G)** The percentage of latencies greater than 2.5 s were much higher during alcohol sessions. HP reward latencies over 2.5 s occurred significantly more during the second half of **(H)** alcohol, but not **(I)** SM sessions. LP reward latencies were similar between the first and second half during **(J)** alcohol and **(K)** SM sessions. The number of trials per session half is 14 for HP-Alc, 35 for HP-SM, 13 for LP-Alc, and 32 for LP-SM. n = 24 for panel **(A–D)**, n = 48 for panel **(E–G)**, n = 17–21/group for panel **(H–K)**. *$p < 0.05$, **$p < 0.01$ for group effect. All data are expressed as ± standard error mean. †removed one outlier (over 200 s).

for alcohol, not strawberry milk, in high-preference mice occur more within the first half of a session" and **Supplementary discussion**).

When reward latencies for alcohol were examined by consumption, HC and LC mice were not different during early-stage (**Supplementary Figure 2A5**, HCvLC: $F_{1,46}$ = 0.0213, p = 0.8846), late-stage (**Supplementary Figure 2B5**, HCvLC: $F_{1,46}$ = 0.1225, p = 0.7280; time: $F_{6.30,289.3}$ = 2.159, p = 0.0441), or during strawberry milk sessions (**Supplementary Figure 2C5**, HCvLC: $F_{1,46}$ = 0.1658, p = 0.6858). Since sorting the mice by preference proved to be more sensitive toward overall performance, consumption analysis was largely discontinued at this point.

Responses for alcohol, not strawberry milk, in high-preference mice occur more within the first half of a session

As noted in section "Reward latency is increased during alcohol sessions, but not in strawberry milk, due to occasional longer reward latency trials," changes in responding across a session may indicate altered drive for reward, e.g., where time-related shifts in reward latency in **Figure 3** might relate to satiety, and such motivational changes across a session could be expressed in other measures such as less responding and/or less accurate responding. Representative trial-by-trial sessions visually describe clear differences in performance based on preference and reward (alcohol or SM, **Supplementary Figure 7**). Thus, we investigated differences in correct, incorrect, and omissions in the first versus second half of each session to confirm any behavioral shifts. Mice that averaged at least 10 correct responses across the last five alcohol late-stage training sessions were included in this analysis, in order to more clearly assess time-related changes in performance for alcohol or SM.

High-preference mice displayed significantly more correct responses in the first half versus second half of sessions (**Supplementary Figure 6A1**, 1st-v-2nd-half: $F_{1,38}$ = 9.025, p = 0.0047) whereas LP mice had similar correct responses in both halves (**Supplementary Figure 6A2**, 1st-v-2nd-half: $F_{1,32}$ = 0.6845, p = 0.4142). In contrast, the number of incorrect responses were similar between the first and second halves for both HP mice (**Supplementary Figure 6C1**, 1st-v-2nd-half: $F_{1,38}$ = 0.0008, p = 0.9773) and LP mice (**Supplementary Figure 6C2**, 1st-v-2nd-half: $F_{1,32}$ = 0.6345, p = 0.4316; time: $F_{3.56,114}$ = 4.254, p = 0.0043). In addition, while there were fewer overall omissions by later training, both HP (**Supplementary Figure 6E1**, 1st-v-2nd-half: $F_{1,38}$ = 23.08,

$p < 0.0001$) and LP (Supplementary Figure 6E2, 1st-v-2nd-half: $F_{1,32} = 4.482$, $p = 0.0421$) mice displayed higher omissions in the second half then the first half. In contrast to alcohol, during SM sessions there were no differences between first and second halves in correct responding in HP (Supplementary Figure 6B1, 1st-v-2nd-half: $F_{1,38} = 1.232$, $p = 0.2739$; time: $F_{3.34,126.9} = 8.077$, $p < 0.0001$) or LP mice (Supplementary Figure 6B2, 1st-v-2nd-half: $F_{1,32} = 0.1309$, $p = 0.7199$; time: $F_{2.35,75.24} = 19.42$, $p < 0.0001$), or in incorrect SM responses in HP (Supplementary Figure 6D1, 1st-v-2nd-half: $F_{1,38} = 2.895$, $p = 0.0970$; time: $F_{3.38,128.6} = 6.412$, $p = 0.0002$) or LP mice (Supplementary Figure 6D2, 1st-v-2nd-half: $F_{1,32} = 2.195$, $p = 0.1483$; time: $F_{2.98,95.44} = 12.96$, $p < 0.0001$). However, HP mice displayed more omissions in the second half (Supplementary Figure 6F1, 1st-v-2nd-half: $F_{1,38} = 5.703$, $p = 0.0220$; time: $F_{2.833,107.7} = 4.249$, $p = 0.0081$) and LP mice did not (Supplementary Figure 6F2, 1st-v-2nd-half: $F_{1,32} = 2.195$, $p = 0.1483$; time: $F_{2.803,89.71} = 8.125$, $p = 0.0001$; interaction: $F_{4,128} = 2.507$, $p = 0.0453$); however, the omissions difference in HP mice when responding for alcohol was $p < 0.0001$, while the comparable difference for SM was $p = 0.0220$. Together, these findings concur with reward latency results (Figure 3) that alcohol engagement in HP mice was greater during the first half of the session, which was overall not seen in LP mice or for SM responding in HP or LP, and we speculate that the second half decline in HP performance could reflect intoxicating effects of alcohol, satiety, or other factors (see Supplementary discussion).

Intermittent alcohol exposure enhances behavioral engagement especially in previously low-engagement individuals

For Phase 2 of our studies, mice were allowed to drink alcohol under an Intermittent Access two-bottle choice (IA2BC) drinking paradigm, with 24-h access to 20% alcohol (versus water), three times a week, for 3 weeks. We were particularly interested in the possibility that IA2BC would not only enhance overall performance for alcohol, but specifically increase performance of LP mice. This could indicate that excessive consumption is particularly hazardous for individuals who innately have lower engagement with alcohol (while higher-engagement individuals already have greater risk for developing problem drinking).

Overall, HP and LP mice (defined by their alcohol behavior in initial DID sessions) had similar IA2BC consumption (Supplementary Figure 4O, HPvLP: $F_{1,46} = 0.8358$, $p = 0.3654$; time: $F_{3.93,178.3} = 37.81$, $p < 0.0001$) and preference (Supplementary Figure 4N, HPvLP: $F_{1,46} = 3.373$, $p = 0.0728$; time $F_{7.81,355.8} = 11.25$, $p < 0.0001$). However, HP did have greater preference during the first five sessions of IA2BC (Supplementary Figure 4N, HPvLP: $F_{1,46} = 4.479$, $p = 0.0398$; time $F_{3.24,149.1} = 20.91$, $p < 0.0001$), although consumption levels did not differ (Supplementary Figure 4O, HPvLP: $F_{1,46} = 1.417$, $p = 0.2401$; time $F_{1.89,87.01} = 49.28$, $p < 0.0001$).

To better assess how IA2BC drinking might influence responding in HP and LP mice, we averaged measures in the last 3 late-stage alcohol sessions and compared them with the average of the last 3 post-IA2BC sessions. While pre-IA2BC versus post-IA2BC correct responses were not different for HP (Figure 4A1, $p = 0.0995$), IA2BC experience greatly increased correct responses in LP mice (Figure 4A2, $p < 0.0001$). In addition, the change in correct responses (pre versus post IA2BC) was significantly different between LP and HP mice (Figure 4A3, $U = 176$, $p = 0.0202$). In contrast, HP mice had lower correct responses during SM sessions (Figure 4B1, $p = 0.0208$), while LP mice did not (Figure 4B2, $p = 0.2367$), nor were there differences in relative change in correct (Figure 4B3, $p = 0.4170$). Thus, after IA2BC, LP showed significantly greater alcohol engagement, as indexed by number of correct responses, while HP mice showed less impact of IA2BC experience in this measure.

Similar to correct, premature responses for alcohol were unaffected by IA2BC in the HP (Figure 4C1, $p = 0.6017$) but were increased in LP (Figure 4C2, $p = 0.0002$) mice, with LP mice having significantly greater premature responding after IA2BC compared to HP (Figure 4C3, LP $U = 141.5$, $p = 0.0019$). SM premature were unaffected by IA2BC in HP (Figure 4D1, $p = 0.2832$) and LP (Figure 4D2, $p = 0.9908$), and with no differences in the change in performance after IA2BC between HP and LP (Figure 4D3, student's t-test, $t(46) = 0.7155$, $p = 0.4779$). While premature responding can indicate increased impulsivity, we speculate that increased number of premature actions may occur in parallel with overall greater responding in the task, i.e., that premature responses may also reflect the level of behavioral engagement (and that increased premature in LP mice after IA2BC emphasizes their newfound readiness to respond for alcohol compared to pre-IA2BC, see section "Discussion").

Finally, IA2BC did increase omissions for HP (Figure 4E1, $p = 0.0126$), with the opposite trend for LP (Figure 4E2, $p = 0.1434$), and LP had less loss of engagement for alcohol relative to HP [Figure 4E3, student's t-test, $t(46) = 3.24$, $p = 0.0022$], indexed by these omissions. No changes were found in HP (Figure 4F1, $p = 0.2076$) or LP (Figure 4F2, $p = 0.2182$) during SM sessions, and relative performance changes were also similar between the groups (Figure 4F3, $U = 208$, $p = 0.1015$). Additional measures are detailed in Supplementary results and Supplementary Figure 8.

Together, these data strongly suggest that IA2BC overall increased engagement with alcohol responding significantly more in LP versus HP mice. Interestingly, in LP mice, IA2BC promoted responding even in mice who, pre-IA2BC, had barely responded (5 or fewer correct pre-IA2BC, increasing to

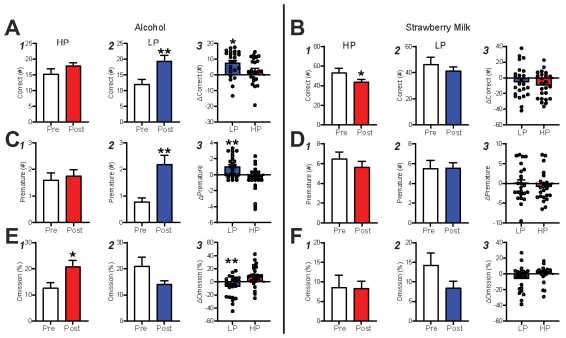

FIGURE 4
Intermittent alcohol exposure promotes responding in the 5-CSRTT. **(A)** LP mice gave more correct responses after IA2BC and had an overall greater change in correct than HP mice. **(B)** HP mice displayed a decrease in correct and had a similar change in performance as LP mice during SM sessions. **(C)** LP premature responding greatly increased post-IA2BC and had a greater performance change than HP mice, this was not seen during **(D)** SM sessions. **(E)** % Omissions were greater in HP post-IA2BC and LP mice had a greater performance change than HP mice. **(F)** No differences in % omission was found during SM sessions. n = 24/group, $*p < 0.05$, $**p < 0.01$ for effect of group. All data are expressed as ± standard error mean.

∼15 correct post-IA2BC). IA2BC also had limited impact on SM sessions, with only HP mice displaying decreased correct SM responses (although no decrease in alcohol responses). However, this SM decrease may also suggest decreased sucrose seeking behaviors after intermittent alcohol exposure as seen in previous studies (Starski et al., 2020). The effect of intermittent alcohol exposure on performance, regardless of preference, opens many avenues of investigation.

Discussion

Excessive alcohol consumption is a widely prevalent activity that may promote the development of AUD, where alcohol intake becomes a necessity for the individual and becomes a considerable barrier to treatment. Increased motivation and abnormal attentiveness for alcohol are often signs of AUD, and individuals with higher trait impulsivity are also at greater risk for AUD (see section "Introduction"). Thus, it is critical, when seeking to develop novel treatments, to discover biological mechanisms that promote these forms of behavioral engagement for alcohol. In the current study, we used the 5-CSRTT, a behavioral paradigm that can measure a number of facets of behavioral engagement (e.g., attention, impulsivity, and motivation) in the same session. Importantly, we, for the first time, adopted the 5-CSRTT to train mice to respond for alcohol as reward. All previous studies examined how alcohol exposure alters 5-CSRTT responding for sugar, but we wanted the mice to associate the task with alcohol, rather than sugar, so that future implementation of more challenging forms of the task (e.g., to assess impulsivity under variable timing), will reflect their motivation and overall performance for alcohol. Interestingly, using this novel alcohol 5-CSRTT paradigm, we found that alcohol preference was a more sensitive indicator of performance for alcohol in the 5-CSRTT, rather than consumption. HP mice learned the task faster and had greater participation than LP mice. Further differences were found within sessions, where HP mice had more correct responses and faster latencies in the first half of the session, perhaps suggesting a form of "front-loading" behavior and/or satiety later in the session (see below). "Front-loading" is typically observed in alcohol drinking paradigms where the majority of intake occurs within a short period, usually 30 min, of initial alcohol access (Rhodes et al., 2007; Griffin et al., 2009; Barkley-Levenson and Crabbe, 2012; Maphis et al., 2022; Wilcox M. V. et al., 2014). In the current study, we contextualize front-loading as more correct responding within the first half of the session as the total session time is 30 min. Finally, we found that 3 weeks of alcohol drinking (IA2BC) greatly promoted responding for alcohol in LP mice, with lesser or no change in HP related to IA2BC. This suggests that IA2BC produced a greater increase in motivation for alcohol in mice that innately began with lower preference, while innately higher preference mice were already more alcohol-responsive. Importantly, IA2BC overall had little effect on SM sessions, further underscoring the specificity in IA2BC effects on increasing alcohol engagement in LP individuals.

Studies have used the 5-CSRTT to compare treatment effects on motivation, attention, and impulsivity for sugar (Torregrossa et al., 2012; Fletcher et al., 2013). Indeed, alcohol vapor, liquid diet, and gavage treatment have all been used to induce increase premature responding or attentional errors in the 5-CSRTT when responding for sugar (Semenova, 2012; Irimia et al., 2015; Broos et al., 2017; Starski et al., 2020). However, to date there are no studies we are aware of that exclusively use an intoxicant as reward for 5-CSRTT. One goal of this study was to effectively train mice in this complex task for an alcohol-only reward (10% alcohol), with no other additives (such as saccharin or

even saccharin fade). This important advancement has allowed us to observe how motivated an animal was to wait during the intertrial interval, give a correct response, then retrieve the alcohol. Previous studies have trained animals to respond for alcohol in other behaviors such as differential reinforcement of low rates of responding (DRL) and progressive ratio tasks (Deehan et al., 2011; Somkuwar et al., 2018). While progressive ratio is valuable for measuring motivation, and DRL for impulsivity, the 5-CSRTT is designed to assess a broader range of measures in the same session. However, training animals in the 5-CSRTT with alcohol remains largely unexplored. We detail the nuances that come with having an intoxicant as a behavioral reward in this task in the **Supplementary discussion**, potential effects of increasing intoxication. Thus, we suggest that 5-CSRTT with alcohol as the reward can be robustly studied. This method of alcohol responding will likely be invaluable for future studies of how different interacting factors lead to different pathways to excessive drinking, including conditions with higher challenge (e.g., requiring greater attention or waiting) that some variants of 5-CSRTT testing can examine.

Both preference and consumption analyses revealed differences between their respective High/Low groups during early-stage sessions, however only preference analyses yielded consistent differences in number of trials and correct responses whereas all differences found during early-stage sessions disappeared during late-stage. Thus, we find that 5-CSRTT performance was significantly related to alcohol preference rather than consumption, including where HP mice demonstrated significantly more engagement with alcohol compared with LP mice. One speculation is that preference is related to an innate attention to and engagement with some condition. In this model, HP mice may reflect an individual who repeatedly orders a drink when there is an inherent waiting period (bartender order to delivery of alcohol), payment (correct response), and, finally, reward retrieval (consumption of drink). LP mice, however, may be compared with a more social drinker, where they drink when alcohol is easily available (two-bottle choice), but are more likely to be dissuaded if it requires actively work for it (purchasing, waiting, or traveling for alcohol). To further speculate, it is interesting that, in addition to high trait impulsivity, people at risk for binging have higher alcohol preference (Poulos et al., 1995; Crews and Boettiger, 2009; Schumann et al., 2011). However, determining clear relations in human studies remains challenging, and the ability to control factors in rodent studies has given insights into the alcohol-impulsivity relationship. For example, mice genetically selected for high alcohol preference display higher impulsivity in a delay discounting task compared with mice selected for low preference, although some aspects of impulsivity (amphetamine and lithium reduction of impulsivity) are not related to preference (Oberlin et al., 2010; Halcomb et al., 2013). In addition, mice genetically selected for high alcohol consumption displayed impaired response inhibition in a Go/No-go task but were not different from low consumption in delay discounting (Wilhelm et al., 2007). However, we should note that consumption level is still a very important factor, and several studies have assessed differential neural mechanisms that underlie higher versus lower intake level (Juarez et al., 2019; Lei et al., 2019). We recently performed BECs in C57BL/6 mice during limited access alcohol paradigms, and they reach binge level on average of 1.6 g/kg for a 30 min session for 20% alcohol (Lei et al., 2016; Wegner et al., 2017; Kwok et al., 2021), this amount approximates 85–100 mg% BEC. Mice in this study had access to roughly 1.3–1.5 g/kg of 10% alcohol which the mice may reach approximately 70–80 mg% BEC. This BEC level is near the 100 mg% that suggests behavioral changes from intoxication (Crabbe, 2012). It is necessary in future studies to strategically collect BEC levels in mice shortly after a behavioral session within our paradigm. Thus, we emphasize that HP and LP had similar drinking levels that, based on blood alcohol assessments in our previous mouse studies (Lei et al., 2016; Kwok et al., 2021), both HP and LP drank sufficient alcohol to on average reach binge level. In addition, premature responding in the 5-CSRTT is an indicator of impulsivity, and we found that HP mice had higher premature responses during early-stage training. This may suggest that their propensity for alcohol promotes error in the ability to wait (for the stimulus), although we speculate that greater premature responses along with greater overall responding could in some cases reflect greater engagement rather than impulsivity *per se*. Future studies will be needed to further dissect these and other aspects of behavior that promote excessive drinking. Cumulatively, the field continues to make substantial strides toward understanding the relationship between impulsivity, alcohol preference and intake, but the interconnection of these remains ambiguous.

Impulsivity is a complex construct that has come under scrutiny due to the wide breadth of behaviors it encompasses (Meda et al., 2009; MacKillop et al., 2016; Strickland and Johnson, 2021). Risk-taking tasks, delay-discounting, DRL, reversal learning, Go/No-Go, and, here, the 5-CSRTT are examples of impulsivity-related tasks, however they all measure impulsivity in different ways. Impulsive behaviors have traditionally been separated into two domains, impulsive action and Impulsive choice (Winstanley et al., 2006; Perry and Carroll, 2008; Winstanley, 2011; Jentsch et al., 2014). Under impulsive action, Go/No-Go and reversal learning measure action inhibition while the 5-CSRTT measures the ability to wait. In contrast, impulsive choice tasks measure sensitivity to delayed (e.g., delay-discounting) and risky choices (e.g., Balloon analogue risk task). We have chosen the 5-CSRTT since the task allows assessment of premature responses (related to impulsivity) as well as omissions and accuracy (more clear indicators of engagement). However, premature responses during standard training sessions may describe participation opposed to impulsivity. Future studies will utilize randomized

waiting periods to truly test impulsive responding. Thus, with 5-CSRTT we have the ability to measure impulsivity (among other measures) related to voluntary alcohol acquisition, especially in future (and ongoing) studies using 5-CSRTT variants that more clearly assess (e.g., variable timing of reward presentation). Importantly, even with the broad nature of impulsivity, clinical studies using impulsivity tasks often shown its relationship to alcohol use (Dick et al., 2010; Lejuez et al., 2010; Adams et al., 2013; Elder et al., 2019; Herman and Duka, 2019) and the 5-CSRTT has been used clinically to predict higher alcohol intake in highly impulsive individuals (Sanchez-Roige et al., 2014a). Here, we attempted to bridge a much-needed gap in rodent alcohol-impulsivity studies so that future work can focus on impulsive action for voluntary alcohol consumption, and related behavioral indicators of excessive intake, to decipher potential patterns and biomarkers that mirror clinical findings.

Here we focus on mice that have reached adulthood and continued to observe their progression toward behavioral engagement for alcohol. In humans, the average age of first drink is just over 17 (Caetano et al., 2014), which is approximately equivalent to the age of the mice at inception of the experiment (8 weeks) (Dutta and Sengupta, 2016; Wang et al., 2020), thus we are able to observe the motivation of a translational timepoint in which individuals begin to drink. In addition, while we did not explicitly examine age, other important and interesting studies have shown that intermittent alcohol exposure starting during earlier adolescence can have stronger changes in affect- and motivation-related behavior than alcohol-exposed adults (Van Skike et al., 2015; Kim et al., 2019; Nentwig et al., 2019; Healey et al., 2022). Here, using the intermittent access paradigm, we described a stark change in lower preference, less alcohol-engaged mice, where intermittent alcohol was related to a behavior shift toward higher alcohol responding and more impulsivity. Thus, these mice might model a lower trait risk in humans, which nonetheless can be shifted to more maladaptive responding with sufficient alcohol drinking. It is also important to note that, during adolescence, animal models involving repeated alcohol exposure (alcohol vapor) find increased impulsivity and decreased attention within the 5-CSRTT, in addition to other cognitive behavioral problems (Coleman et al., 2011, 2014; Semenova, 2012; Seemiller and Gould, 2020). Thus, it remains to be determined whether the same aspect of adolescence is a factor in own studies.

A limitation of this study is the inclusion of only male mice since female mice may present differences in alcohol engagement within the 5-CSRTT. Recently, the number of women with problem alcohol drinking has increased and these problems can be more severe (Brady and Randall, 1999; Erol and Karpyak, 2015; White et al., 2015; Becker and Koob, 2016; Grant et al., 2017; Carvalho et al., 2019). E.g., women can exhibit greater deficits in inhibitory control as a result from heavy drinking, and comorbidities, such as stress, promote drinking behavior more than in men (Peltier et al., 2019). Further, women with a family history of AUD exhibit higher error in go/no-go tasks (Saunders et al., 2008; Nederkoorn et al., 2009; Weafer et al., 2015). Sex differences in delay discounting tasks have conflicting reports (Beck and Triplett, 2009; Weafer and de Wit, 2014), however it was observed that women discounted alcohol more compared with monetary rewards than males (Yankelevitz et al., 2012). In rodent studies, sex differences have been found in the modulation of noradrenaline on attention and impulse control where a noradrenaline reuptake inhibitor was more effective in reducing impulsivity in male rats than female (Mei et al., 2021). Alcohol drinking and impulsive action behavior was found to be strongly associated only in male rats (Hammerslag et al., 2019). Additionally, transgenic BDNF changes induce female-only changes in impulsivity in 5-CSRTT and alcohol self-administration (Hogan et al., 2021). Thus, potential sex differences in mechanisms that underlie impulsivity, behavioral engagement for alcohol, as well as related divergences in underlying cortical circuitry and motivation drives (Barker and Taylor, 2019; Flores-Bonilla and Richardson, 2020), make it critical to use both sexes in future studies.

Several studies have shown how disruptions in various cortical areas can alter behavioral engagement detected through the 5-CSRTT (Chudasama et al., 2003; Pisansky et al., 2019; Starski et al., 2019). The anterior cingulate cortex (ACC), a region involved in higher-level cognitive function, modulates accuracy and omissions (Chudasama et al., 2003; Norman et al., 2021) and is required for top-down action control within the 5-CSRTT (White et al., 2018). Further, errors in decision-making after chronic alcohol use have been linked to ACC (Mashhoon et al., 2014; Smith et al., 2017; Zakiniaeiz et al., 2017). Additionally, the anterior insula (aINS) has been implicated in impulsive behaviors (Kayser et al., 2012; Pattij et al., 2014; Centanni et al., 2021), including where premature responding in the 5-CSRTT correlates with aINS thinning in rat (Belin-Rauscent et al., 2016). The aINS has also been heavily associated with attention (Rolls, 2016; Haaranen et al., 2020a,b; Happer et al., 2021), as a critical part of the salience network, and been implicated in problem alcohol drinking (Menon and Uddin, 2010; Cardenas et al., 2011; Seif et al., 2013; Grodin et al., 2018; Campbell et al., 2019). Further, motivational aspects of alcohol have been strongly linked with particular brain circuits including nucleus accumbens dopamine and glutamate signaling (Brodie et al., 1990; Gonzales and Weiss, 1998; Spiga et al., 2014; Sutera et al., 2016; Brancato et al., 2017, 2021) and future work is required to examine the brain mechanisms underlying the responding for alcohol we examine here. Though there are many regions associated with behavioral engagement, the ACC and aINS may be future targets of investigation linking intermittent alcohol intake and changes in engagement for alcohol.

Currently, investigators may be hesitant to replace the typical appetitive reward with an intoxicant. However, in the present study, we observed the satisfactory performance of mice

while training in the 5-CSRTT for an alcohol reward. This paradigm displays the relationship between motivation for freely accessible alcohol (DID, two-bottle choice) and for when mice must perform an attention-based task (5-CSRTT) for alcohol. Interestingly, HP mice were more likely to perform the task than their LP counterparts, while protracted alcohol consumption increased engagement with alcohol in LP mice significantly more than HP, while SM responding was higher than for alcohol and overall not different in HP versus LP or before and after IA2BC. In addition, differences in performance found within HP sessions emphasize the importance of doing a trial-by-trial analysis to maximize the efficacy and interpretability of the data. Though we do not have a non-IA2BC control, the differences between HP and LP mice after IA2BC found within this study detail the risk intermittent alcohol intake has on problem drinking even in those that did not prefer alcohol. Thus, our behaviorally focused series of experiments set a foundation to answer important neurological hypotheses that will be featured in future studies. Cumulatively, our findings offer a novel insight into preference-related motivation for and engagement with alcohol, that can be used to identify behavioral patterns and brain mechanisms among the different factors (including attention, reward motivation, impulsivity, and perseveration) that could come together in different ways to promote excessive alcohol drinking and its substantial harms.

Data availability statement

The raw data supporting the conclusions of this article will be made available upon request to the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Indiana University Institutional Animal Care and Use Committee.

Author contributions

PS, DM, and HM designed the behavioral schedule and collected the data. PS and DM analyzed the data. PS and FH wrote the manuscript. All authors provided critical review of the content and approved of the final version for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.968359/full#supplementary-material

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Adolescent alcohol exposure alters threat avoidance in adulthood

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Adolescent binge-like alcohol exposure impairs cognitive function and decision making in adulthood and may be associated with dysfunction of threat avoidance, a critical mechanism of survival which relies upon executive function. The present study investigated the impact of binge-like alcohol exposure during adolescence on active avoidance in adulthood. Male and female rats were subjected to adolescent intermittent ethanol (AIE) exposure by vapor inhalation and then tested in adulthood using a platform-mediated avoidance task. After training to press a lever to receive a sucrose reward, the rats were conditioned to a tone that co-terminated with a foot-shock. A motivational conflict was introduced by the presence of an escape platform that isolated the rat from the shock, but also prevented access to the sucrose reward while the rat was on the platform. During the task training phase, both male and female rats exhibited progressive increases in active avoidance (platform escape) in response to the conditioned tone, whereas innate fear behavior (freezing) remained relatively constant over training days. A history of AIE exposure did not impact either active avoidance or freezing behavior during task acquisition. On the test day following platform acquisition training, female rats exhibited higher levels of both active avoidance and freezing compared to male rats, while AIE-exposed male but not female rats exhibited significantly greater levels of active avoidance compared to controls. In contrast, neither male nor female AIE-exposed rats exhibited alterations in freezing compared to controls. Following 5 days of extinction training, female rats continued to display higher levels of active avoidance and freezing during tone presentation compared to males. Male AIE-exposed rats also had higher levels of both active avoidance and freezing compared to the male control rats. Together, the results demonstrate that female rats exhibit elevated levels of active avoidance and freezing compared to males and further reveal a sex-specific impact of AIE on threat responding in adulthood.

KEYWORDS

adolescence, alcohol, fear, platform-mediated avoidance, sex differences, foot shock, active avoidance, freezing

Introduction

Adolescence is a period of developmental change that includes increased risk-taking and sensation-seeking. It is therefore, not surprising that adolescence is also a period during which individuals typically begin experimenting with alcohol. A recent analysis of results from the Monitoring the Future National Survey reported that 3.4% of individuals aged 14 and 14% of individuals aged 18 reported having engaged in binge drinking behavior (defined as consumption of 5 or more drinks in a row) at least once within the past 2 weeks (Patrick and Terry-McElrath, 2019). In addition, a significant proportion of these individuals (1% of

14-year-old and 5.4% for 18-year-old individuals) also report having engaged in high-intensity binge drinking (defined as >10 drinks in a row) during this same time. Of particular concern are the potential long-term consequences of adolescent binge drinking on brain development. For example, increasing evidence from preclinical models demonstrates that a history of binge-like alcohol exposure during adolescence can lead to persistent changes in cognitive function in adulthood (Spear, 2018; Crews et al., 2019; Lees et al., 2020; Salmanzadeh et al., 2020; Sicher et al., 2022).

The ability to adaptively respond to threats in the environment is an important aspect of behavioral flexibility. While modulation of threat avoidance is essential for survival, an inability to appropriately evaluate and respond to threats is prevalent in many psychiatric disorders, including major depression and substance abuse (LeDoux and Daw, 2018; Mobbs, 2018). Preclinical models of Pavlovian auditory fear conditioning is a commonly used approach for investigating the role of learning and memory in threat responding that involves repeated pairing of an aversive stimulus (typically a foot shock) with a tone. After the formation of a learned association of the tone and the aversive shock, subsequent presentation of the tone alone elicits a defensive response. The most common readout of defensive responding in auditory fear conditioning is innate freezing behavior observed upon presentation of the fear-conditioned cue. However, while classic fear conditioning paradigms that assess freezing have been instrumental in advancing our understanding of fear learning and memory, this approach has a number of limitations for understanding the regulation of more complex forms of avoidance behaviors. Behavioral threat responding can be roughly categorized as being either goal-directed or non-goal-directed. Goal-directed avoidance is typically an action-outcome form of responding that is decision-based, while non-goal-directed responses are typically innate and passive in nature and include freezing and startle behaviors (LeDoux and Daw, 2018; Ball and Gunaydin, 2022). A threat response that involves performing a learned action to avoid harm is an active form of avoidance. This is in contrast to passive avoidance behaviors that do not require a response to avoid harm. In fact, in classic fear conditioning procedures, the innate freezing response has no impact on whether or not the animal will receive a shock.

The platform-mediated avoidance task (PMA) was developed as a means to access decision-based avoidance under conflict (Bravo-Rivera et al., 2014). An important feature of the PMA task is the incorporation of reward-seeking into the procedure that introduces a motivational conflict between reward and harm avoidance. While the animal learns that it can avoid a foot-shock by moving onto an escape platform in response to presentation of conditioned warning signal, it does so at the cost of forfeiting the opportunity to obtain a reward since it can physically no longer access the reward lever and reward magazine. The PMA task thus serves as a model of risky decision-making in which the animal must develop a response strategy that weighs the cost-benefits of obtaining a reward vs. harm avoidance. Since adolescent alcohol exposure has been previously shown to impact cognitive function and decision-making in adulthood (Spear, 2018; Crews et al., 2019), the present study utilized the PMA task to examine the effects of adolescent binge alcohol exposure on subsequent threat responding in adult male and female rats.

Materials and methods

Animals

Long-Evans dams were obtained from Envigo (Indianapolis, IN) and shipped with 10 pups of both sexes that were postnatal day (PD) 22 upon arrival. At PD 24, pups were weaned and pair-housed with same sex littermates in standard polycarbonate cages. Rats were maintained on a 12 h/12 h reverse light/dark cycle with *ad libitum* access to tap water and food (Teklad 2918, Envigo, Indianapolis, IN, USA) and water. All animal procedures were conducted in accordance with the National Institute of Health Guidelines under Institutional Animal Care and Use Committee approved protocols at the Medical University of South Carolina.

AIE exposure

Adolescent male and female Long-Evans rats underwent five successive 2-day cycles of vapor ethanol exposure according to previously described methods (Gass et al., 2014). As depicted in **Figure 1A**, adolescent rats were subjected to five cycles of Air or ethanol vapor between PD 28–44, which is roughly equivalent to 10–18 years of age in humans (Spear, 2015). Each exposure cycle consisted of two consecutive days of intermittent exposure to ethanol vapor followed by two ethanol-free days. Each exposure day of a cycle consisted of 14 h in the vapor chambers followed by a 10 h ethanol-free period outside of the chamber. Each 2-day cycle was followed by 1–2 non-exposure day(s). Control rats were treated similarly to AIE-exposed rats but were only exposed to air. Behavioral intoxication scores were taken immediately upon the removal of the rats from the vapor chambers. Intoxication rating involved a subjective 5-point scale where 1 = no signs of intoxication; 2 = slight motor impairment; 3 = obvious motor impairment but able to walk; 4 = dragging abdomen, loss of righting reflex; and 5 = loss of righting and eyeblink reflexes (Nixon and Crews, 2002; Gass et al., 2014). Tail blood was collected immediately after intoxication scoring on the second day of each cycle for measurement of blood ethanol concentrations (BECs) using an Analox alcohol analyzer (Analox Instruments, Atlanta, GA).

Platform-mediated avoidance (PMA) task

Threat responding was assessed using a platform-mediated avoidance (PMA) task as previously described (Bravo-Rivera et al., 2014). As depicted in **Figure 1B**, 1 week following AIE or Air exposure, rats were food restricted to 85%–90% of free-feeding body weight. On PD 60, rats began operant training to lever press for a reward using a variable interval schedule of reinforcement that averaged 30 s (VI30). During all phases of training and testing, sucrose pellets were available on the VI30 schedule. Rats were trained in standard operant chambers (29.53 × 24.84 × 18.67 cm; Med Associates, Burlington, VT, USA) located in sound attenuating cubicles (Med Associates). Reward lever training was conducted over a period of 7–10 days. Once a criterion of 10 lever presses/min was reached, platform-mediated avoidance training began. Three rats were excluded from the study because they did not meet the criteria.

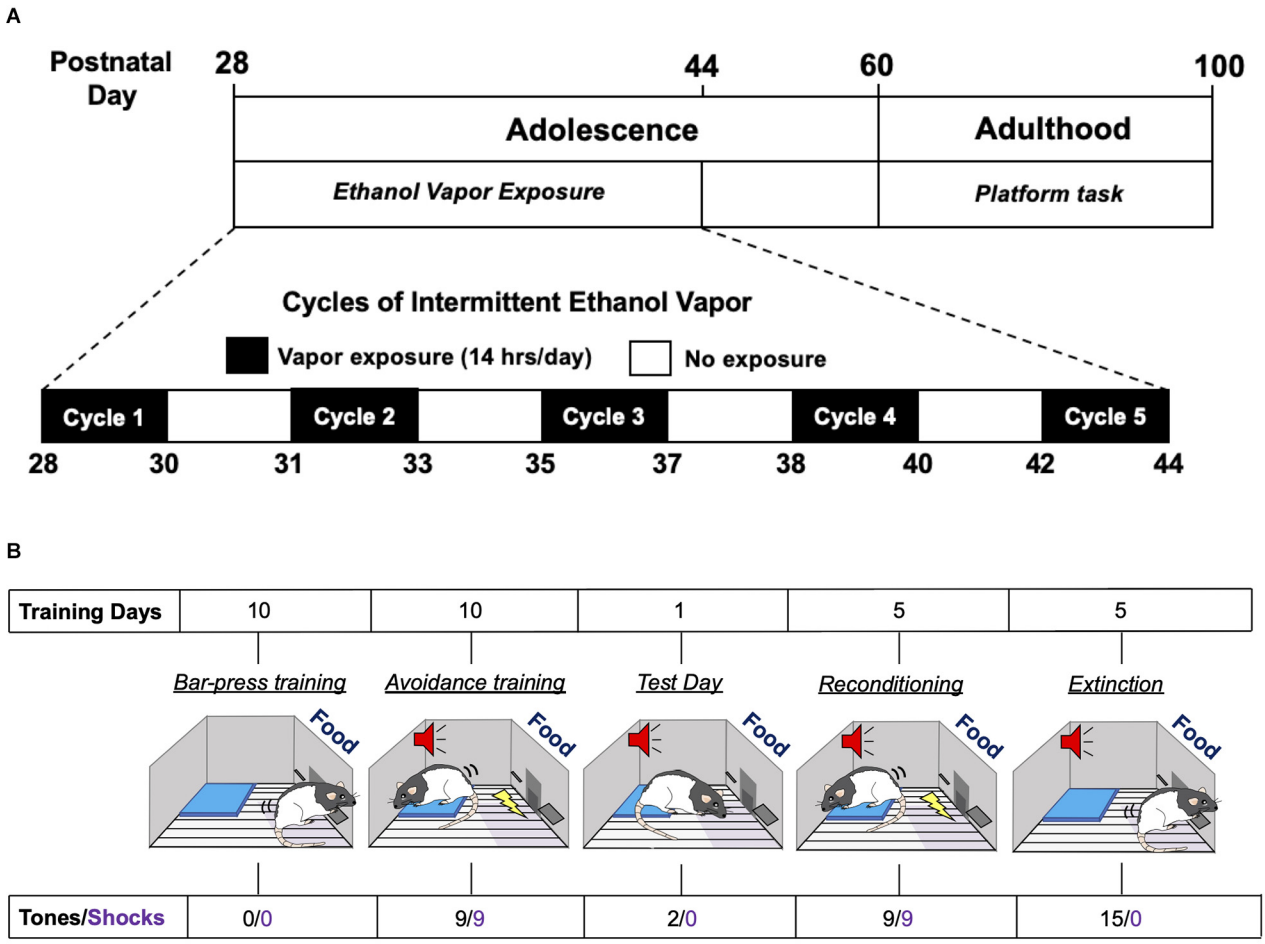

FIGURE 1
Experimental time-line of adolescent intermittent ethanol exposure and the platform-mediated avoidance task. **(A)** Schematic depiction of the daily sequence of adolescent intermittent ethanol (AIE) exposure by vapor inhalation followed by platform-media avoidance testing in adulthood. **(B)** Schematic depiction of the sequence for the different phases of the platform-mediated avoidance task for assessment of approach-avoidance behavior.

During the platform-mediated avoidance training phase, rats were conditioned to a tone (30 s, 4 kHz, 75 dB) that co-terminated with a foot shock delivered through the metal floor grids (2 s, 0.4 mA). Tone-shock pairings were separated by a variable inter-trial interval that averaged 3 min. Rats received three tone-shock pairings per session, with a 5-min free-press interval in between sessions. Free-press intervals included time without shock or tone presentation while the reward was still available in order to reduce fear and suppression of lever pressing. Rats received three sessions per day, for a total of nine tone-shock pairings per day. During all phases of training and testing, including extinction, an immobilized acrylic square platform (14.0 × 14.0 × 0.33 cm) was located in the far corner of the floor opposite the active lever. During avoidance training, rats were able to avoid shock by moving onto the platform. Due to the platform's location, rats must leave the safety of the platform in order to lever press to receive a sucrose reward pellet. Avoidance training was continued for a period of 10 days in order to attenuate freezing and lever press suppression when the tone was on. Active avoidance was defined as the rat having at least two paws on the platform and unable to reach the lever or food magazine. This was assessed at 1-s intervals across the entire period of tone presentation. On a subsequent retrieval test day (day 11), rats received two tones that did not co-terminate with shock. Rats were then re-conditioned to the tone-shock pairing and then underwent 5 days of extinction training with the platform in place, with each extinction session consisting of 15 tones per day that were not paired with shock. Testing and extinction days consisted of two or 15 sequential tone presentations, respectively, without any free-press intervals. At the beginning of lever press training, the average body weight of the rats was 267 g for the males and 204 g for the females. At the end of extinction training, the average body weight for the males was 324 g and 203 g for the females. There were no differences in body weights between the Air control and AIE groups for either sex.

Data analysis

Data were recorded using digital cameras (Logitech, Newark, CA, USA), and freezing behavior was analyzed with AnyMaze software Version 7.00 (Stoelting Co., Wood Dale, IL, USA). The freezing function of AnyMaze automatically detects periods when the animal is freezing. The freezing score in AnyMaze is calculated using an "on" and "off" threshold. Using a training set of videos, the threshold

parameters within AnyMaze were iteratively adjusted until automated freezing detection matched the freezing that was observed by visual inspection of the videos. The minimum duration for a freezing episode in AnyMaze was set at 750 ms. For active avoidance behavior, platform location detection in AnyMaze was confirmed by manually reviewing the video recordings to assess whether a rat was positioned "on" or "off" the platform during each second of the 30-s tones. A rat was assigned a score of 0% if it was "off" of the platform and a score of 100% if it was positioned "on" the platform. Data across time were averaged into 3-s bins and across each session per trial day. Unless otherwise stated, data were analyzed using a 2-way ANOVA with Sidak's *post-hoc* comparisons. The average percent time rats spent on the platform during the 30-s tone was analyzed using a mixed-effects ANOVA. Analysis of the percentage of rats that remained on the platform during the shock interval (SI) at the end of the tone was analyzed using a chi-squared analysis. For these studies, in order to identify whether a rat was considered to have remained on or off the platform during the SI, rats that had received an average score higher than 50% were considered on the platform during the session. The percentage of rats that remained on the platform during the SI was compared across groups.

Results

The present study utilized a well-characterized model of AIE exposure by vapor inhalation that is designed to simulate repeated episodes of binge intoxication. The procedure involved subjecting adolescent rats to intermittent cycles of ethanol vapor between PD28–44. Behavioral intoxication and blood ethanol concentration (BEC) were assessed at the end of each exposure cycle. The average intoxication score using the 1–5 point rating scale was 1.78 ± 0.10 for male rats and 2.16 ± 0.14 for female rats, which corresponds to a modest level of intoxication. The corresponding BEC values were 250 ± 12.0 mg/dl for the male rats and 246 ± 24.6 for the female rats. Statistical analysis (unpaired *t*-test) revealed that intoxication scores in the female rats were significantly higher than in the males ($p = 0.0309$), whereas there were no significant differences in the BEC values between the male and female rats.

At PD60, rats began training on the PMA task. During the 10 days of task acquisition training, active avoidance (platform escape) was assessed by calculating the average percent time the rats spent on the platform during a 30-s tone that predicted delivery of a shock during the final 2 s of the tone. For analysis of task acquisition by male rats (**Supplementary Figure S1A**), a mixed-effects ANOVA of active avoidance behavior (expressed as time spent on the platform) revealed a main effect of time (acquisition days) [$F_{(9,174)} = 9.639$, $p < 0.001$]. While there was a non-significant trend of treatment (Air vs. AIE) [$F_{(1,22)} = 3.442$, $p = 0.077$], there was a significant time × treatment interaction [$F_{(9,174)} = 2.126$, $p = 0.029$]. Freezing behavior was also assessed in the same animals during task acquisition by measuring the percent of time the rats froze during tone presentation (**Supplementary Figure S1C**). The ANOVA revealed there was no main effect of time [$F_{(9,174)} = 0.940$, $p = 0.491$] or treatment [$F_{(1,22)} = 0.532$, $p = 0.473$], nor was there a significant time × treatment interaction [$F_{(9,174)} = 1.766$, $p = 0.077$]. Analysis of total lever presses during each session across task acquisition (**Supplementary Figure S1E**) revealed a main effect of time [$F_{(9,174)} = 8.568$, $p < 0.001$]. There was no significant effect of treatment [$F_{(1,22)} = 3.523$, $p = 0.073$] or a time × treatment interaction [$F_{(9,174)} = 1.187$, $p = 0.306$].

For the acquisition of active avoidance behavior in the female rats (**Supplementary Figure S1B**), a mixed-effects ANOVA revealed a significant main effect of time [$F_{(9,201)} = 5.842$, $p < 0.001$], indicating that similar to male rats, Air and AIE-exposed female rats also increased their time spent on the platform during the tone at similar rates during task acquisition. There was no main effect of treatment [$F_{(1,25)} = 2.978$, $p = 0.096$] or of a time × treatment interaction [$F_{(9,201)} = 1.727$, $p = 0.084$]. Analysis of freezing across acquisition days by the female rats (**Supplementary Figure S1D**) revealed a significant main effect of time [$F_{(9,201)} = 2.342$, $p = 0.015$], but no significant effect of treatment [$F_{(1,25)} = 0.5705$, $p = 0.457$] or of a time × treatment interaction [$F_{(9,201)} = 0.8990$, $p = 0.5271$]. Analysis of total lever presses by the female rats during task acquisition (**Supplementary Figure S1F**) revealed a significant effect of time [$F_{(9,198)} = 3.192$, $p = 0.001$] and a time × treatment interaction [$F_{(9,198)} = 2.008$, $p = 0.040$], but no main effect of treatment [$F_{(1,25)} = 0.036$, $p = 0.850$].

Taken together, the above analyses revealed that both male and female rats exhibited progressive increases in the acquisition of active avoidance across training days as they learn to avoid the shock by retreating to the platform. In contrast, innate freezing behavior was relatively low from the outset and did not change across acquisition days. The results also indicate that a history of AIE did not impact either active avoidance or freezing behaviors during the task acquisition phase.

Active avoidance and freezing during the test phase of the task

Following the 10-day task acquisition phase, rats underwent a single test session in which they were exposed to two tones that did not co-terminate with a shock. Analysis of the total percent time the rats were on the platform during this 5-min test session (**Figure 2A**) revealed there was a main effect of treatment (Air vs. AIE) [$F_{(1,45)} = 6.669$, $p = 0.013$], no main effect of sex [$F_{(1,45)} = 0.042$, $p = 837$], but a significant interaction between sex and treatment [$F_{(1,45)} = 5.236$, $p = 0.027$]. A Sidak's multiple comparison *post-hoc* test revealed a significant difference between Air and AIE-exposed male but not female rats ($p = 0.012$). When the analysis was restricted to the time the rats were on the platform during the 30-s tone (**Figure 2B**), the ANOVA indicated there was a main effect of both treatments [$F_{(1,45)} = 12.38$, $p = 0.001$] and sex [$F_{(1,45)} = 6.413$, $p = 0.014$], and a significant treatment × sex interaction [$F_{(1,45)} = 5.225$, $p = 0.027$]. Sidak's *post-hoc* test revealed that the time spent on the platform during the tone by the Air male rats was significantly different from all other groups (all p values < 0.01). A chi-squared analysis was used to assess the percentage of rats considered to be on the platform >50% of the time during the 2-s shock intervals (SI) averaged across each session (**Figure 2C**). This revealed that the percentage of rats on the platform during the SI was not different across the groups ($p = 0.724$).

We next examined the time-course of when the rats were on the platform during the tone (averaged across both tones). For the male rats (**Figure 2D**), a mixed-effect ANOVA revealed there was a main

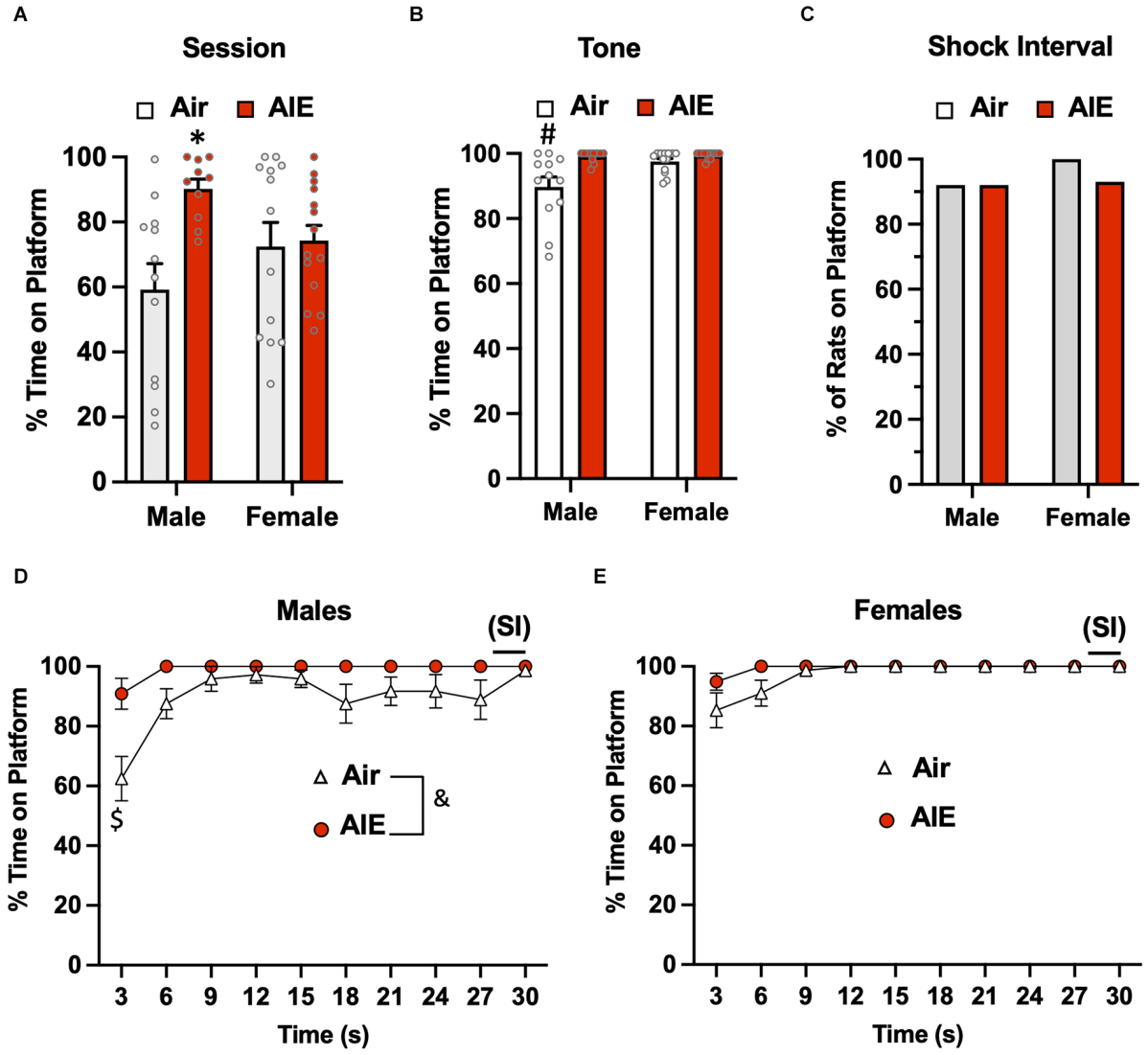

FIGURE 2
Active avoidance behavior assessed on the test day of the platform-mediated avoidance task. **(A)** When examined over the entire test session, AIE-exposed male rats spent significantly more time on the platform compared to the male Air control animals. **(B)** When the analysis of time on the platform was restricted to the periods of the 30-s tone, both male and female rats were located on the platform to a much greater extent compared to the tone-off periods. In addition, the significant difference in time on the platform between the male Air and male AIE-exposed rats was still observed. **(C)** Chi-square analysis revealed there was no significant difference in the percentage of rats that remained located on the platform during the 2-s shock interval (SI) at the end of the tone. Analysis of the time-course in 3 s bins of when the male **(D)** and female **(E)** rats were positioned on the platform during the tone revealed a significant main effect of AIE in the male rats. This was primarily driven by the significant difference observed at the initial 3 s time point. Data represent the means ± sem (except for the contingency date in panel **C**; n = 12–13/group). *indicates significant difference from male Air rats, $p = 0.013$; $^{\#}$indicates significant difference from all other groups, all p values < 0.01; $^{\$}$indicates significant difference of Air and AIE at the 3 s time point, $p = 0.000$; $^{\&}$indicates significant main effect of AIE, $p = 0.009$.

effect of time [$F_{(9,189)} = 7.656$, $p < 0.001$] and treatment (Air vs. AIE) [$F_{(1,21)} = 8.272$, $p < 0.009$], and a time × treatment interaction [$F_{(9,189)} = 2.730$, $p = 0.005$]. A Sidak's multiple comparisons *post-hoc* test revealed that male AIE-exposed rats spent significantly less time on the platform compared to Air control rats during the first 3 s of the tone ($p = 0.000$). For the female rats (Figure 2E), the analysis revealed a significant main effect of time [$F_{(9,220)} = 6.161$, $p = 0.000$], but no main effect of treatment [$F_{(1,220)} = 0.4865$, $p = 0.486$] or of a time × treatment interaction [$F_{(9,220)} = 0.4865$, $p = 0.882$].

Passive avoidance behavior during the 5-min test session was assessed by measuring the percentage of time rats froze during the presentation of the two conditioned tones (Figure 3A). The ANOVA revealed there was a main effect of sex [$F_{(1,43)} = 5.748$, $p = 0.020$] in which female rats exhibited higher levels of freezing than males. While there was no significant main effect of treatment (Air vs. AIE) [$F_{(1,43)} = 0.834$, $p = 0.366$], there was a non-significant trend towards a sex × treatment interaction [$F_{(1,43)} = 3.927$, $p = 0.053$]. When analysis of freezing to the tone was restricted to when the rats were on the platform (Figure 3B), the ANOVA indicated there was no main effect of sex [$F_{(1,47)} = 1.530$, $p = 0.222$] or treatment [$F_{(1,47)} = 1.377$, $p = 0.246$], and no sex × treatment interaction [$F_{(1,47)} = 0.083$, $p = 0.374$]. Analysis of lever pressing during the 5 min test session (Figure 3C), revealed a main effect of treatment [$F_{(1,42)} = 7.094$, $p = 0.010$]. A follow-up multiple comparisons *post-*

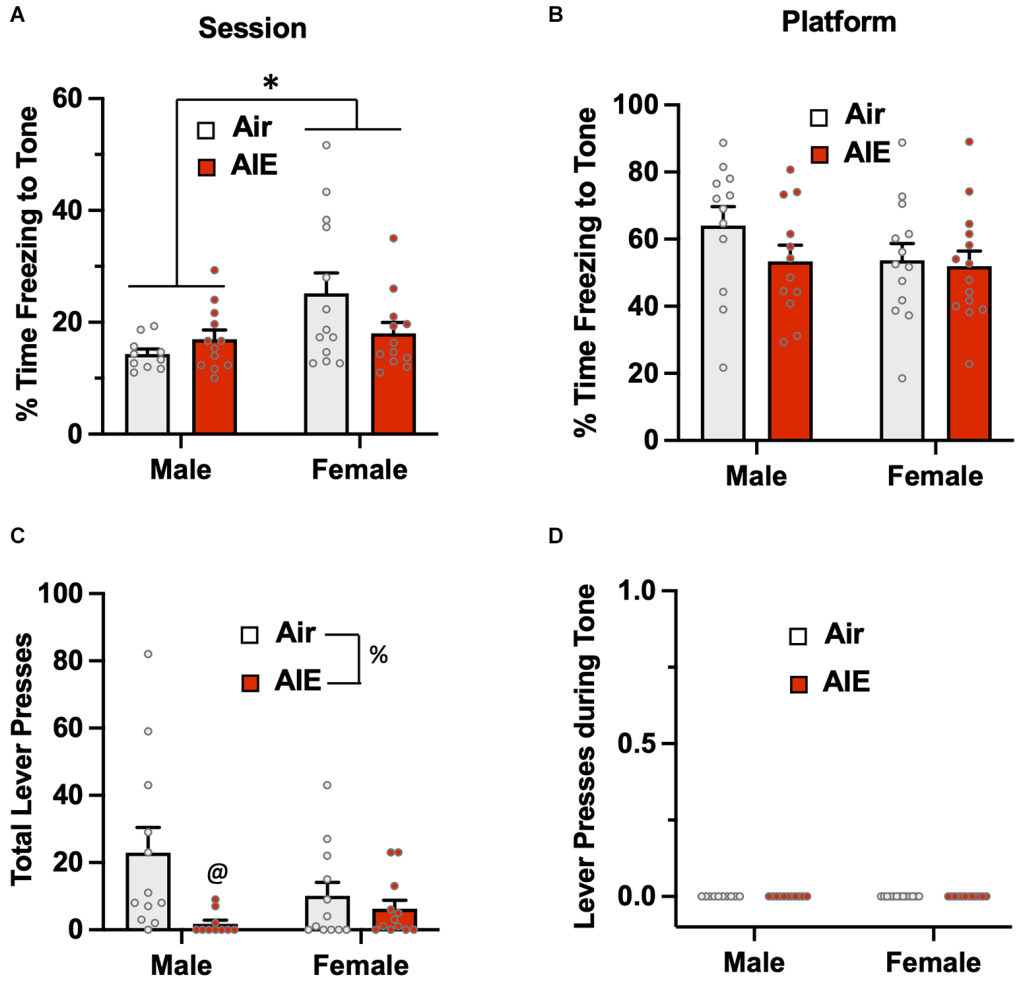

FIGURE 3
Freezing behavior and lever pressing assessed on the test day of the platform-mediated avoidance task. **(A)** When freezing to the tone on the test day was examined irrespective of location, female rats exhibited higher levels of freezing compared to the male rats. AIE exposure did not significantly alter freezing in either sex. **(B)** When analysis of freezing to the tone was restricted to when the rats were positioned on the platform, the sex difference in freezing was no longer observed. **(C)** Examination of lever responding during the test session revealed total lever pressing by AIE-exposed male rats was significantly reduced. **(D)** No lever pressing during the tone was observed. Data represent the means ± sem (n = 12–13/group). *indicates significant main effect of sex, p = 0.02; @indicates significant difference from the male Air rats, p = 0.010; %indicates significant main effect of AIE, p = 0.010.

hoc test indicated that male AIE-exposed rats pressed the lever significantly less compared to the male Air control rats. While there was no main effect of sex on total lever presses [$F_{(1,42)}$ = 0.800, p = 0.375], there was a non-significant trend of a sex × treatment interaction [$F_{(1,42)}$ = 3.404, p = 0.072]. For lever pressing during the tone, there were no differences across sex or AIE exposure as no rats lever pressed during this period (Figure 3D).

Taken together, the above analysis of the test phase of the task indicates that female rats exhibit higher levels of both active and passive avoidance behavior compared to male rats. In addition, it further revealed that a history of AIE exposure is associated with sex-specific alteration in active but not passive avoidance.

Active avoidance and freezing during the extinction phase of the task

The third phase of the PMA task involved the examination of the extinction of avoidance behavior over five consecutive days, with each extinction day consisting of 15 tone presentations but no shock co-termination. For active avoidance on the first day of extinction (Figure 4A), ANOVA analysis of the percent time rats spent on the platform during the entire session revealed there was no main effect of sex [$F_{(1,47)}$ = 0.8664, p = 0.356] or treatment [$F_{(1,47)}$ = 0.4696, p = 0.496], nor was there a significant sex × treatment interaction [$F_{(1,47)}$ = 0.1437, p = 0.706]. When the analysis was restricted to the time on the platform during the 30-s tone (Figure 4B), the ANOVA again revealed no main effect of treatment [$F_{(1,47)}$ = 0.5062, p = 0.480] or sex [$F_{(1,47)}$ = 0.9208, p = 0.342], nor was there a treatment × sex interaction [$F_{(1,47)}$ = 0.5062, p = 0.480]. Chi-squared analysis of the percentages of rats on the platform during the 2-s SI averaged across each session (Figure 4C) also revealed no group differences (p = 0.507). For analysis of the time-course of when the male rats were on the platform during the 30-s tone (Figure 4D), there was a main effect of time [$F_{(9,186)}$ = 8.461, p = 0.000] and a significant time × treatment interaction [$F_{(9,186)}$ = 2.821, p = 0.003], but no main effect of treatment [$F_{(1,22)}$ = 0.3997, p = 0.533]. With the female rats (Figure 4E), there was a main effect of time [$F_{(9,195)}$ = 14.55, p = 0.000]

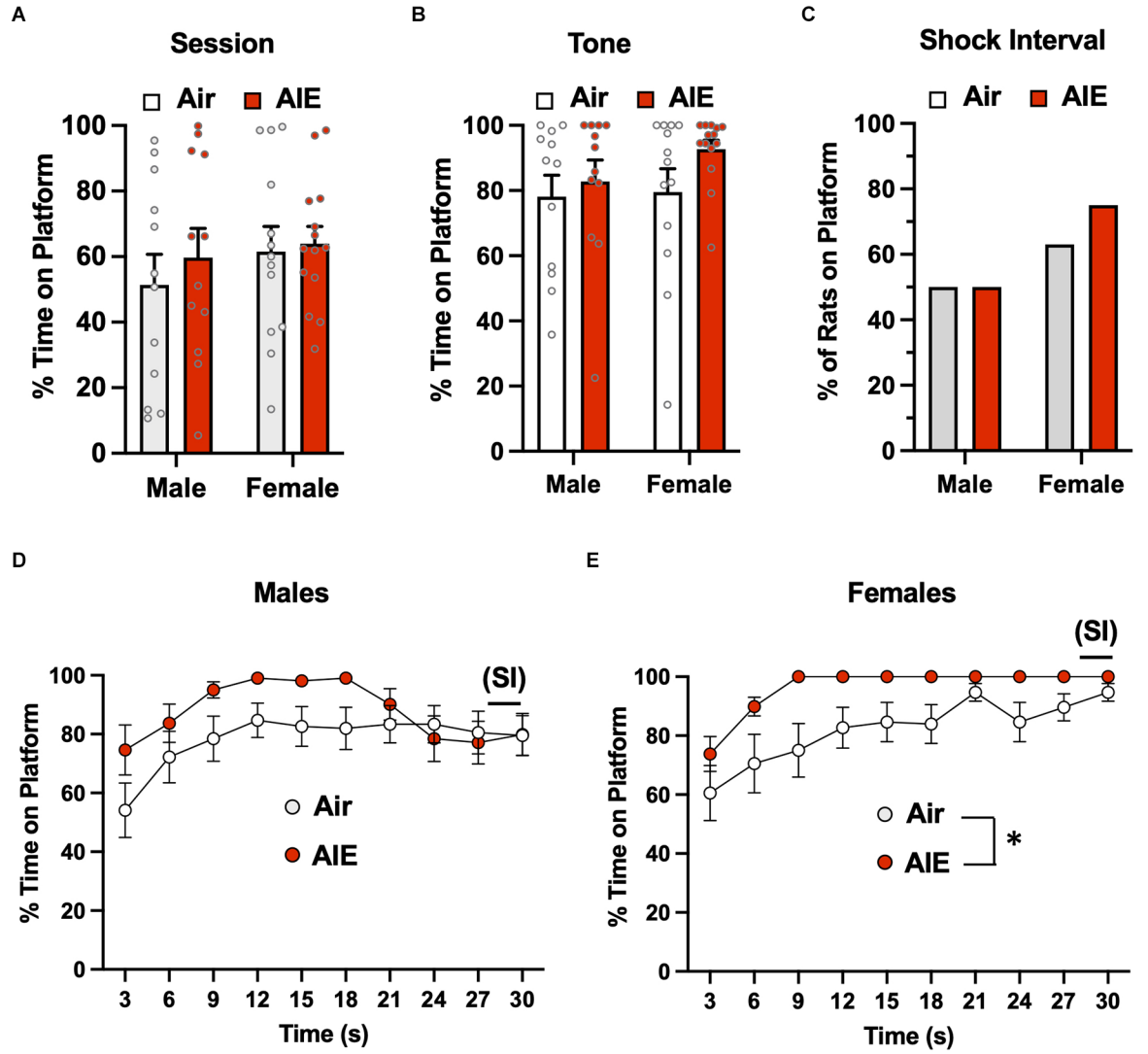

FIGURE 4
Active avoidance behavior assessed on the first day of extinction of the platform-mediated avoidance task. When the percentage of time the rats were positioned on the platform was examined over the entire session **(A)** or only during the tone presentations **(B)**, there were no significant difference across sex or AIE. **(C)** Chi-square analysis indicated there were no differences in the percentage of rats considered to have remained on the platform during the 2-s shock interval (SI). Analysis of the time-course in 3 s bins of when the male **(D)** and female **(E)** rats were on the platform during the tone presentations revealed a significant main effect of time and a significant main effect of AIE in the female rats. Data represent the means ± sem (except for the contingency date in panel **C**; n = 12–13/group). *indicates a significant main effect of AIE, p = 0.046.

and of treatment [$F_{(1,25)} = 4.384$, $p = 0.046$], but no time × treatment interaction [$F_{(9,195)} = 0.7436$, $p = 0.668$].

Analysis of passive avoidance behavior on the first day of extinction (Figure 5A) indicated there was a significant main effect of sex [$F_{(1,46)} = 8.715$, $p = 0.005$]. Follow-up *post-hoc* analysis revealed freezing to the tone by the female Air rats was significantly greater compared to all other groups (all p values < 0.05). While there was a trend towards a main effect of treatment [$F_{(1,46)} = 3.850$, $p = 0.055$] and towards an interaction of sex × treatment [$F_{(1,46)} = 3.586$, $p = 0.064$], neither reach the level of statistical significance. When the analysis was restricted to freezing while on the platform (Figure 5B), there was no main effect of sex [$F_{(1,47)} = 0.826$, $p = 0.368$] or treatment [$F_{(1,47)} = 1.149$, $p = 0.289$], and no treatment by sex interaction [$F_{(1,47)} = 0.1956$, $p = 0.660$]. Analysis of lever pressing during the Day 1 extinction session (Figure 5C) revealed a main effect of sex [$F_{(1,47)} = 6.189$, $p = 0.016$], but no effect of treatment [$F_{(1,47)} = 0.3659$, $p = 0.548$] or of a sex × treatment interaction [$F_{(1,47)} = 0.4237$, $p = 0.518$]. When this analysis was restricted to lever presses only during the tone (Figure 5D), there continued to be a main effect of sex [$F_{(1,43)} = 4.520$, $p = 0.039$], but no treatment [$F_{(1,43)} = 0.2895$, $p = 0.593$] or sex × treatment interaction [$F_{(1,43)} = 0.3968$, $p = 0.532$].

Overall, the above analysis of avoidance behaviors during the first day of extinction indicates that while a history of AIE exposure did not significantly impact passive avoidance on the first day of extinction, female rats tend to display higher levels of passive avoidance than their male counterparts and exhibit reductions in lever pressing to obtain a sucrose reward.

Analysis of the time spent on the platform during the entire session of the 5th day of extinction (Figure 6A) revealed a significant main effect of treatment [$F_{(1,46)} = 5.137$, $p = 0.028$], but no main effect of sex [$F_{(1,46)} = 2.175$, $p = 0.147$] or of a treatment × sex interaction [$F_{(1,46)} = 1.550$, $p = 0.219$]. Sidak's *post-hoc* test indicated

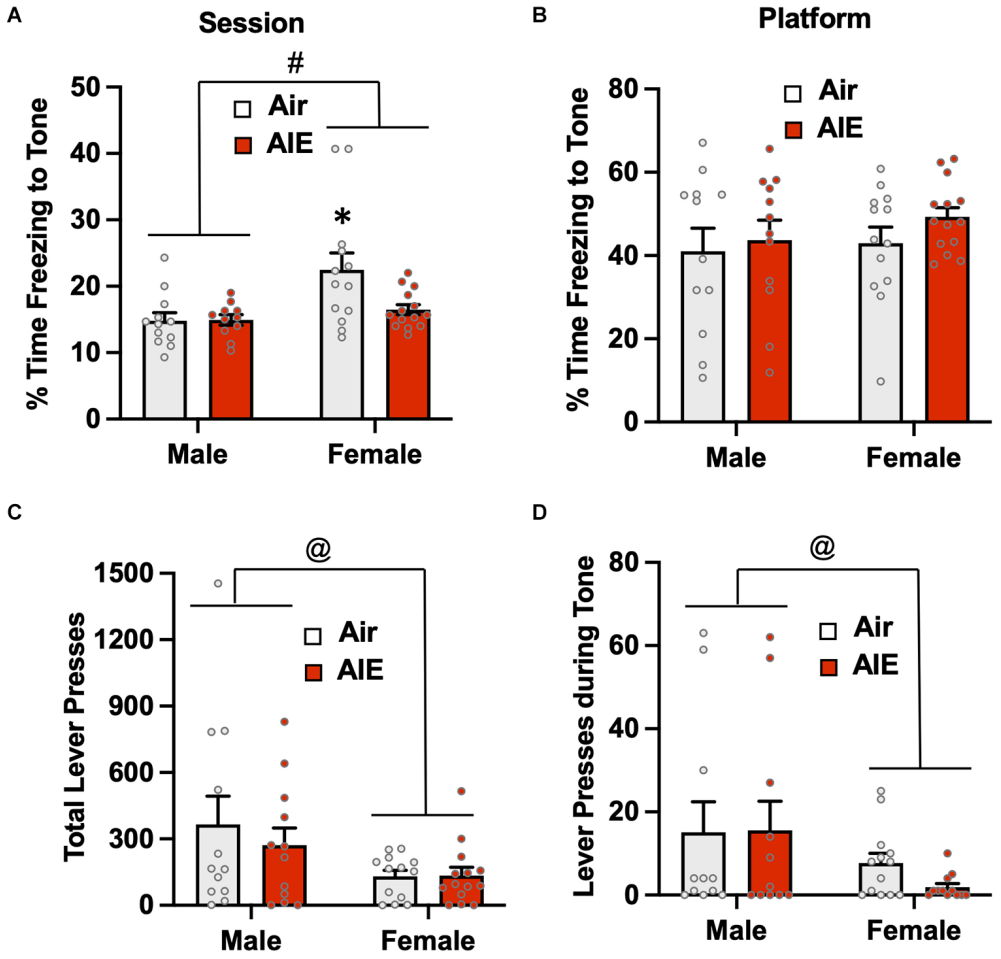

FIGURE 5
Freezing behavior and lever pressing assessed on the first day of extinction of the platform-mediated avoidance task. **(A)** When freezing during tone presentation on the first day of extinction was examined irrespective of the location, female rats exhibited higher levels of freezing compared to the males. A prior history of AIE exposure significantly reduced freezing to the tone in the female rats. **(B)** When analysis of freezing during tone presentation was restricted to when the rats were located on the platform, the sex difference and effect of AIE exposure on freezing was no longer observed. **(C)** Examination of total lever responding during the test session revealed lever pressing by female rats was significantly reduced compared to male rats. **(D)** The sex-specific reduction in lever pressing was also observed during presentation of the tone. Data represent the means ± sem (n = 12–13/group). #indicates significant difference between males and females, p = 0.005; *indicates significant difference from all other groups, all p values < 0.05; @indicates significant differences between males vs. females, all p values < 0.05.

that the percent time spent on the platform by the male Air rats was significantly lower compared to the male AIE (p = 0.018) and female AIE rats (p = 0.011). When the analysis was restricted to the 30-s tone period (Figure 6B), the ANOVA again indicated a main effect of sex [$F_{(1,47)}$ = 37.17, p = 0.000], but no main effect of treatment [$F_{(1,47)}$ = 0.5348, p = 0.468] nor was there a treatment × sex interaction [$F_{(1,47)}$ = 2.551, p = 0.116]. Sidak's multiple comparisons *post-hoc* test revealed that both groups of male rats were significantly different from both groups of female rats (all p values < 0.01). Whether the rats were considered to have remained on the platform during the 2-s SI were averaged across each session for extinction day 5 (Figure 6C). A Chi-squared analysis revealed a significant difference in the percentage of rats on the platform during the shock interval (p = 0.000). Specifically, the percentage of rats from each group considered to be on the platform during the shock interval ranged from 0% for male Air rats, 20% for male AIE-exposed rats, 63% for female Air rats, and 57% for female AIE-exposed rats. Examination of the time-course of when male rats were on the platform during the 30-s tone (Figure 6D) revealed there was a non-significant trend of a main effect of time [$F_{(9,197)}$ = 1.858, p = 0.060], and no significant effect of treatment [$F_{(1,22)}$ = 1.974, p = 0.174] or of a time × treatment interaction [$F_{(9,197)}$ = 1.602, p = 0.116]. With female rats, analysis of the time-course of when they were on the platform during the tone (Figure 6E) revealed a main effect of time [$F_{(9,117)}$ = 2.032, p = 0.041], but no main effect of treatment [$F_{(1,13)}$ = 0.7117, p = 0.414] or of a time × treatment interaction [$F_{(9,99)}$ = 0.7732, p = 0.641].

The next set of analyses examined extinction-dependent changes in percent time on the platform in the Air control male and female rats as a function of Test day (baseline), Extinction Day 1, and Extinction Day 5 (Supplemental Figure S2). Mixed effect one-way ANOVAs with Tukey's *post-hoc* tests revealed significant reductions in the percent time on the platform for both males and females when compared across the 3 days [Males: $F_{(1.979,21.77)}$ = 10.19, p = 0.0008; Ext Day 5 significantly different from Ext Day 1 (p = 0.0130) and Test Day (p = 0.0049); Females: $F_{(1.526,18.31)}$ = 8.929, p = 0.0035; Ext Day 5 significantly different from Test Day (p = 0.0066)] and when assessed specifically during tone presentation [Males: $F_{(1.313,14.44)}$ = 71.93,

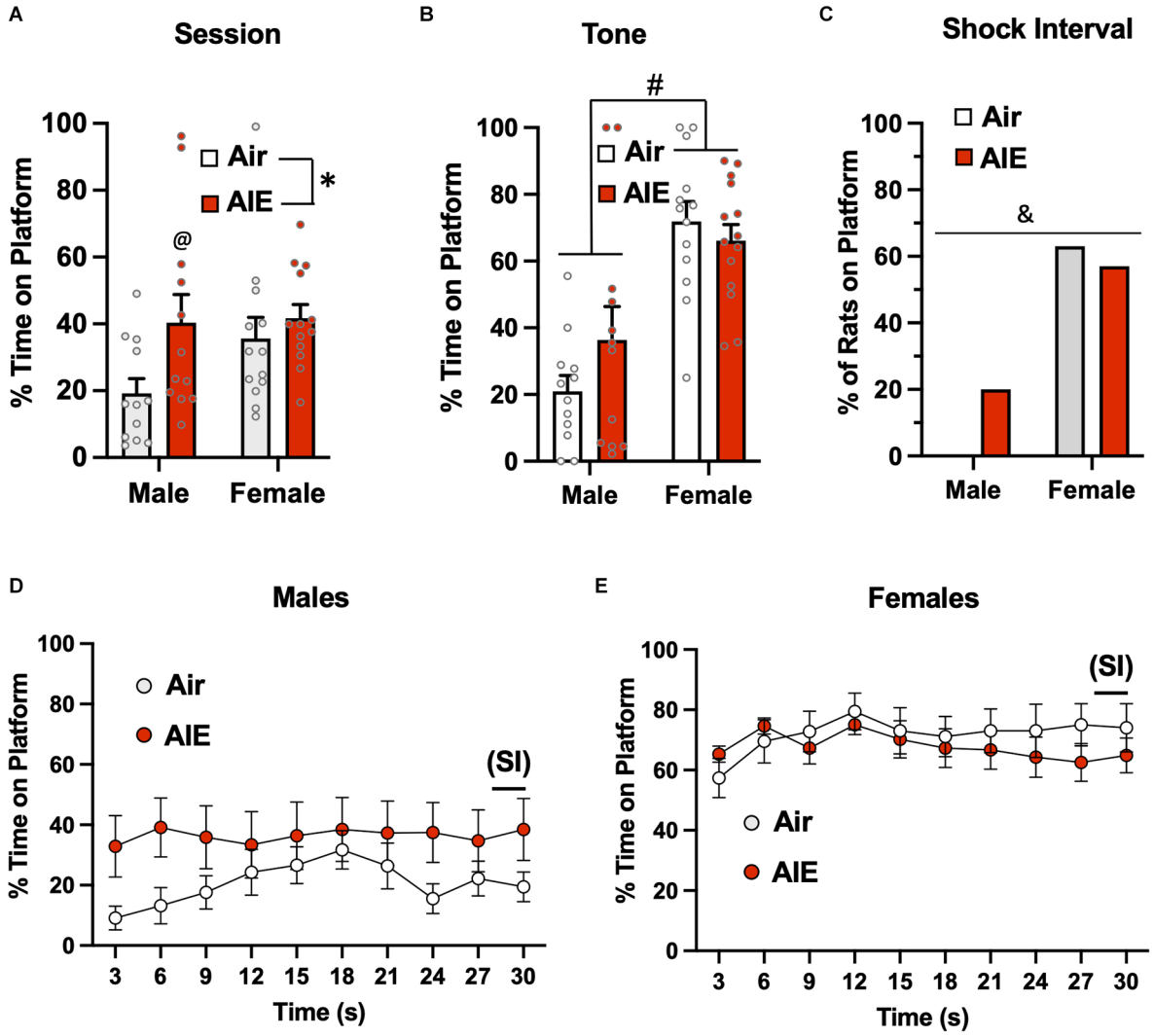

FIGURE 6
Active avoidance behavior assessed on the last day of extinction of the platform-mediated avoidance task. **(A)** When the percentage of time the rats were located on the platform was examined over the entire extinction session, male AIE-exposed rats exhibited significant increases in time on the platform compared to the male Air rats. **(B)** When analysis of time on the platform was restricted to the period of the tones, the female rats continued to spend significantly more time on the platform. **(C)** Chi-square analysis also revealed there was a significant difference in the percentage of rats considered to have remained on the platform during the 2-s shock interval (SI). Analysis of the time-course in 3 s bins of when the male **(D)** and female **(E)** rats were located on the platform during the tone presentations revealed a significant main effect of time in the female rats. Data represent the means ± sem (except for the contingency date in panel **C**; n = 12–13/group). *indicates significant difference between Air and AIE, p = 0.028; $^{@}$indicates significant difference between the male Air and male AIE rats, p = 0.018; $^{\#}$indicates significant difference between male and female rats, p = 0.000; $^{\&}$indicates significant group difference, p = 0.000.

$p < 0.001$; Ext Day 5 significantly different from both Test Day ($p < 0.001$) and Ext Day 1 ($p < 0.001$).

Analysis of the percent of time the rats froze to the tone on the last day of extinction (Figure 7A) revealed there was no main effect of sex [$F_{(1,42)} = 0.1358$, $p = 0.713$], but there was a main effect of treatment [$F_{(1,42)} = 7.37$, $p = 0.009$] and a treatment × sex interaction [$F_{(1,42)} = 10.80$, $p = 0.002$]. A Sidak's multiple comparisons *post-hoc* test indicated that this effect was likely driven by increased freezing of male AIE-exposed rats compared to the male Air rats ($p < 0.001$). When the analysis was restricted to freezing during the tone while the rats were on the platform (Figure 7B), there was a main effect in the of sex [$F_{(1,47)} = 44.91$, $p = 0.000$], but no effect of treatment [$F_{(1,47)} = 1.717$, $p = 0.196$] or of a sex × treatment interaction [$F_{(1,47)} = 1.829$, $p = 0.182$]. Sidak's multiple comparisons test revealed both groups of male rats froze significantly less than both groups of female rats (all p values < 0.01). Analysis of lever pressing during the Day 5 extinction session (Figure 7C) revealed that female rats lever pressed significantly less than male rats [$F_{(1,47)} = 20.28$, $p < 0.001$], but there was no effect of treatment [$F_{(1,47)} = 0.01$, $p = 0.910$] nor was there a sex × treatment interaction [$F_{(1,47)} = 0.2721$, $p = 0.604$]. A Sidak's multiple comparisons *post-hoc* test revealed that lever pressing in both groups of male rats was significantly greater than lever pressing in both groups of female rats (all p values < 0.05). When the analysis was restricted to lever pressing during the tone (Figure 7D), there was a significant main effect of treatment [$F_{(1,46)} = 8.014$, $p = 0.006$], but no main effect of sex [$F_{(1,46)} = 3.029$, $p = 0.088$] nor was there a treatment × sex interaction [$F_{(1,46)} = 2.936$, $p = 0.093$]. *Post-hoc* analysis revealed that the AIE-exposed male ($p = 0.017$) and

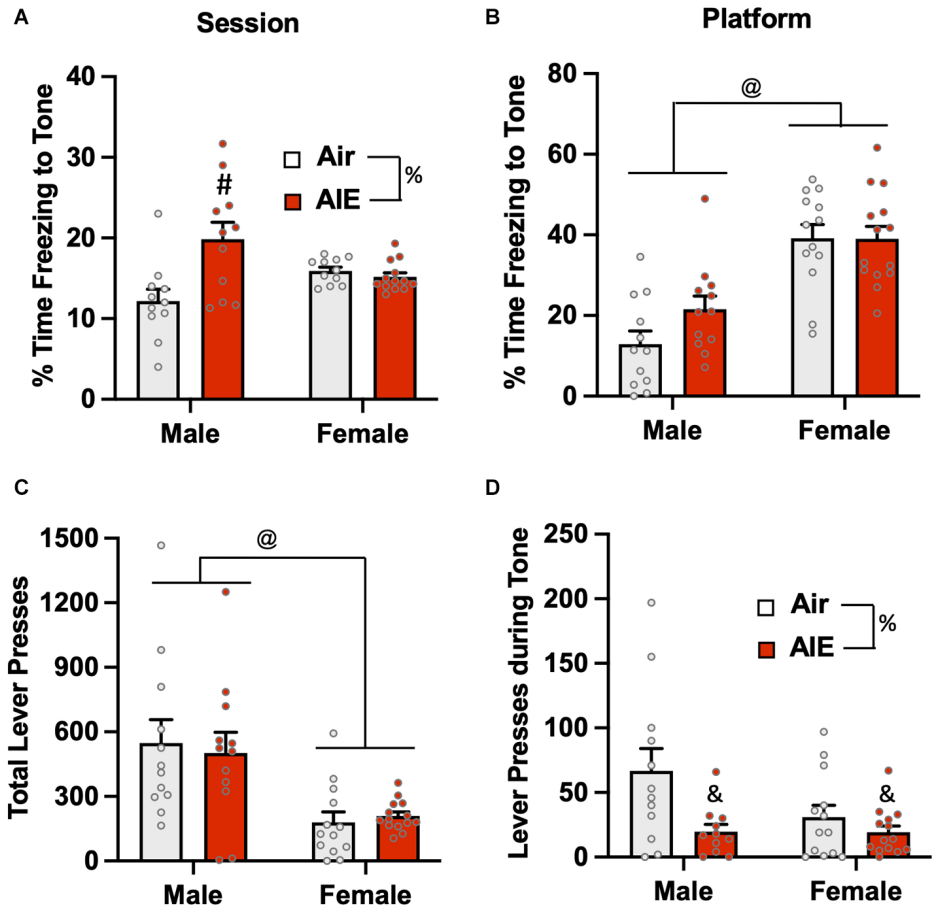

FIGURE 7
Freezing behavior and lever pressing on the last day of extinction of the platform-mediated avoidance task. **(A)** When freezing during tone presentation on the last day of extinction was examined irrespective of platform location, male AIE-exposed rats froze significantly more compared to male Air rats. **(B)** When analysis of freezing to the tone was restricted to the period when the rats were positioned on the platform, female rats froze significantly more than male rats. During the period, the significant increase in freezing in the male AIE-exposed rats compared to the male Air rats was no longer observed. **(C)** Examination of total lever responding during the test session revealed lever pressing by female rats was significantly reduced compared to male rats. **(D)** AIE-exposed male and female rats also exhibited reductions in lever pressing during presentation of the tone. Data represent the means ± sem (n = 12–13/group). $^{\%}$indicates a significant main effect of AIE, all p values < 0.01. $^{@}$indicates a significant difference between male and female rats, p = 0.000; $^{\&}$indicates significant difference of AIE group compared the their respective Air group, all p values < 0.05; $^{\#}$indicates significant difference between mail Air and AIE rats, p = 0.002.

female (p = 0.009) rats exhibited significantly lower levels of lever pressing during the tone compared to their respective Air control groups.

The final set of analyses examined extinction-dependent changes in percent time freezing in the Air control male and female rats as a function of Test day (baseline), Extinction Day 1, and Extinction Day 5 (**Supplemental Figure S3**). Mixed effect one-way ANOVAs with Tukey's *post-hoc* tests revealed no significant changes in freezing in either male or female rats when compared across days [Males: $F_{(1.409,13.39)}$ = 1.392, p = 0.2726; Females: $F_{(1.394,23.69)}$ = 2.868, p = 0.0922]. When assessed specifically during tone presentation, male but not female rats exhibited significant time-dependent reductions in freezing [Males: $F_{(1.767,19.44)}$ = 28.38, p < 0.0001; Ext Day 5 significantly different from both Test Day (p = 0.0001) and Ext Day 1 (p < 0.0035), Test Day significantly different from Ext Day 1 (p = 0.0055)] [Females: $F_{(1.862,22.35)}$ = 3.753, p = 0.4908].

Together, the results from analysis of the last day of extinction revealed significant differences in avoidance behavior as a function of both sex and a history of AIE. Both active and passive avoidance in female rats was extinguished to a much less degree than in the male rats, which was also reflected by a reduction in lever pressing for the reward. Conversely, AIE exposure reduced the extinction of both active and passive avoidance only in male rats.

Discussion

Preclinical studies investigating the long-term effects of adolescent alcohol exposure indicate that adult rats that had been subjected to repeated episodes of binge-like alcohol intoxication during adolescence display signs of increased anxiety-like behaviors and deficits in decision-making (Nasrallah et al., 2009; Clark et al., 2012; Gass et al., 2014; Schindler et al., 2014; Pandey et al., 2015; Crews et al., 2016; Barker et al., 2017; Chandler et al., 2022). There is also evidence that certain types of experiences during critical periods

of adolescence can alter brain development and circuit maturation and lead to permanent changes in adult behavior, including the expression of threat responding (Dow-Edwards et al., 2019; Gerhard et al., 2021). The present study examined the impact of adolescent binge-like alcohol exposure in male and female rats on behavioral responding in a reward-avoidance conflict task. This task required animals to balance the competing motivation to retrieve a reward against the motivation to avoid receiving a shock. Our results revealed that female rats exhibit elevated levels of both active avoidance and innate freezing behavior compared to male rats, and further revealed a sex-specific effect of AIE on threat responding in adulthood (summarized in **Table 1**).

The active avoidance task we employed involved an initial training period during which the rats learned to avoid a foot-shock by moving onto an escape platform in response to the presentation of a warning signal (tone). During training, both male and female rats rapidly acquired active avoidance as evidenced by a progressive increase in time spent on the platform during the presentation of the shock predicting tone. There were no differences between males and females or Air and AIE-exposed rats in the percentage of time spent on the platform, the percentage of time spent freezing, or the total number of reward lever presses. This lack of effect of AIE on these behavioral measures during task training is in agreement with previous studies indicating that AIE exposure minimally impacts basic learning processes (including responding to sucrose reward) that do not tax higher-order cognitive resources (Coleman et al., 2011; Semenova, 2012; Acheson et al., 2013; Risher et al., 2013; Gass et al., 2014; Towner and Spear, 2021). While studies using the PMA task have previously observed a reduction in freezing during the acquisition of platform avoidance (Bravo-Rivera et al., 2014), we did not observe changes across training in the percentage of time rats froze, nor did we see a change in total reward lever pressing across training. Since rats are unable to access the reward lever while positioned on the platform, it is interesting that there was not a reduction in reward lever pressing across training in spite of the progressive increase over training days in the time spent on the platform. A likely explanation for this is that the rats compensated for a reduction in the time of lever access by increasing reward-seeking during the inter-trial intervals, which may reflect the optimization of a reward-avoidance behavioral strategy.

Following the completion of active avoidance training, a test session was conducted to examine retrieval of the avoidance memory. Analysis of time spent on the platform during the entire test session or during the tone period only revealed that AIE-exposed male rats spent significantly more time on the platform compared to male Air control rats. In contrast, the percentage of rats on the platform during the shock interval indicated that all groups of rats equally remained on the platform during the period of time when the shock would have been delivered. The fact that AIE-exposed male rats spent the majority of their time on the platform during the retrieval test session is consistent with a significant reduction in reward-seeking in these rats compared to the male Air control rats. The time-course of platform escape during the conditioned tone also revealed treatment and sex differences in recall of learned active avoidance behavior. This difference was primarily observed during the initial period of the tone when the male Air rats displayed a delay in moving onto the platform. This was in contrast to the male AIE-exposed animals that either rapidly relocated to the platform upon tone presentation or were already positioned on the platform prior to initiation of the tone. As expected based upon the fact that the rats were on the platform nearly the entire time of the tone presentation, reward lever pressing during this period was virtually non-existent.

It is of interest that the results of previous studies regarding the effects of adolescent alcohol exposure on anxiety-like behaviors in adult rats appear to depend upon the strain of the rat being studied. For example, adult male Sprague-Dawley rats that had been subjected to AIE exhibited increased time in the closed arms of the elevated plus maze (EPM; Pandey et al., 2015; Kyzar et al., 2019), whereas adult male Wistar and Long-Evans rats that had undergone AIE exposure displayed increased time in the open arms of the EPM (Gilpin et al., 2012; Gass et al., 2014). A potential explanation for these seemingly opposing observations is that while AIE exposure may have enhanced anxiety across rat strains, there are strain-specific differences in their threat-coping mechanisms. The increase in open arm time on the EPM by AIE-exposed Long-Evans rats may reflect a bias towards disinhibition and increased escape responding. Such an effect would be consistent with the AIE-induced enhancement of active avoidance in the current study that was also carried out in Long-Evans rats.

Extinction of conditioned fear is a form of behavioral flexibility commonly investigated in animal models (Bouton et al., 2021). When a conditioned stimulus (e.g., auditory tone) is presented repeatedly in the absence of the unconditioned stimulus (e.g., foot-shock), extinction learning is observed as a gradual reduction of the conditioned response (Myers and Davis, 2007). Extinction is not simply the forgetting of a previously learned association, but instead involves the formation of a new extinction memory that competes with the original fear memory for the control of behavior (Quirk and Mueller, 2008; Gass and Chandler, 2013; Bouton et al., 2021). The relative strengths of the fear and extinction memories can be manipulated to influence which memory controls behavioral

TABLE 1 Summary of effect of sex and AIE exposure on active avoidance and freezing behaviors across acquisition, test day, and extinction.

Sex/Condition	Behavior	Acquisition	Test Day	Extinction day 1	Extinction day 5
Males:	Avoidance	-	↑	-	↑
Effect of AIE	Freezing	-	-	-	↑
Females:	Avoidance	-	-	-	-
Effect of AIE	Freezing	-	-	↓	-
Females compared	Avoidance	-	-	-	↑
to Males	Freezing	-	↑	↑	↑

Significant increases or decreases as a function of treatment (AIE compared to Air) or sex (females compared to males) are indicated by upward or downward arrows, respectively ($p < 0.05$). Active avoidance represents changes time on the platform during the entire session or during the tone. Freezing represents changes in freezing during tone presentation across the entire session or while on the platform.

responding. For example, increasing the number of extinction trials increases the relative strength of the extinction memory compared to the strength of the original fear memory. In the case of the PMA task, there is also a motivational conflict between the desire to avoid a shock and the motivation to obtain a reward, and this motivational component can impact extinction learning. It should be noted that in order for the rats to undergo avoidance extinction in the PMA task, they must learn that the shock is no longer being delivered, which can only occur if the animal is not on the platform during the shock interval. Thus, the additional component of motivated reward-seeking in the PMA task may facilitate the rate of extinction learning by enhancing the incidence of platform off-time during the shock interval (i.e., more opportunities to extinguish the CS-US association). The fact that the percentage of rats on the platform during the shock interval rapidly declined on the first day of extinction (compared to the preceding test day) indicates that rats were able to quickly assess that a shock was no longer being delivered at the end of the conditioned tone.

Our results also reveal a striking difference in the extinction of active avoidance between male and female rats in the PMA task. On the first day of extinction training, both male and female Air control rats spent a similar percentage of time on the platform during the conditioned tone (78.1% for males and 79.5% for females). However, by the last day of extinction, male rats exhibited a large reduction in the percent time spent on the platform during the tone, while the percentage of time the female rats were on the platform did not significantly change (21.0% for males and 71.9% for females). These sex differences were also reflected in the percentage of rats that remained on the platform during the SI. The percentage of male Air control rats on the platform during the SI dropped from 50% on the first day of extinction to 0% on the last day of extinction. In contrast, the percentage of female Air control rats on the platform during the shock interval did not change during extinction training (63% on both the first and last day of extinction). Taken together, these observations indicate that female rats were more resistant to the extinction of active avoidance compared to male rats. While the percentage of time male and female Air control rats spent freezing during each daily session did not change across training, the percent freezing by males was significantly lower when they were on the platform. In contrast, there was no change in the freezing across extinction by females when assessed during either the session or while located on the platform. It should be noted that an interpretation of sex-dependent differences in the extinction of freezing behavior specifically during tone presentation may be confounded by the fact that male rats spent comparatively little time on the platform by the end of extinction training. Our results also revealed sex-specific effects of AIE exposure on extinction learning. While there were no differences in active avoidance between Air and AIE-exposed rats on the first day of extinction training, by the last day of training the AIE-exposed male (but not female) rats spent significantly more time on the platform compared to the Air male rats. A similar sex-specific effect was observed with the extinction of freezing behavior. Together, our observations suggest that AIE exposure promoted extinction-resistant avoidance behavior that was specific to males. However, the interpretation of a selective effect of AIE on extinction learning in males may also by confounded by the fact that female rats, regardless of treatment, were substantially more resistant to extinction.

As preclinical studies of fear-related behaviors have traditionally been carried out in male rats, there is a substantial gap in our knowledge of sex differences in threat responding. While the limited number of studies involving traditional fear-conditioning and extinction paradigms have yielded some conflicting conservations (Velasco et al., 2019), they tend to suggest that female rats display a reduction in the acquisition of contextual fear conditioning compared to males (Maren et al., 1994; Pryce et al., 1999; Wiltgen et al., 2001; Kosten et al., 2005; Gresack et al., 2009; Ribeiro et al., 2010). In agreement with this, we recently reported that female Long-Evans rats exhibited lower levels of freezing during fear conditioning, more rapid extinction of freezing behavior, and lower levels of freezing during fear recovery compared to male rats (Chandler et al., 2022). With active avoidance paradigms, some studies suggest that females rats may adopt more active coping strategies compared to male rats and that female rats exhibit an increase in risk-aversive behavior, which may promote increases in active avoidance compared to male rats (Beatty and Beatty, 1970; Steenbergen et al., 1990; Lalanza et al., 2015; Orsini et al., 2016, 2021; Shanazz et al., 2022). Although speculative, these increases may contribute to enhanced vulnerability to anxiety-related disorders observed in women (Li and Graham, 2017; Day and Stevenson, 2020). The results of the present study utilizing the PMA task are consistent with the idea that female rats exhibit increased active avoidance of a threat compared to male rats. Our results also suggest that AIE may have sex-specific effects on threat-coping strategies in males such that the AIE-exposed male adult rats resemble females in adopting higher levels of active avoidance behavior. However, a caveat to this interpretation is that there may have been a ceiling effect in female rats that prevented any further enhancement of active avoidance responding.

Heightened threat appraisal is thought to be a major driver of maladaptive behavior that includes extinction-resistant avoidance. Enhanced fear and anxiety, such as has been observed following AIE, have been associated with overestimation of the danger posed by a threat (Ball and Gunaydin, 2022). Therefore, the interpretation of extinction-resistant avoidance exhibited by the male rats could also have been confounded by an apparent increase in threat avoidance that was observed on the test day prior to the initiation of extinction training. Thus, an increase in threat appraisal in association with a stronger avoidance memory might be expected to be more difficult to extinguish. However, the fact that both Air and AIE-exposed rats were equally on the platform during the SI may argue against an AIE-induced increase in threat appraisal. In addition to threat appraisal, another potential contributing factor in the apparent expression of extinction-resistant avoidance could be the development of habitual avoidance behavior (Pittig et al., 2020; Manning et al., 2021; Ball and Gunaydin, 2022). Habitual avoidance can emerge during extended periods of training if the avoidance response is repeatedly reinforced, as might occur across the 10-day training period of the PMA task. Using a modified version of the PMA task that involved "extinction-with-response prevention," it was recently reported that rats that had undergone prolonged avoidance training displayed habit-like impairments in extinction avoidance (Martinez-Rivera et al., 2020). We and others have previously shown that AIE exposure facilitates the development of habitual responding for a natural reward in female but not male rats (Barker et al., 2017; Towner and Spear, 2021). It is tempting to speculate that the extinction-resistant avoidance that we observed in female rats compared to male rats may reflect the development of habitual avoidance across the 10-day training period. However, while it is difficult to disentangle the relative contributions of threat

appraisal and habitual avoidance to extinction given the experimental design of the PMA task used in the current study, the fact that the percent time freezing to the tone on the last day of extinction was greater in the female rats compared to the male rats may argue for heightened threat appraisal instead of increased habitual behavior as a contributing factor to extinction-resistant avoidance of the female rats.

In summary, the present study demonstrated that female rats exhibit elevated levels of active avoidance and freezing compared to males, and a sex-specific impact of AIE on threat responding in adulthood. These observations add to a growing body of evidence obtained from both human and animal studies that adolescent alcohol exposure can result in long-term alterations in adult behavior.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary materials**, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee approved protocols at the Medical University of South Carolina.

Author contributions

LC and JL jointly designed the experiments, performed the statistical analysis, interpreted the data, generated the figures, and wrote the manuscript. JL performed the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.1098343/full#supplementary-material.

SUPPLEMENTARY FIGURE 1
Active avoidance and freezing behavior assessed across the acquisition period of the platform-mediated avoidance task. **(A,B)** Male and female rats exhibited progressive increases in active avoidance (percent time located on the platform) over the time-course of training days. In contrast, the percent time freezing during tone presentation **(C,D)** and lever pressing **(E,F)** remained relatively stable over the time-course of training. A history of AIE exposure had no effect on either active or passive avoidance, or on lever pressing across training days. Data represent the means ± sem (n = 12–13). *indicate a significant main effect of time (acquisition days), all p values < 0.01.

SUPPLEMENTARY FIGURE 2
Time-course of reduction in active avoidance across extinction training in male and female Air rats. Male and female rats exhibited progressive reductions in percent time on the platform when assessed across baseline (Test day), Extinction Day 1, and Extinction Day 5. This reduction was observed in the percent time on the platform during the entire session **(A,B)** and during the tone presentation period only **(C,D)**. Data represent the means ± sem (n = 12–13). %indicates significantly different from all other days, all p values < 0.05; *indicates significantly different from Test day only, all p values < 0.05; #indicates significantly different from all other days, all p values < 0.001.

SUPPLEMENTARY FIGURE 3
Differential changes in freezing across extinction training in male and female control rats. When assessed during the entire session, there were no significant changes in percent freezing during tone presentation when accessed across baseline (Test day), Extinction Day 1, and Extinction Day 5 in either male **(A)** or female **(B)** rats. When was assessed when the rats were located on the platform, freezing during tone presentation was significantly reduced in male **(C)** but not female **(D)** rats. Data represent the means ± sem (n = 12–13). *indicates significantly different from all other days, all p values < 0.001.

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Sexually dimorphic development of the mesolimbic dopamine system is associated with nuanced sensitivity to adolescent alcohol use

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Alcohol use remains a major public health concern and is especially prevalent during adolescence. Adolescent alcohol use has been linked to several behavioral abnormalities in later life, including increased risk taking and impulsivity. Accordingly, when modeled in animals, male rats that had moderate alcohol consumption during adolescence exhibit multiple effects in adulthood, including increased risk taking, altered incentive learning, and greater release of dopamine in the mesolimbic pathway. It has been proposed that alcohol arrests neural development, "locking in" adolescent physiological, and consequent behavioral, phenotypes. Here we examined the feasibility that the elevated dopamine levels following adolescent alcohol exposure are a "locked in" phenotype by testing mesolimbic dopamine release across adolescent development. We found that in male rats, dopamine release peaks in late adolescence, returning to lower levels in adulthood, consistent with the notion that high dopamine levels in adolescence-alcohol-exposed adults were due to arrested development. Surprisingly, dopamine release in females was stable across the tested developmental window. This result raised a quandary that arrested dopamine levels would not differ from normal development in females and, therefore, may not contribute to pathological behavior. However, the aforementioned findings related to risk-based decision-making have only been performed in male subjects. When we tested females that had undergone adolescent alcohol use, we found that neither risk attitude during probabilistic decision-making nor mesolimbic dopamine release was altered. These findings suggest that different developmental profiles of the mesolimbic dopamine system across sexes result in dimorphic susceptibility to alcohol-induced cognitive and motivational anomalies exposure.

KEYWORDS

adolescent alcohol use, decision making, dopamine, adolescent development, nucleus accumbens

Introduction

Alcohol remains the most frequently used substance among adolescents, and in this age group, there is a prevalence of high levels of binge drinking (Lees et al., 2020). During adolescence, cortical and limbic regions undergo major plastic changes that are sensitive to the harmful effects of toxic substances like alcohol (Spear, 2000; Chambers et al., 2003; Crews et al., 2007, 2016; Bava and Tapert, 2010; Marinelli and McCutcheon, 2014) resulting in long-lasting behavioral changes (Casey and Jones, 2010). Specifically, adolescent alcohol use (AAU) is associated with later-life deficits in adaptive decision making, impulsivity, and reward valuation (Goudriaan et al., 2007; Johnson, 2008; Brevers et al., 2014) and so it has been proposed that AAU arrests neural development, locking in adolescent phenotypes (Spear, 2000; Crews et al., 2019). Some of these behavioral alterations have been modeled in rodents. For example, male rats with a history of AAU have higher risk attitudes when tested on probabilistic reward tasks (Nasrallah et al., 2009). Notably, the same level of alcohol exposure during adulthood did not produce a change in risk attitude (Schindler et al., 2014), supporting the notion that there is a unique window of vulnerability during adolescence. Commensurate to this behavioral change, increased mesolimbic dopamine signaling has been reported during reward-related behaviors (Nasrallah et al., 2011; Spoelder et al., 2015), and in response to physiological stimulation (Schindler et al., 2016; Kruse et al., 2017).

Based upon this premise, we hypothesized that dopamine transmission peaks during adolescence in normal development but, following AAU, remains high into adulthood, promoting risk taking (and potentially other impulsive behaviors). Therefore, we tested evoked mesolimbic dopamine release across developmental time points, using stimulation procedures that discern neuronal terminal or cell-body mechanisms of potential age differences.

Methods

Animal and housing

Sprague-Dawley rats (39 females, 32 males; Charles River, Hollister, CA, USA) began experimental procedures at Post Natal Day (PND) 25 (gavage) or 27 (gelatin) for animals exposed to alcohol, or PND 30, 50, or 120 for neurochemical experiments without alcohol exposure. Rats were housed in polycarbonate tubs on a 12-h light/dark cycle (lights on a 06:00) for one week before this date. Water and rodent chow (Teklad, Harlan, Kent, WA, USA) was available *ad libitum* except as noted. All work in this manuscript was approved by the Institutional Animal Care and Use Committee of the University of Washington.

Alcohol preparation, administration, and withdrawal

Alcohol administration through the voluntary consumption of gelatin containing alcohol was presented to adolescent rats (PND 30–50) in a gel matrix consisting of distilled water (76.67%), Knox Gelatin (3.33%), polyose (10%), and 190 proof ethanol (10%), whereas the control gelatin contained distilled water in place of ethanol. Preparation was as described (Rowland et al., 2005; Nasrallah et al., 2011; Schindler et al., 2014). Gels were available 24 h a day with *ad libitum* access to food and water. Gel intake levels were measured daily and expressed in g/kg of body weight. All rats had access to only control gelatin for the first three days; after which rats were divided into either ethanol or control gelatin groups matched by weight and baseline intake for 20 days of assigned gelatin intake.

While this mode of administration produces enduring effects on cognition (Nasrallah et al., 2009, 2011; Schindler et al., 2014, 2016; Spoelder et al., 2015; Kruse et al., 2017), it does not achieve blood-alcohol concentrations that model heavy episodic drinking in adolescents. Therefore, we also utilized a second model of AAU that produces higher blood-alcohol concentrations (Crews et al., 2016). Adolescent intermittent ethanol administration via intragastric (IG), alcohol was presented to adolescent rats (PND 25–55) as a mixture of 190-proof ethanol and distilled water (16 g/kg, 20% ethanol, weight over volume). One cohort of rats received a single daily IG administration of ethanol and the other cohort received a single daily IG administration of distilled water (comparable volumes of water) on a 2-day on/off schedule Animals were then weighed and monitored daily.

For both IG and gelatin methods, after the last day of administration, the animals underwent three to four weeks of withdrawal and were monitored daily for withdrawal symptoms (e.g., seizures, weight loss, lack of grooming, and anxious behavior). It is important to mention that no overt signs of withdrawal were observed. Once the withdrawal period was completed, the rats began a food restriction diet of 90 $\pm$ 2% of their bodyweight and were exposed to 45 mg sucrose pellets (Bio-Serv, Frenchtown, NY) in their home cage to reduce neophobia. Additionally, prior to the start of the behavioral tasks, the rats underwent one magazine-training session in a standard operant chamber (Med Associates, St. Albans, VT) where they were given 15 min to consume 10 sucrose pellets in the magazine tray.

Probabilistic decision-making task

Risk attitude was tested in female and male rats using a probabilistic decision-making task. After magazine training was completed, rats were trained on an operant fixed ratio (FR) schedule to a criterion of >23 presses out of 20 total trials where one pellet was delivered following the depression of the left or right lever. Rats then underwent auto-shaping, where the rats were required to first nose-poke the magazine tray to begin the trail where the intertrial interval was increased from 0 to 15 s, the time to perform the trail initiating the poke was decreased to 10 s, and the intertrial interval was increased to 30 s.

Detailed methods for these and the following tasks can be found in previous publications (Nasrallah et al., 2009, 2011; Clark et al., 2012; Schindler et al., 2014). During the task, rats were presented with two levers flanking the magazine tray where one lever represented the certain lever (low-risk) and the other the uncertain lever (high-risk). The low-risk lever was associated with a certain (1.00) delivery of two sucrose pellets and the uncertain lever was associated with the probabilistic

(1.00, 0.75, 0.50, 0.25, and 0.00) delivery of four sucrose pellets. Each session consisted of 24 forced trials followed by 24 free choice trials where each probability presented on a different day decreased in descending order with an intertrial interval of 45 s. During the forced choice trials and following the trial initiation, a single lever would extend and the pressing of that lever resulted in the illumination of the tray light signaling the delivery of the associated reward based on the certainty of that lever and probability of that day; whereas following trial initiation during the free choice trials, both levers were extended with a total of 10 s for that rat to choose between the two levers.

After the probabilistic decision-making was completed, female control and ethanol rats underwent anesthetized surgeries with fast-scan cyclic voltammetry to measure pedunculopontine tegmental nucleus (PPT) and medial forebrain bundle (MFB) stimulated dopamine release in the nucleus accumbens (NAcc) as follows.

Non-survival voltammetry surgeries

To test dopamine transmission across adolescent development, dopamine release was evoked by MFB or PPT stimulation and measured with FSCV during non-survival surgeries in females and males during early adolescence (PND 30–35), late adolescence (PND 50–55) or adulthood (PND 120–125). Female adult rats from control and gelatin decision-making behavioral groups were also tested this way.

Rats were anesthetized with a 1.5 g/kg urethane (i.p.) and head-fixed in a Kopf stereotaxic instrument. The skull was exposed and burr holes were drilled targeting the NAcc (relative to bregma: 1.3 mm anterior and 1.3 mm lateral, MFB (relative to bregma: 4.6 mm posterior and 0.8 mm lateral), and PPT (relative to bregma: 8.0 mm posterior and 2.0 mm lateral). Another burr hole was drilled on the contralateral side for placement of the reference electrode (Ag/AgCl). A carbon-fiber microelectrode was centered above the NAcc burr hole and lowered 6.8–7.2 mm ventral from the top of the brain.

On completion of the experiment, current was passed through the voltammetry electrode to produce a lesion to aid histological identification of the recording location. Animals were then sacrificed using phenytoin/pentobarbital (Bethanasia) and their brains were harvested for histological analysis of the recording and stimulating electrode placement. On analysis of these data using one-way analysis of variance (ANOVA), there were no significant differences in electrode placement between experimental groups. Specifically, no differences were observed ($p < 0.05$) between working electrode placement in the NAcc (dorsal/ventral: $F_{(1,61)\ group} = 0.522$; medial/lateral: $F_{(1,60)\ group} = 0.9984$; anterior/posterior: $F_{(1,61)\ group} = 1.275$), stimulating electrode placement MFB (dorsal/ventral: $F_{(1,37)\ group} = 0.7424$; medial/lateral: $F_{(1,37)\ group} = 0.7336$; anterior/posterior: $F_{(1,37)\ group} = 0.8156$) or stimulating electrode PPT (dorsal/ventral: $F_{1,44)\ group} = 0.7081$; medial/lateral: $F_{(1,44)\ group} = 1.315$; anterior/posterior: $F_{(1,44)\ group} = 0.4166$) between experimental groups.

Fast-scan cyclic voltammetry recording

For recordings, a triangular waveform was applied to the carbon fiber starting at a potential of −0.4 V, ramping up to 1.3 V, and back down to −0.4 V (vs Ag/AgCl) at a rate of 400 V/s and a 10 Hz (held at −0.4 V between scans; Wanat et al., 2013; Schindler et al., 2016). A bipolar stimulating electrode was then incrementally lowered into either the PPT or MFB and NAcc dopamine release was evoked by electrical stimulation of the bipolar stimulating electrode at 60 pulses (p), 60 Hz, and 200 μA. Once maximum stimulated dopamine release was achieved, stimulations occurred at varying currents, pulses, and frequencies, and corresponding input-output curves were recorded.

For recording of the first input-output curve, the stimulation current was varied at 25, 50, 100, 150, 200, 300, and 400 μA respectively, while the pulse was held at 60 p and the frequency was held at 60 Hz. Next, the stimulation pulses were varied from 48, 42, 30, 18, 12, 6, and 3 p respectively, while the stimulation current was held at 400 μA and the frequency at 60 Hz. Stimulations and their corresponding recordings were performed with 5 min between each variation, including 5 min in between manipulation of pulse and current.

Statistical analyses

All statistical analyses were conducted using Prism 6 (GraphPad).

Stimulated dopamine release was analyzed with two-way mixed-measures ANOVA with stimulation current or the number of pulses as a within-subject, and age or treatment as a between-subject factor. Behavioral data for decision-making sessions were analyzed using a two-way mixed-measures analysis of variance using probability as a within-subject and treatment as a between-subject factor. For intragastric alcohol administration, data were analyzed using three-way mixed-measures ANOVA with probability as a within-subject and treatment and sex as between-subject factors. All data are presented as mean ± SEM and threshold for statistical significance was set at $p < 0.05$ within correction for multiple comparisons as appropriate.

Results

Stimulated dopamine release across development

To investigate developmental changes in the excitability of mesolimbic dopamine neurons, we measured dopamine release in the NAcc in rats aged 30 (early adolescence), 50 (late adolescence), or 120 (adulthood) days postnatally. First, we evoked dopamine release with electrical stimulation of the MFB using a range of stimulation parameters. Dopamine release was reliably detected, with increasing peak dopamine concentration to higher simulation current or number of pulses in males ($F_{(1.576,15.76)\ current} = 93.25$, $p < 0.0001$, **Figure 1A**; $F_{(1.540,15.40)\ current} = 80.27$, $p < 0.0001$, **Figure 1B**) and females ($F_{(1.266,12.66)\ current} = 31.79$, $p < 0.0001$,

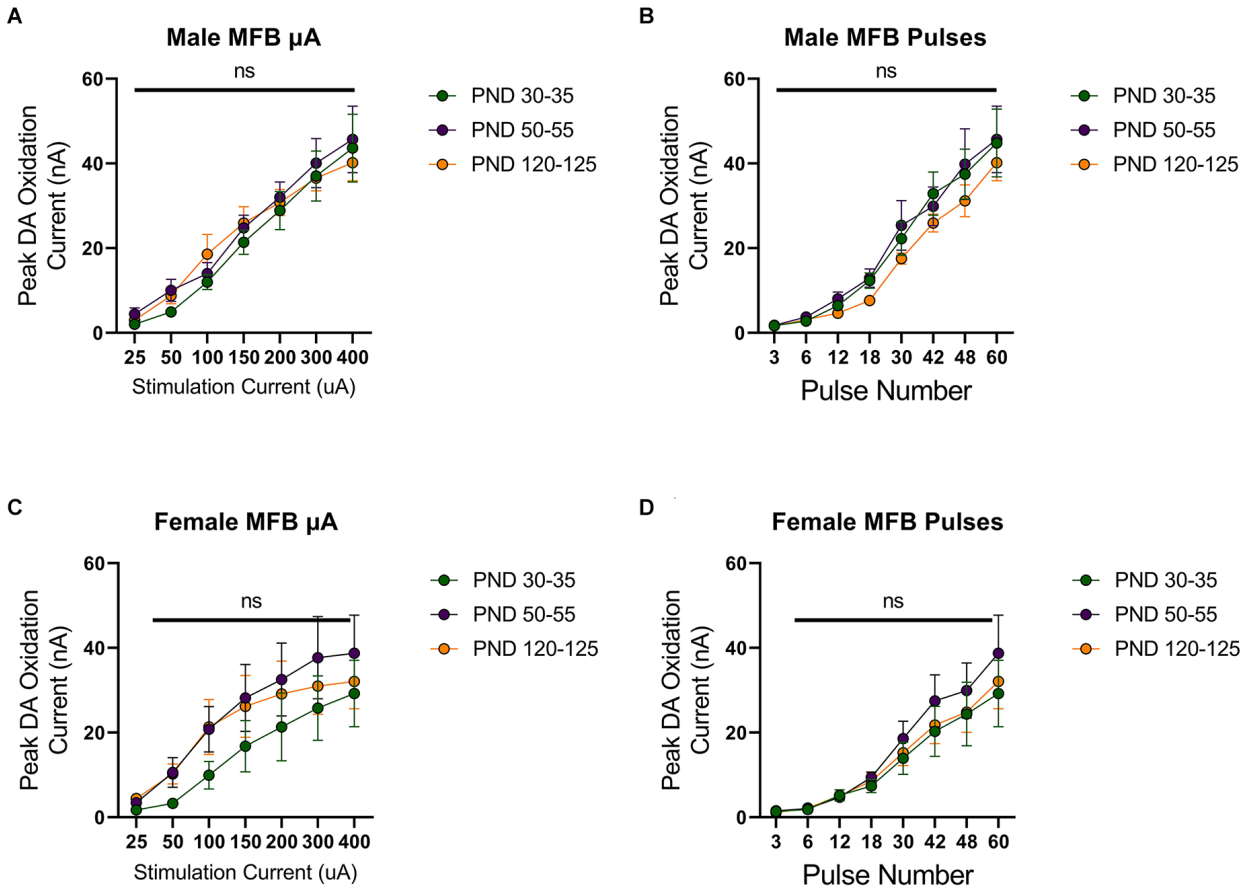

FIGURE 1
Peak dopamine (DA) oxidation current (nA) recordings in the nucleus accumbens (NAcc) over time after a single electrical stimulation of the medial forebrain bundle (MFB) in Males **(A,B)** and Females **(C,D)** averaged over varying stimulation currents **(A,C)** and pulses **(B,D)** at various ages. **(A)** Males' PND 50–55 (n = 5) did not significantly differ from PND 30–35 (n = 4) and PND 120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the MFB with increasing stimulation current. **(B)** Males' PND 50–55 (n = 4) did not significantly differ from PND 30–35 (n = 4) and PND 120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the MFB with increasing pulse number. **(C)** Females' PND 50–55 (n = 6) did not significantly differ from PND 30–35 (n = 4) and PND 120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the MFB with increasing stimulation current. **(D)** Females' PND 50–55 (n = 6) did not significantly differ from PND 30–35 (n = 4) and PND 120–125 (n = 6) in phasic DA release in the NAcc after stimulation of the MFB with increasing pulse number. All data are presented as mean ± SEM for the peak DA oxidation current according to stimulation current (25, 50, 100, 150, 200, 300, and 400 nA) and pulse number (3, 6, 12, 18, 30, 42, 48, and 60). ns $p > 0.05$.

Figure 1C; $F_{(1.942,19.42)\ current} = 35.09$, $p < 0.0001$, Figure 1D). However, these evoked dopamine-release profiles exhibited no significant differences across the tested developmental time points in males (current: $F_{(2,10)\ age} = 0.2164$, $p > 0.05$, $F_{(12,60)current \times age} = 0.5093$, $p > 0.05$, Figure 1A; pulses: $F_{(2,10)\ age} = 0.5778$, $F_{(14,70)current \times age} = 0.3545$, $p > 0.05$, Figure 1B) or females (current: $F_{(2,10)\ age} = 0.7016$, $F_{(12,60)current \times age} = 0.4490$, $p > 0.05$, Figure 1C; pulses: $F_{(2,10)\ age} = 0.4182$, $F_{(14,70)current \times age} = 0.3201$, $p > 0.05$, Figure 1D).

Stimulation of the MFB activates ascending dopamine axons and provides an assessment of presynaptic (terminal) function in the control of dopamine release. To also assess the excitability of dopamine neurons to afferent input, we stimulated the PPT to evoke dopamine release by transsynaptic stimulation. PPT stimulation consistently increased dopamine release in a current and pulse-number sensitive manner in males ($F_{(1.557,21.79)\ current} = 35.27$, $p < 0.0001$, Figure 2A; $F_{(1.259,12.59)\ current} = 29.33$, $p < 0.0001$, Figure 2B) and females ($F_{(2.015,28.21)\ current} = 30.13$, $p < 0.0001$, Figure 2C; $F_{(1.362,17.70)\ current} = 56.40$, $p < 0.0001$, Figure 2D). Under these conditions, differences emerged across the developmental time points. Specifically, there was not a main effect of age on dopamine release in males ($F_{(2,14)\ age} = 3.157$, $p > 0.05$, Figure 2A; $F_{(2,10)\ age} = 2.413$, $p > 0.05$, Figure 2B) but the pattern of dopamine release for increasing simulation current differed across development ($F_{(12,78)current \times age} = 2.598$, $p < 0.01$, Figure 2A) and pulse number ($F_{(14,70)current \times age} = 2.572$, $p < 0.01$, Figure 2B), with the highest dopamine release during late adolescence (PND 50–55). However, there were no significant differences between developmental time points in females (current: $F_{(2,14)\ age} = 1.249$, $p > 0.05$, $F_{(12,84)current \times age} = 0.7699$, $p > 0.05$, Figure 2C; pulses: $F_{(2,13)\ age} = 1.823$, $p > 0.05$, $F_{(14,91)current \times age} = 1.267$, $p > 0.05$, Figure 2D). When directly comparing females and males, significant sex differences were not observed for MFB stimulation ($F_{(1,20)\ sex} = 2.431$, $p > 0.05$, $F_{(2,20)\ age} = 0.4258$, $p > 0.05$, $F_{(2,20)sex \times age} = 0.1330$, $p > 0.05$, Figure 3A). For

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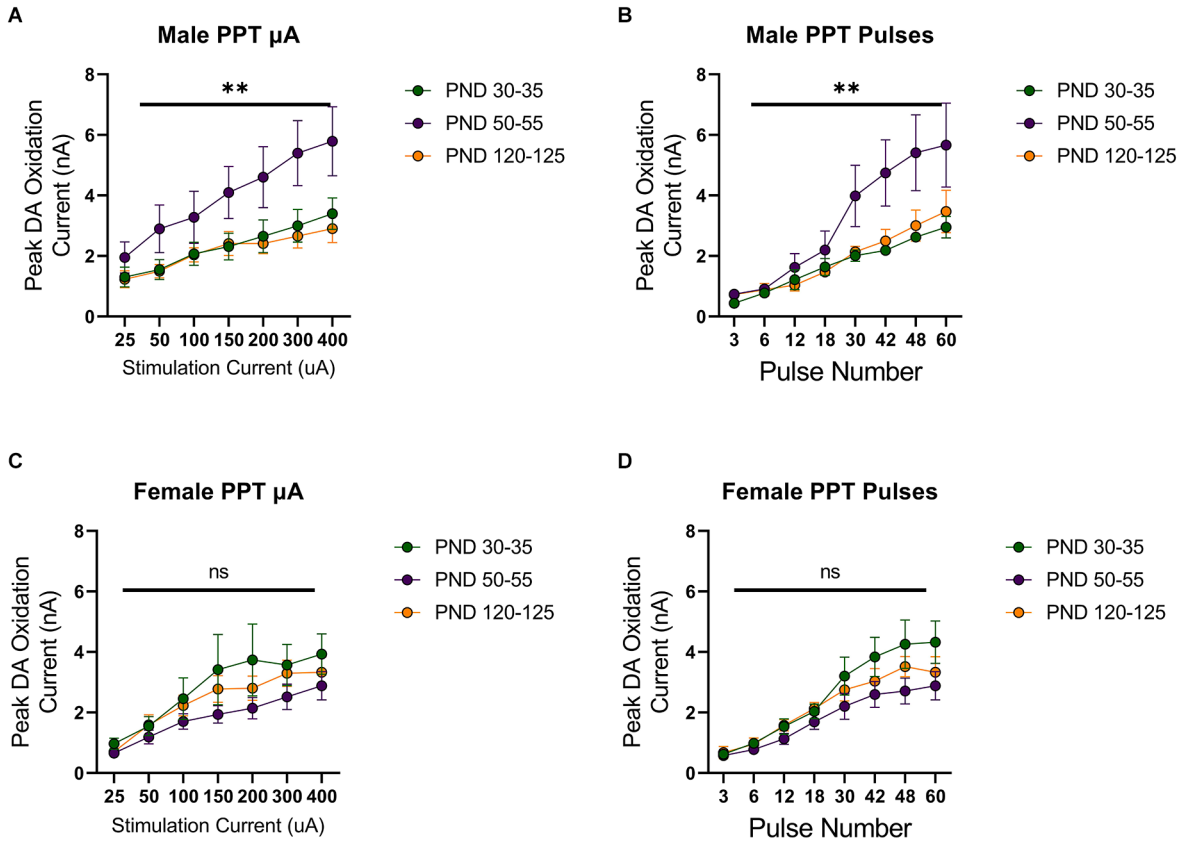

FIGURE 2
Peak DA oxidation current (nA) recordings in the NAcc over time after a single electrical stimulation of the pedunculopontine tegmental nucleus (PPT) in Males **(A,B)** and Females **(C,D)** averaged over varying stimulation current **(A,C)** and pulses **(B,D)** at various ages. **(A)** Males' PND 50–55 (n = 6) significantly differed from PND 30–35 (n = 7) and PND 120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the PPT with increasing stimulation current. **(B)** Males' PND 50–55 (n = 5) significantly differed from PND 30–35 (n = 4) and PND 120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the PPT with increasing pulse number. **(C)** Females' PND 50–55 (n = 6) significantly differed from PND 30–35 (n = 4) and P120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the PPT with increasing stimulation current. **(D)** Females' PND 50–55 (n = 5) significantly differed from PND 30–35 (n = 4) and PND 120–125 (n = 4) in phasic DA release in the NAcc after stimulation of the PPT with increasing pulse number. All data are presented as mean ± SEM for the peak DA oxidation current according to stimulation current (25, 50, 100, 150, 200, 300, and 400 nA) and pulse number (3, 6, 12, 18, 30, 42, 48, and 60). $^{**}p < 0.01$ and ns $p > 0.05$.

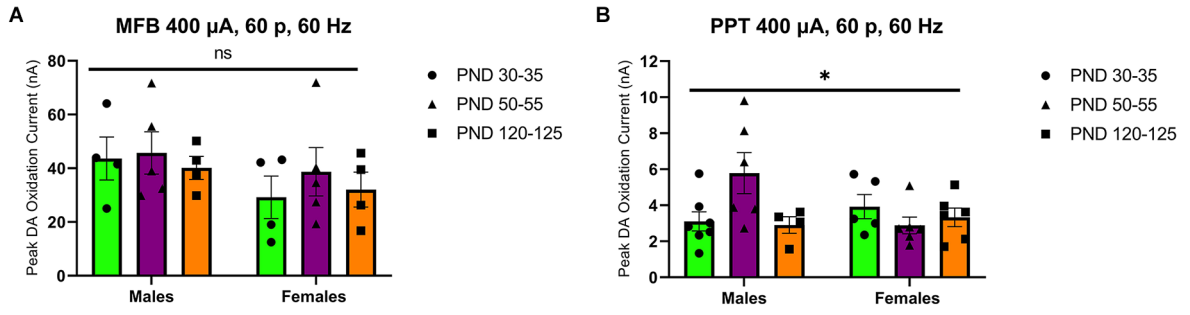

FIGURE 3
Peak DA oxidation current (nA) recordings in the NAcc for electrical 60-pulse stimulation (60 Hz, 400 µA) of the MFB **(A)** or PPT **(B)**. The pattern of NAcc dopamine release across development did not significantly differ between sexes (n = 13 per sex) for MFB stimulation **(A)** but was significant between females (n = 14) and males (n = 14) following PPT stimulation **(B)**. All data are presented as mean ± SEM. $^{*}p < 0.05$ and ns $p > 0.05$ (sex × age interaction).

PPT stimulation, there were no significant main effects of sex ($F_{(1,28)\,\text{sex}} = 0.9110$, $p > 0.05$) or age ($F_{(2,28)\,\text{age}} = 1.552$, $p > 0.05$), but there was a significant interaction between these factors ($F_{(2,28)\text{sex} \times \text{age}} = 4.444$, $p < 0.05$, Figure 3B). These data indicate sex differences in the developmental profile of dopamine neuron excitability which peaks in late adolescence in males but remains stable across the tested developmental time points in females.

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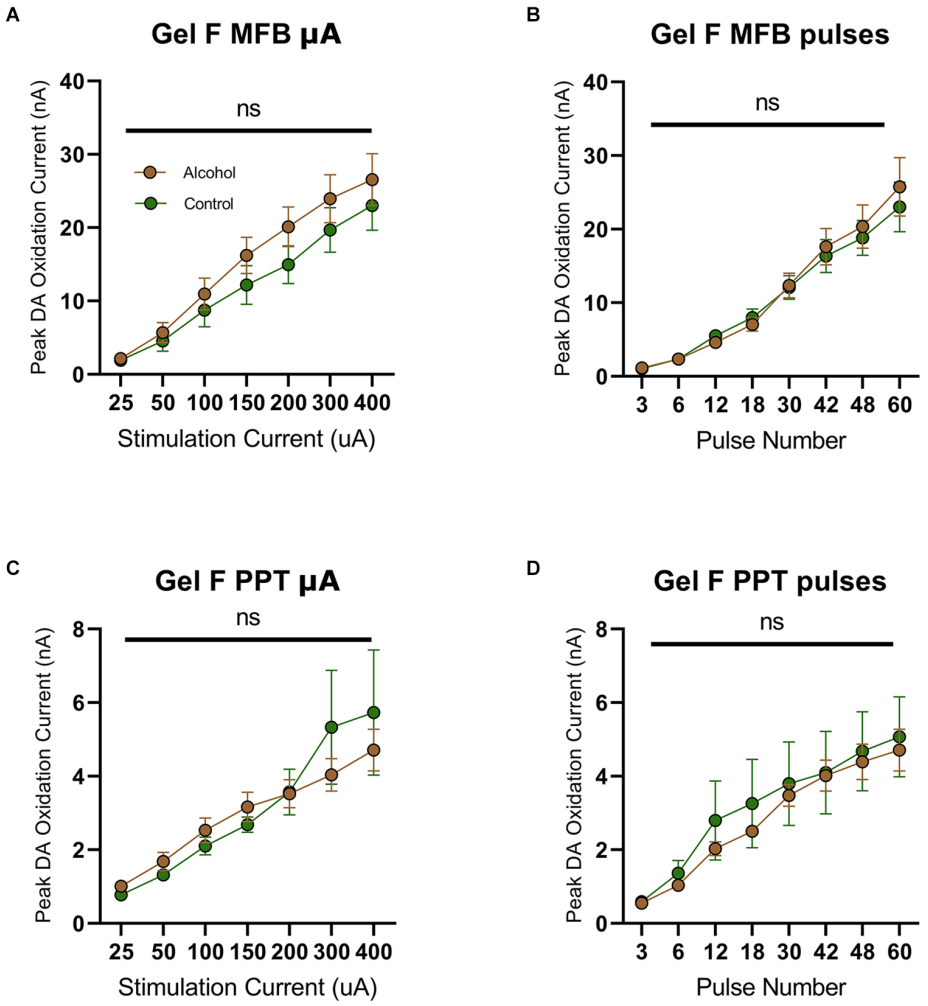

FIGURE 4
Peak DA oxidation current (nA) recordings in the NAcc over time after a single electrical stimulation of the MFB **(A,B)** and PPT **(C,D)** averaged over varying stimulation currents, pulses, and stimulation frequency for females. **(A)** Alcohol-exposed (n = 10) and control (n = 7) female animals did not significantly differ in phasic DA release in the NAcc after stimulation of the MFB with increasing current **(B)** Alcohol-exposed (n = 10) and control (n = 7) female animals did not significantly differ in phasic DA release in the NAcc after stimulation of the MFB with increasing pulse number. **(C)** Alcohol-exposed (n = 10) and control (n = 7) female animals did not significantly differ in phasic DA release in the NAcc after stimulation of the PPT with increasing stimulation current. **(D)** Alcohol-exposed (n = 10) and control (n = 7) female animals did not significantly differ in phasic DA release in the NAcc after stimulation of the PPT with increasing pulse number. All data are presented as mean ± SEM for the peak DA oxidation current according to stimulation current (25, 50, 100, 150, 200, 300, and 400 nA) and pulse number (3, 6, 12, 18, 30, 42, and 48). ns $p > 0.05$ for all.

Effect of adolescent ethanol exposure on stimulated dopamine release in adulthood in female rats

Previously, we observed increased dopamine release during adulthood in male rats that consumed alcohol during adolescence (Schindler et al., 2016). This effect has been attributed to the notion of "arrested development" where adolescent phenotypes are locked in following AAU (Crews et al., 2019). However, based on the current findings, an interesting dilemma arises since dopamine release was not elevated in females during adolescence (**Figure 3**). Following this line of reasoning, we would not anticipate elevated dopamine release in adult females following AAU. To test this hypothesis, we measured phasic dopamine release in the NAcc of adult female rats with a history of AAU. Female rats were given access to gelatin containing 10% ethanol or vehicle 24 h a day for 20 continuous days (PND 30–50) and then dopamine release was assessed by MFB or PPT stimulation during adulthood (PND 90–110). Similar to experiments in male rats. stimulation of the MFB ($F_{(1.706,25.59)\ \text{current}} = 51.09$, $p < 0.0001$, **Figure 4A**; $F_{(1.091,\ 16.36)\ \text{pulses}} = 70.77$, $p < 0.0001$, **Figure 4B**) or PPT ($F_{(1.171,17.57)\ \text{current}} = 22.71$, $p < 0.0001$, **Figure 4C**; $F_{(2.111,\ 31.96)\ \text{pulses}} = 36.96$, $p < 0.0001$, **Figure 4D**) reliably elicited phasic dopamine release in the NAcc. However, MFB stimulation did not evoke a significantly higher dopamine release in female rats with a history of AAU when compared to controls in response to increasing stimulation current ($F_{(1,15)\ \text{treatment}} = 1.031$, $p > 0.05$, $F_{(6,90)\text{current} \times \text{treatment}} = 0.5739$, $p > 0.05$, **Figure 4A**) or pulse number ($F_{(1,15)\ \text{treatment}} = 0.05355$, $p > 0.05$, $F_{(7,105)\text{pulse} \times \text{treatment}} = 0.3920$, $p > 0.05$, **Figure 4B**). Likewise, there was no significant difference in electrically evoked dopamine release in the NAcc due to PPT stimulation

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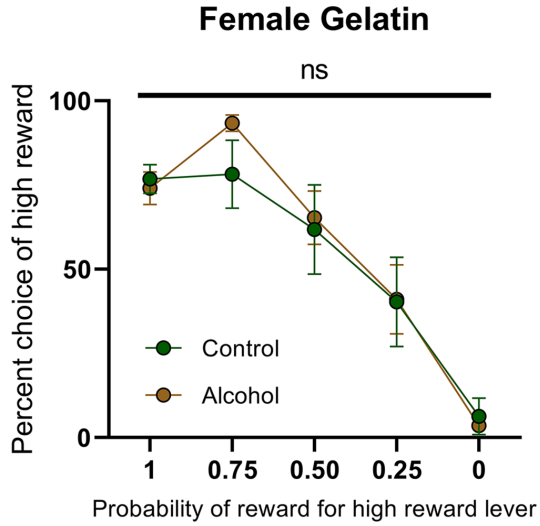

FIGURE 5
Psychometric function for choice over probability from alcohol-exposed and control animals. Alcohol-exposed (n = 8) and control (n = 8) animals did not differ in choice behavior over the probability of reward for high reward lever or in total reinforcements received. All data are presented as mean ± SEM for the percent of high choice reward according to the probability of reward for high reward lever of 1.00, 0.75, 0.50, 0.25, and 0.00. ns $p > 0.05$.

in female rats who had a history of AAU in response to increasing stimulation current ($F_{(1,15)\ \text{treatment}} = 0.03726$, $p > 0.05$, $F_{(6,90)\text{current} \times \text{treatment}} = 1.203$, $p > 0.05$, Figure 4C) or pulse number ($F_{(1,15)\ \text{treatment}} = 0.2302$, $p > 0.05$, $F_{(6,90)\text{pulse} \times \text{treatment}} = 0.2803$, $p > 0.05$, Figure 4D). These data demonstrate that females exposed to AAU do not show a comparable increased release of PPT stimulated dopamine to that previously observed in males (Schindler et al., 2016).

Effect of adolescent ethanol exposure on probabilistic decision-making in adult female rats

This failure to observe enduring changes in dopamine release following AAU in females provides an additional challenge on previous interpretations of AAU on cognition. The elevated dopamine in males is concomitant with an increased risk attitude during probabilistic decision making. However, the lack of elevated dopamine release in females questions whether the same behavioral changes would take place. Consequently, we trained female rats with AAU history (gelatin exposure described above) to perform probabilistic decision-making where animals choose between deterministic small rewards (two food pellets) and probabilistic large rewards (four food pellets). The probability of delivery of the large reward when chosen was systematically descended during each behavioral session (1.00, 0.75, 0.50, 0.25, 0.00). The frequency of choosing the large reward varied according to its probability of delivery ($F_{(4,55)\ \text{probability}} = 31.40$, $p < 0.0001$, Figure 5), but this pattern did not significantly differ between adult female rats with a history of AAU and their controls ($F_{(1,55)\ \text{treatment}} = 0.2968$, $p > 0.05$, $F_{(4,55)\ \text{treatment} \times \text{probability}} = 0.7999$, $p > 0.05$, Figure 5). This result is surprising given that significant differences have consistently been observed across numerous cohorts of male rats (Nasrallah et al., 2009, 2011; Clark et al., 2012; Schindler et al., 2014, 2016) and, therefore, identifies a potentially important sexual dimorphism in the impact of AAU.

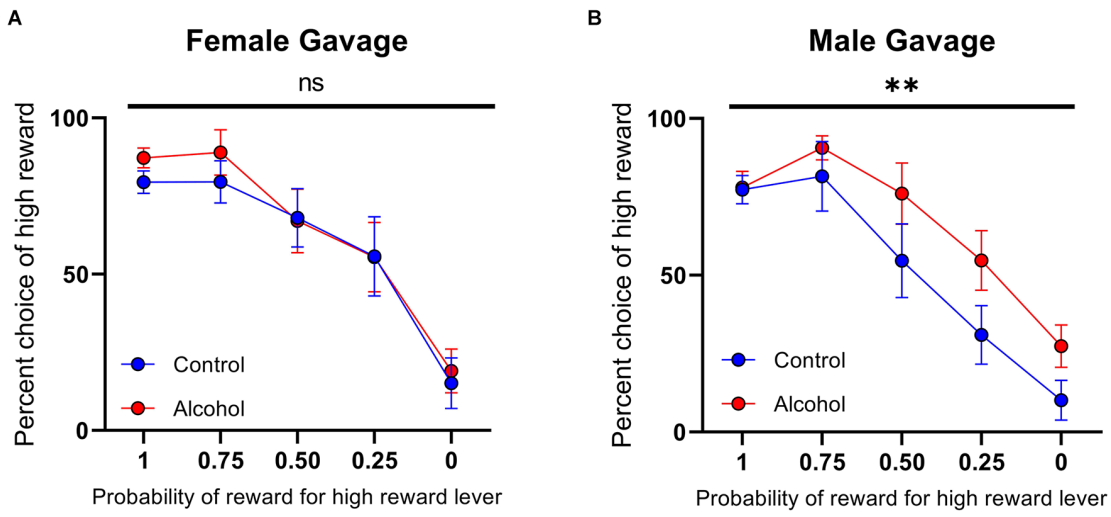

FIGURE 6
Psychometric functions for males and females receiving alcohol or vehicle via intragastric (IG). **(A)** Alcohol-exposed (n = 10) and control (n = 8) female animals did not differ in choice behavior over the probability of reward for high reward lever or in total reinforcements received. **(B)** Alcohol-exposed (n = 8) and control (n = 7) male animals did significantly differ in choice behavior over the probability of reward for high reward lever, but not in total reinforcements received. All data are presented as mean ± SEM for the percent of high choice reward according to the probability of reward for high reward lever of 1.00, 0.75, 0.50, 0.25, and 0.00. ** $p > 0.01$, ns $p > 0.05$.

Adolescent ethanol administration

Females that underwent the gelatin AAU model consumed 30.32 ± 1.96 g/kg/day (n = 12) of alcohol during adolescence. This value is considerably higher than male rats who underwent comparable training. For example, Schindler et al. (2016) reported 9.0 ± 1.2 g/kg/day in males ($t_{(28)}$ = 9.833, $p < 0.0001$, unpaired t-test vs. females in the current study). Nonetheless, this model results in only moderate blood ethanol concentrations (Schindler et al., 2014) without reaching levels of heavy episodic drinking. Therefore, to test whether the lack of effect of AAU on risk taking in females was due to a dosing issue we next used a different model of alcohol administration that produces blood-alcohol concentrations at binge levels. Ethanol (20% w/v) was administered intermittently (cycles of two days on and two days off) via intragastric gavage between developmental days PND 25 to PND 55. Again, animals' choices were sensitive to the probability of the high reward (females: $F_{(4,80)\ \text{probability}}$ = 20.91, $p < 0.0001$, **Figure 6A**; males: $F_{(4,65)\ \text{probability}}$ = 22.35, $p < 0.0001$, **Figure 6B**). Consistent with the gelatin model, the risk preference of female rats exposed to this treatment did not significantly differ from control rats on the probabilistic decision-making task ($F_{(1,80)\ \text{treatment}}$ = 0.5446, $p > 0.05$, $F_{(4,80)\text{probability} \times \text{treatment}}$ = 0.1471, $p > 0.05$, **Figure 6A**). In contrast, male rats that received ethanol by gavage during adolescence exhibited a significantly different pattern of decision-making with increased preference for large probabilistic rewards over small deterministic rewards ($F_{(1,65)\ \text{treatment}}$ = 7.764, $p < 0.01$, $F_{(4,65)\text{probability} \times \text{treatment}}$ = 0.6704, $p > 0.05$, **Figure 6B**). Accordingly, when females and males were compared directly, we found a significant effect of sex ($F_{(1,145)\ \text{sex}}$ = 5.920, $p < 0.05$). These data demonstrate that, regardless of the method of ethanol administration and the amount received, AAU leads to risky decisions making in adult males, but not in females.

Discussion

Here, we measured dopamine excitability across normal adolescent development. The experiments were designed to provide a platform to investigate the hypothesis that behavioral perturbations in adulthood arising from AAU are caused by alcohol-arresting post-adolescent neural development.

We observed a significant peak in dopamine neuronal excitability during the adolescent period in males. Therefore, if AAU arrests the development of this system then we would anticipate higher levels of dopamine during adulthood than in controls where dopamine excitability drops following adolescence. This prediction is consistent with our previous work demonstrating greater dopamine excitability during adulthood in animals that underwent AAU as compared to controls (Schindler et al., 2016). Moreover, the changes in dopamine across development were a result of mechanisms in the cell bodies rather than the terminals, as differences were not observed following axonal stimulation. Again, this change was consistent with altered dopamine release in adults following AAU (Schindler et al., 2016).

Conversely, in females, we did not observe changes in dopamine excitability across development. This lack of a peak in adolescent females was surprising, and seemingly refutes the idea that altered behavior in later life could be a result of arrested development of the mesolimbic dopamine system. However, this position assumes that AAU increases risk attitude during probabilistic decision-making in females like it does in males (Nasrallah et al., 2009, 2011; Clark et al., 2012; Schindler et al., 2014, 2016). In actual fact, the effect of AAU on probabilistic decision-making had never been tested in female rats. Indeed, in the current work, we failed to observe any difference in risk attitude between females that underwent AAU and controls. Because we were concerned that the lack of behavioral perturbation in females may be a dosing effect, then we repeated the experiment with an alternative method of alcohol administration that produced higher blood-alcohol concentrations. However, this approach again failed to produce altered risk-based decision-making in females despite being effective in male subjects. Therefore, females do not have elevated dopamine excitability in late adolescence; and alcohol use during this time does not produce elevated dopamine excitability, nor does it alter risk attitude, in adulthood. This pattern contrasts with males where dopamine excitability is elevated during late adolescence and drops in adulthood, but with alcohol use during this time, the elevated excitability is sustained into adulthood and animals exhibit a higher risk attitude during probabilistic (economic) decision making. While these concomitant neurochemical and behavioral findings are correlational, there has been a wealth of reports linking the NAcc (Kuhnen and Knutson, 2005; Zalocusky et al., 2016) and dopamine transmission (Fiorillo et al., 2003; Clark et al., 2012; Hart et al., 2015; Mortazavi et al., 2023) to risk taking, providing well-founded evidence for a causal relationship.

Our findings identify clear sex differences in the mesolimbic dopamine system during adolescent development and their correlation with the effect of AAU on later life physiology and behavior. However, it is not clear whether the dimorphic effects of alcohol are categorical, or whether they are quantitative effects. With regard to the dose of alcohol, females consumed a larger quantity of alcohol, during gelatin-based self-administration, than males in previous studies (Nasrallah et al., 2009, 2011; Clark et al., 2012; Schindler et al., 2014, 2016). Moreover, even with the much higher dosing achievable with gavage administration, females did not exhibit the altered risk taking observed in males undergoing the same alcohol dosing regimen. Therefore, it is unlikely that the sex differences are simply due to an insufficient dose of alcohol in the females. Another potential quantitative difference could be in the window of administration. Alcohol exposure took place over the same postnatal days in females and males, even though adolescent development is more advanced in females (Spear, 2000). However, alcohol administration by gavage was initiated earlier (PND 25) and extended to thirty days and still did not reveal an effect on risk-based decision-making in females. Of course, it is possible that this behavior in females may be susceptible to alcohol in the pre-adolescent postnatal period, but this developmental window was not tested in the current work. Nonetheless, given that males initiate alcohol use earlier on average than females—a trend that appears to be narrowing but is still significant in 2020 (White, 2020)—even if the female vulnerability is earlier in development, it would afford a distinct advantage to females at the population level.

Sex differences in the enduring effects of AAU have previously been observed (Bava and Tapert, 2010; Crews et al., 2016; Robinson et al., 2021; Maldonado-Devincci et al., 2022). Along with the current work, these data provide growing evidence for the nascent hypothesis that AAU can lock in adolescent cortical and limbic phenotypes and their downstream behaviors into adulthood, even when they are not comparable between sexes. This is particularly intriguing for traits that are sexually dimorphic in adolescence, but then normally converge in later life, since AAU could perpetuate these sex differences. Therefore, if the current findings translate to humans then the implication would be that females are protected against the effects of AAU on at least one cognitive process, specifically risk attitude.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by University of Washington Institutional Care and Use Committee.

Author contributions

LK, AS, and JC conceived the studies. AA, LK, and AS performed the studies. AA analyzed the data. AA and PP wrote the manuscript. AA, LK, AS, and PP edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Negative and positive allosteric modulators of the α7 nicotinic acetylcholine receptor regulates the ability of adolescent binge alcohol exposure to enhance adult alcohol consumption

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Rationale and Objectives: Ethanol acts directly on the α7 Nicotinic acetylcholine receptor (α7). Adolescent-binge alcohol exposure (ABAE) produces deleterious consequences during adulthood, and data indicate that the α7 receptor regulates these damaging events. Administration of an α7 Negative Allosteric Modulator (NAM) or the cholinesterase inhibitor galantamine can prophylactically prevent adult consequences of ABAE. The goals of the experiments were to determine the effects of co-administration of ethanol and a α7 agonist in the mesolimbic dopamine system and to determine if administration of an α7 NAM or positive allosteric modulator (PAM) modulates the enhancement of adult alcohol drinking produced by ABAE.

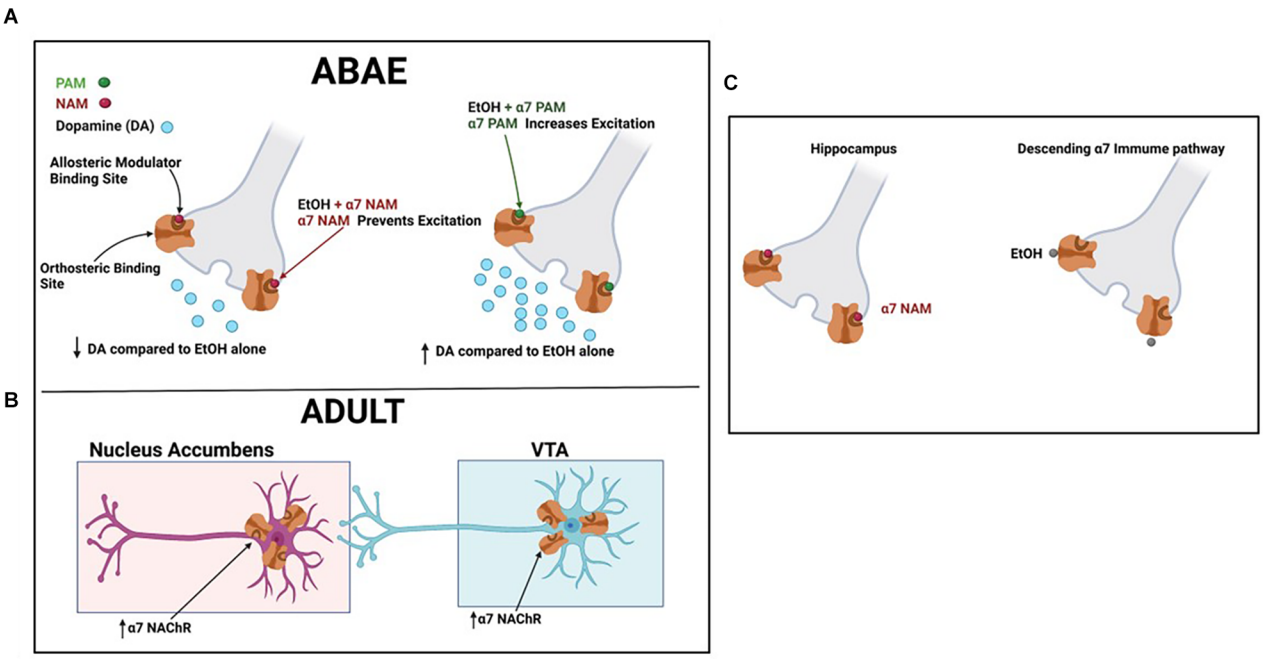
Methods: In adult rats, ethanol and the α7 agonist AR-R17779 (AR) were microinjected into the posterior ventral tegmental area (VTA), and dopamine levels were measured in the nucleus accumbens shell (AcbSh). In adolescence, rats were treated with the α7 NAM SB-277011-A (SB) or PNU-120596 (PAM) 2 h before administration of EtOH (ABAE). Ethanol consumption (acquisition, maintenance, and relapse) during adulthood was characterized.

Results: Ethanol and AR co-administered into the posterior VTA stimulated dopamine release in the AcbSh in a synergistic manner. The increase in alcohol consumption during the acquisition and relapse drinking during adulthood following ABAE was prevented by administration of SB, or enhanced by administration of PNU, prior to EtOH exposure during adolescence.

Discussion: Ethanol acts on the α7 receptor, and the α7 receptor regulates the critical effects of ethanol in the brain. The data replicate the findings that cholinergic agents (α7 NAMs) can act prophylactically to reduce the alterations in adult alcohol consumption following ABAE.

KEYWORDS

alpha7 acetylcholine receptor, dopamine, alcohol, adolescence, prevention



GRAPHICAL ABSTRACT
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Introduction

One could argue that the largest, most continuous voluntary neurodevelopment experiment has been humankind's alcohol consumption during adolescence. Throughout the world, multiple intervention programs have been conducted to reduce adolescent alcohol consumption, but the data indicate that the rate of adolescent alcohol consumption has remained a major public health concern (Patrick et al., 2017; Substance Abuse and Mental Health Services Administration (SAMHSA), 2018; Eisenberg et al., 2022; Kreski et al., 2022; Lines et al., 2022). The major epidemiological alteration in adolescent alcohol consumption is the increase in the overall rate of binge drinking [i.e., 4+/5+ drink/occasion (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2004)] during the transition from late adolescent/young adulthood into adulthood (Bell et al., 2014; Jager et al., 2015). The increased rate of binge drinking in adolescents/young adults led to the need to characterize high-intensity and extreme-intensity drinking in adolescents (Jager et al., 2015; Patrick and Terry-McElrath, 2017; Aiken et al., 2022). During the transition window from adolescence to young adult (21–26), 30% of US residents report recent bouts of binge drinking (Patrick et al., 2017; Kreski et al., 2022; Lines et al., 2022), while 11% report high-intensity drinking and 5% report extreme-intensity drinking (Patrick et al., 2017; Keyes et al., 2022).

The deleterious effects of adolescent/young adult alcohol binge drinking are numerous, diverse, and not limited to altering alcohol-related behaviors. Traditionally, the alcohol field has focused upon the well-replicated finding that adolescent alcohol consumption enhances adult alcohol consumption and the rate of adult alcohol use disorder (Chou and Pickering, 1992; Hingson et al., 2008). Specifically, epidemiological analyses have indicated that the risk of developing alcohol use disorder (AUD) is increased 1.3–1.6 times in individuals who initiate alcohol consumption before the age of 15 (Dawson et al., 2008). The effects of adolescent-binge alcohol exposure (ABAE) is not specific to alcohol consumption behaviors. Excessive adolescent alcohol consumption is associated with higher risk of developing mood disorders (depression and anxiety disorders), neurodegenerative diseases [e.g., Alzheimer's disease (AD), Parkinson's disorder (PD), other dementia-related Illnesses], other mental health disorders (schizophrenia), and auto-immune diseases during adulthood (Harwood et al., 2010; Langballe et al., 2015; Schwarzinger et al., 2018; Coleman et al., 2019; Barnett et al., 2022; Tucker et al., 2022). Therefore, developing interventions that prevent ABAE will benefit society by reducing adult rates of AUD and reducing the risk factors of several other disorders.

Neurologically, ABAE disrupts the normal remodeling of cortical and limbic regions that occurs during adolescence (Geidd, 2004). Replicable effects of adolescent alcohol consumption on the adult brain (Hauser et al., 2021a,b; Rahman and Bell, 2021) include alterations in the dopaminergic (hyper-dopaminergic) and cholinergic (reduction in ChAT, increase expression of the *Chrna7* in multiple brain regions), persistent modification of epigenetic factors, and alterations in the neuro- and peripheral immune systems (Spear and Swartzwelder, 2014; Mulholland et al., 2018; Hauser et al., 2019a, 2021a,b; Swartzwelder et al., 2019). The "time window" of ABAE is being worked out, and the present work is significant as it might extend several studies that suggest the effects of early-life exposures to environmental and psycho-social factors on later-life development of cognitive and neurological disorders, e.g., AD (Maloney and Lahiri, 2016). The initial perturbation is maintained and later further triggered through epigenetic mechanisms, i.e., the Latent Early-life Associated Regulation (LEARn) pathway, derived from studies

in rodents and primates (Lahiri et al., 2009; Wu et al., 2011).

The ABAE-induced adult hyper-dopaminergic system has been observed in multiple studies using distinct methodological approaches. Adolescent ethanol consumption or peripheral administration of ethanol increases basal dopamine levels and/or dopamine reuptake in the AcbSh during adulthood (Sahr et al., 2004). In contrast, comparable adult EtOH exposure does not produce similar effects (Pascual et al., 2007, 2009). Similarly, adolescent voluntary EtOH consumption resulted in a hyperdopaminergic response to nicotine during adulthood which was not observed following comparable adult EtOH consumption (Waeiss et al., 2019). In Wistar and alcohol-preferring (P) rats, ABAE results in a leftward and upward shift of the dose-response curve (increased sensitivity) for EtOH to stimulate dopamine release in the AcbSh after posterior ventral tegmental area (VTA) microinjection (Hauser et al., 2021b). ABAE-induced hyper-dopaminergic system during adulthood is thought to be part of the biological basis for the enhanced adult EtOH consumption observed following ABAE. Activation of the mesolimbic dopamine pathway is considered critical for the development of alcohol consumption and the progression to AUD (Rodd et al., 2004).

Exposure to a variety of drugs of abuse (alcohol, nicotine, opioid, and cocaine) during adolescence results in reduced ChAT expression during adulthood (Wilson et al., 1994; Abreu-Villaça et al., 2004). Recent data examining compensatory alterations in nicotinic acetylcholine receptors (NAChR) in response to the ABAE-induced reduction in ChAT has indicated an increased expression of *Chrna7* (and other NAChRs) in the posterior VTA and AcbSh (Hauser et al., 2019a, 2021b). Voluntary adolescent EtOH consumption in P rats increases by 2-fold the number of homomeric α7-immunoreactive (IR) NAChR neurons in the posterior VTA and increases the protein expression of the α7 receptors in the posterior VTA (Waeiss et al., 2019). Convergently, ABAE induces a significant increase in the *Chrna7* (α7) gene expression in the posterior VTA and the AcbSh (Hauser et al., 2019a, 2021b). In addition, adolescent intermittent ethanol caused a persistent increase in adult histone methylation at histone 3 lysine 9 dimethylation (H3K9me2) near the NTRK1 neurotrophic receptor tyrosine kinase 1 (NTRK1) gene and DNA methylation in promoter regions of ChAT, both of which were remediated by wheel running (Vetreno et al., 2020).

Research characterizing the α7 receptor has reported unique properties of the receptor. In humans, a chimeric gene (*CHRFAM7A*) that encodes a protein that acts as an innate α7 NAM (Araud et al., 2011). Polymorphisms of the *CHRFAM7A* gene (reduction in function) results in increase susceptibility to several diseases (e.g., schizophrenia; Akbarian and Kundakovic, 2015) observed following ABAE. Conversely, over-expression of the α7 is associated with a number of neuropsychiatric disorders. An increase in the expression of the α7 (approximately 30%, comparable to what is observed preclinically following ABAE treatment in rats) results in alterations in the expression of epigenetic factors and other genes, differential regulation of developmental signaling, and altered neurogenesis and synaptogenesis (c.f., Meganathan et al., 2021).

The α7 receptor is associated with non-canonical activation of neurons, immune, and other cells. On leukocytic T cells, the α7 receptor increases Ca^{2+} signaling through activation of a protein complex and TCR/CD3 (increasing protein tyrosine kinase; Razani-Boroujerdi et al., 2007). Phosphocholine-induced inhibition of IL-1b release through metabotropic signaling and protein coupling through the α7 (Richter et al., 2018). In the ventrohippocampal-striatal synapse, activation of the α7 results in sustained increase in glutamate release (Zhong et al., 2008). This α7 mediated effect is considered a main driver of facilitation of glutamatergic synaptic transmission which would increase GABA, acetylcholine, and dopamine release (Zhong et al., 2013). This α7 enhancement of glutamate levels could be the biological basis for the long-term enhancement of glutamate levels in the AcbSh produced by chronic EtOH + nicotine co-use (Deehan et al., 2015). In contrast to the canonical belief that a receptor is either a ligand-gated ion channel or a G-coupled receptor, the α7 is a dual ionotropic and metabotropic receptor. Specifically, activation of the α7 NAChR increases Ca^{2+} influx (ligand-gated ion channel) and/or increases Gαq (sustained release of Ca^{2+}) in the neuron (Kabbani and Nichols, 2018). In addition, α7 desensitization period is short and the recovery period is fast, which makes the α7 very different from other NAChRs (Papke et al., 2009). The properties of the α7 NAChR and the published data indicating the importance of this receptor in mediating the effects of ABAE-induced adult consequences indicate that the α7 NAChR is a valid candidate to develop pharmacotherapeutics to counter ABAE (Rodd et al., 2020).

EtOH directly acts on the α7 receptor. Oocyte determination of EtOH actions at the α7 receptor indicated dual excitatory and inhibitory responses (Doyon et al., 2013a). Similar results were determined using *in vitro* electrophysiological assessment of the action of EtOH on the α7 receptor (Doyon et al., 2013b). However, the complexity of the α7 receptor reduces the confidence in *in vitro* analysis of the effects of EtOH on the receptor (e.g., G-couple protein properties vs. ligand gated ion channels and lack of functional inputs onto selected, stabilized, dissociated neurons). The initial experiment provided the needed data that would examine the *in vivo* effect of activating the α7 receptor on the ability of EtOH to stimulate posterior VTA dopamine neurons. An increase in the activation of the mesolimbic dopamine system suggests the animal is experiencing reinforcing stimulation that could indicate an increased propensity to perform behaviors to obtaining this effect (e.g., alcohol consumption; Rodd et al., 2004).

The effects of adolescent alcohol consumption on adult alcohol consumption can be characterized as a "division of thirds". One third of American adolescents do not drink alcohol while another third consumes alcohol during adolescence but does not develop into adult AUD. The final third are individuals that consume alcohol (binge drinking) during adolescence and are diagnosed with AUD during adolescence or adulthood (Tripodi et al., 2010). There are many types of clinical interventions to treat adolescent alcohol consumption. Cochrane reviews have indicated that the clinical interventions have "poor" outcomes (Foxcroft and Tsertsvadze, 2011; Carney et al., 2016).

Recent preclinical data have indicated possible prophylactic pharmacological intervention to prevent the deleterious

consequences of ABAE on adult alcohol consumption and biological factors. Pretreatment with the α7 negative allosteric modulator (NAM) dehydronorketamine (DHNK) before ABAE prevented the enhancement of alcohol consumption detected during the acquisition of EtOH consumption during adulthood and EtOH relapse drinking produced by ABAE (Rodd et al., 2020). In addition, administration of the α7 receptor agonist AR-R17779 during adolescence mirrored the effects of ABAE in adult ethanol consumption by increasing acquisition and relapse drinking (Rodd et al., 2020). However, this does not indicate that activation of the α7 allosteric binding site mediates the effects of ABAE. Parallel research has indicated that pretreatment with galantamine (a cholinesterase inhibitor) blocked ABAE-induced increases in the expression of genes associated with the innate immune system (*TLR4* and *pNF-κB*) and histones/chromatin related genes (*RGE* and *HMGB1*) and during adulthood (Crews et al., 2021). Preclinical evidence from independent laboratories has indicated that pharmacological intervention can act prophylactically to prevent the deleterious effects of ABAE on adult neurophysiology and behavior. The reported data examined the efficacy of SB-277011-A (an α7 NAM and a D3 antagonist; Zheng et al., 2016) to act prophylactically to prevent ABAE-induced enhancement of adult alcohol consumption.

The allosteric binding site on the α7 receptor could have bi-directional effects on ABAE-induced enhancement of adult alcohol consumption. To date, the parametrics of ABAE (amount of EtOH required to produce the adolescence consequences, length and duration of ABAE, and more) have not been established. A α7 positive allosteric modulator (PAM) could amplify the effects of EtOH during adolescence exposure to produce enhancement of adult alcohol consumption (possible synergistic effects of low-dose EtOH exposure). The current research project also examined the effects of co-administration of the α7 PAM PNU with subthreshold (2.0 g/kg) adolescent EtOH exposure on adult alcohol consumption in male and female Wistar rats.

Methods

Subjects

Wistar rats are maintained at Indiana University School of Medicine as a single-generation colony (IUSM; Indianapolis, IN). The Taconic P (tP) rat is a substrain of the P rat that was maintained at Taconic Farms for over 10 years before being returned to Indiana University (2006). Similar to the Wistar colony, the tP colony complete life cycle (breeding/rearing) was conducted in the same building as the research was performed. Same building care eliminates the stress from shipping adolescents.

Animals care facilities at IUSM are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Research performed in the current experiments were approved by the IUSM Institutional Animal Care and Use Committee (IUSM IACUC) and were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, the NIH, and the Guide for the Care and Use of Laboratory Animals (2011).

Co-Administration of EtOH and the α7 receptor agonist AR-R17779 into the posterior VTA on extracellular dopamine levels in the AcbSh

Microinjection-microdialysis protocol

Detailed methodology for the microinjection-microdialysis procedure has been published elsewhere (**Figure 1**; Deehan et al., 2015; Waeiss et al., 2019, 2020; Hauser et al., 2021b). Surgery was performed in adult rats (>PND 90). Rats were ipsilaterally implanted with guide cannulas aimed at the posterior VTA (AP −5.6 mm, ML +2.1 mm, DV −8.0 mm) and AcbSh (AP +1.5 mm, ML +2.0 mm, DV −5.3 mm). Surgical details are described previously (Waeiss et al., 2019). Because of the delicate nature of the experiment (rats moving their heads can destroy equipment or HPLC assemblies), rats were handled daily after surgery until microdialysis testing. The researchers believe that frequently handling of rats is beneficial to the rats and reduces stress associated with experiments (grasping of rats to insert microinjections, etc.).

Microdialysis analyses of DA levels in the AcbSh were performed using loop-style probes that are manufactured in the laboratory (Waeiss et al., 2020; Hauser et al., 2021b). The production of probes has been described in detail in previous publications (Waeiss et al., 2020; Hauser et al., 2021b). Under isoflurane anesthesia, microdialysis probes were inserted (3.0 mm below the guide cannula) into the AcbSh 24-h before the performance of the microinjection-microdialysis experiment.

During the microinjection-microdialysis experiments, rats are awake and are active in the testing chambers (freely moving). Subjects are connected to the microdialysis apparatus (detailed in Hauser et al., 2021b) and a 90-min washout period (removes residue from probe and stabilizes all neurotransmitter levels) was performed. Samples were collected in 20-min intervals during the five baseline samples and following microinjections into the posterior VTA.

Experimenter controlled microinjections were carried out with an electrolytic microinfusion transducer (EMIT) system (Waeiss et al., 2019; Hauser et al., 2021b). Microinjection of test solutions into the posterior VTA followed a cycle of 5-s microinjection and 15-s timeout period. Microinjections occurred over a 10-min period (30 injections in total, 3 μl total injection volume).

All microdialysis samples were collected and stored for testing using established methods (Waeiss et al., 2019). Samples were analyzed for dopamine content through the use of an high performance liquid chromatography (HPLC) system (Hauser et al., 2021b).

Concentrations of EtOH and AR-R17779

Adult Wistar male rats were microinjected with artificial cerebrospinal fluid (aCSF), EtOH alone (100 or 150 mg%), AR alone (500 nM) or EtOH + AR (50 mg% EtOH + 500 nM AR or 100 mg% EtOH + 500 nM AR). The total number of animals used in the

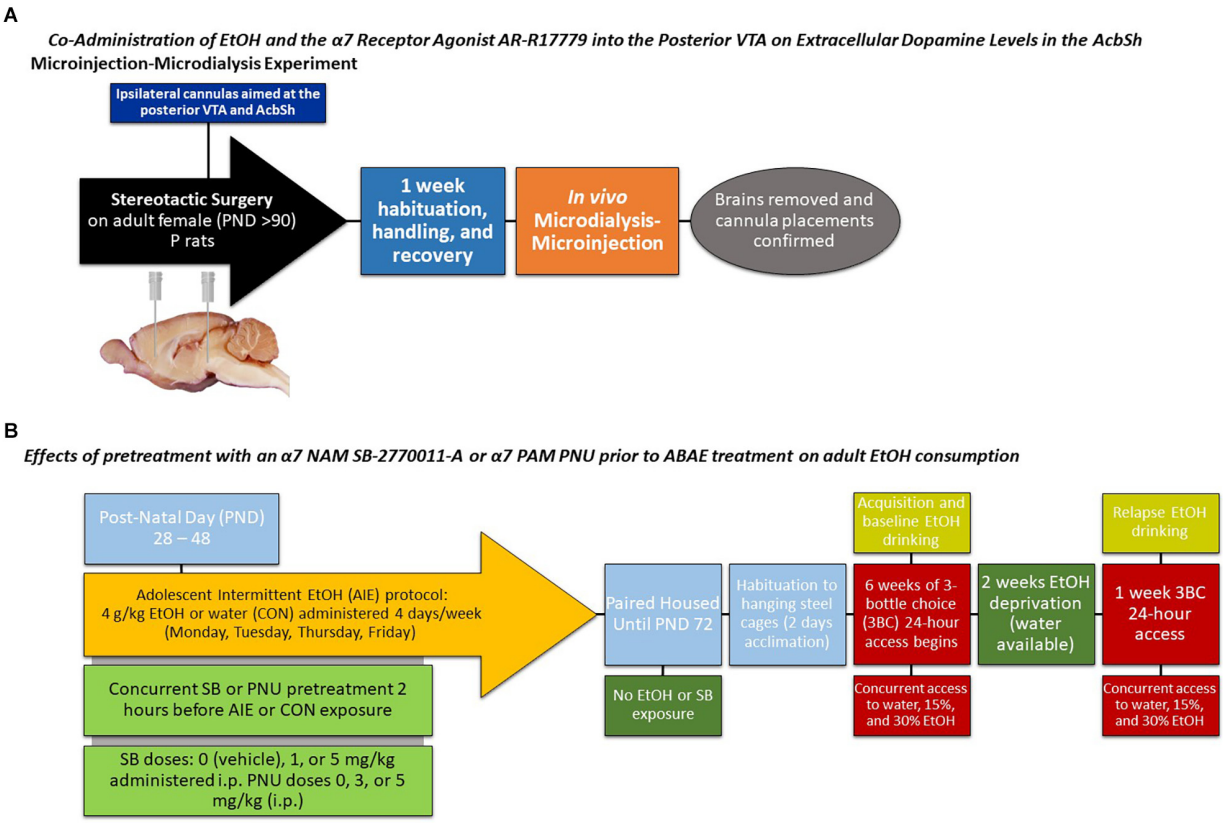

FIGURE 1
Depicts a timeline for the **(A)** microinjection-microdialysis experiments and **(B)** adult alcohol intake experiments.

experiment was 37 (n = 7–9/group). Past research has indicated no difference between male and female rats for the ability of EtOH microinjected into the posterior VTA to stimulate dopamine release in the AcbSh (Hauser et al., 2021b). Female rats were not included in this study because of financial limitations and the low likelihood to observe sex differences.

Histological verification

At the end of the schedule experiment, animals were sacrificed and a solution of 1% bromophenol blue dye was injected into the posterior VTA and AcbSh. Following storage (−80°C), brains were sectioned (40-μm) and placed on slides. Staining with cresyl violet allowed for delineation of brain regions. Site verification was confirmed using the atlas of Paxinos and Watson.

Pretreatment with an α7 NAM SB-2770011-A prior to ABAE treatment on adult EtOH consumption

The method of testing the effects of ABAE on adult alcohol consumption has been described previously (Rodd et al., 2020; Figure 1). In the current experiment, there were six treatment groups. Adolescent male (n = 35) tP rats were used in the experiment. Visual representation of the experimental design has been published (Figure 1). ABAE treatment began on PND28, were intermittent (4 days of the week), and EtOH treatment (4.0 g/kg/day; 25% v/v EtOH) was performed through gavage. Other rats received comparable gavage treatment (equivalent volume) of water (CON rats) on the same days and for the same duration of treatments. ABAE and CON rats were randomly assigned to one of three doses (0, 1, or 5 mg/kg) of SB-277011-A (SB; Sigma-Aldrich, Inc, St. Louis, MO, USA) that was administered 2 h prior to ABAE or CON treatment (PND 28–48; a total of 14 treatments). Rats were not exposed to SB at any other time. Rats were group housed until the testing of adult alcohol consumption. Previous research has indicated no six differences in the ability of a α7 NAM to block/prevent the effects of ABAE on adult alcohol consumption (Rodd et al., 2020). Because of financial limitations on research and the low probability to observe a sex difference, females were excluded from the study.

Adult EtOH consumption

To remove any potential bias, collection of data (fluid intake) was conducted blind to adolescent treatment conditions.

For the most accurate assessment of adult alcohol intake, all rats were placed into individual hanging steel cages 2 days prior to the initial exposure to adult EtOH (PND 73). The hanging steel cages are equipped with features that reduce stress to the rats (unanchored perch in each cage). The rats use the perch to rest, avoid standing on

the wire mesh, and to sleep. Adding a perch to hanging steel cages is required by the IUSM IACUC.

Throughout the experiment, food and water was constantly available to the rats (*ad libitum*). EtOH solutions made available on PND 75 (15 and 30% EtOH v/v) were created from a stock of ethyl alcohol (190 proof; McCormick Distilling Co., Weston, MO, USA).

The research protocol has been employed multiple times in the past to examine acquisition and relapse EtOH consumption in rats (Rodd et al., 2020). Briefly, rats are given free-choice access to water, 15 and 30% EtOH (3 bottle-choice) for 6 weeks (measurements taken daily). EtOH solutions (but not water) are removed for 2 weeks (deprivation/abstinence). EtOH solutions were returned for a 2-week re-exposure period. This protocol is well-established to produce an increase in EtOH consumption upon re-exposure to EtOH (alcohol deprivation effect, ADE; Rodd et al., 2004).

Effects of co-administration of the α7 PAM PNU simultaneously with low-dose ABAE treatment on adult EtOH consumption

Adolescent male (n = 39) and female (n = 41) Wistar rats were used in the experiment. The experimental design was a 2 × 3 between subject protocol. Rats received low-dose ABAE treatment (2 g/kg, gavage) or water which began on PND28 and were simultaneously administered PNU-120596 (0, 3, or 5 mg/kg; Sigma-Aldrich, Inc, St. Louis, MO, USA). ABAE treatment was intermittent (4 days of the week) until PND48. ABAE exposed to 2 g/kg EtOH should have resulted in a peak blood ethanol concentration (BEC) of approximately 35–40 mg% (Vetreno et al., 2020). The primary reason why both sexes were not tested in the two adult conditions was primarily financial. These prolonged experiments are extremely costly. The same real life constraints (and the general unwillingness for funding agencies to support research preventing the negative consequences of ABAE) limited the dose of EtOH exposure during adolescence and dose of PNU tested. Individuals reading scientific manuscripts must ask the following questions; could they obtain funding for the ideal research project they demand from a publication, and if they were a reviewer for a grant application would they support funding for a full parametric analysis?

Males—24-h free-choice drinking

The goal of the experiment was to have Wistar rats consuming pharmacological relevant levels of alcohol. The most reliable manner to obtain significant BECs in Wistar rats is to provide a palatable alcohol solution (beer). McGregor and colleagues have repeatedly published that all rats will consume beer at a level that produces significant BECs (McGregor et al., 2005; Hargreaves et al., 2009a,b, 2011). In addition, adolescent beer drinking in outbred rats increases beer drinking during adulthood (Hargreaves et al., 2011). A difference between our procedure and McGregor's (and others') protocols is that we do not use pilsners. In a taste test of 54 microbrews, we have determined that rats (Wistar and tP, male and female) prefer India Pale Ales (IPA) or Red Ales (and dislike pilsners). Therefore, we used a West Coast IPA (7.7% alcohol) and a Toasted Red Ale (5.2% alcohol).

To remove any potential bias, collection of data (fluid intake) was conducted blind to adolescent treatment conditions. Rats were treated identical to the tP rats. Rats are given free-choice access to water, 5.2% and 7.7% EtOH (3 bottle-choice) for 6 weeks (measurements taken daily). EtOH solutions (but not water) are removed for 2 weeks (deprivation/abstinence). EtOH solutions (beer) were returned for 2-week re-exposure period. This protocol is well-established to produce an increase in EtOH consumption upon re-exposure to EtOH (alcohol deprivation effect, ADE; Rodd et al., 2004).

Females—1-h daily operant beer self-administration

There are many behavioral measures that could be tested under operant conditions that cannot be assessed through free-choice drinking. The current experiment examined the effects of low-dose ABAE and PNU exposure on the acquisition, maintenance, extinction, seeking, and relapse behavior. Briefly, rats were placed in a standard two lever operant chamber (water or 7.7% West Coast IPA as reinforcer).

Without training, female Wistar rats were placed into the operant chambers and allowed to self-administer water or 7.7% West Coast IPA every day during a 1-h session. Detailed methodology is available in other publications (Rodd-Henricks et al., 2002a,b; Hauser et al., 2019b). The fixed ratio (FR) requirement for the beer solution was an FR1 for the initial 4 weeks, FR3 during weeks 5–6, and an FR5 during weeks 7–8 (maintained on this FR for the rest of the experiment).

Female rats were given 8 weeks of beer access. Rats were then exposed to extinction training during weeks 9 (no beer or water available in operant chambers). Rats were then maintained in their homecages for 2 weeks. Rats were then tested for context-induced beer-seeking [Pavlovian Spontaneous Recovery (PSR)] for four sessions. After seeking testing, rats were maintained in their homecages for a week. Rats were then returned to the operant chamber with beer and water available for self-admininstration.

Statistical analysis

The statistical analysis methodology outlined by Keppel and Zedeck (1986) was used. Briefly, mixed factor ANOVAs were conducted for the overall analysis. The between subject factors were Dose of SB and ABAE. The single within subject factor of Week and the dependent measure of weekly average intake were used. The last 3 days of EtOH consumption prior to the abstinence period was used as baseline for the relapse analyses. The overall analysis was a mixed factor ANOVA with between subject factors of SB and ABAE AEH and within subject factors of day (baseline intake compared to intake levels following EtOH re-exposure). *Post-hoc* comparisons for significant differences for between subject variables was the Tukey's b analyses. To avoid violation of the ANOVA assumption of independent measures,

proper within subject analysis (*t*-tests and orthogonal contrasts) were used. We only report the results of the *t*-test analyses. In past publications, we have detailed the correct equations of Type I error rate inflation (Rodd et al., 2020). Given the effect size of the current dataset there is little likelihood of Type 1 error rate inflation for any reported analyses (c.f., Rodger, 1967).

Results

The effects of co-administration of EtOH and the α7 receptor agonist AR-R17779 into the posterior VTA on extracellular dopamine levels in the AcbSh

The overall statistical analysis performed on the dependent measure of the %change of DA levels from baseline in the AcbSh indicated that there was a synergistic effect of combining EtOH and AR (Group × Sample interaction term—$F_{42,186} = 2.9$; $p < 0.001$; **Figure 2**). The significant interaction term was decomposed by examining the effect of Group at each sample time (performing individual ANOVAs). There were no significant Group differences during the three baseline samples and for the 4th–6th samples obtained after the microinjection procedures (Group $F_{4,32}$ values < 1.04; p values > 0.85). There were significant Group differences during the 1st–3rd samples obtained after microinjections of assigned compounds into the poster VTA (Group $F_{4,32}$ values >13.81; p values < 0.002). *Post-hoc* comparisons (Tukey's b) revealed that during the 1st post-microinjection period there were significant group differences; 100 mg% EtOH + 500 nM AR >50 mg% EtOH + 500 nM AR >500 nM AR >100 mg% EtOH and aCSF controls. Preliminary data (Waeiss et al., 2019) indicated that microinjections of 500 nM AR into the posterior VTA did not alter dopamine release in the AcbSh. We did not anticipate that microinjection of 500 nM AR would significantly increase dopamine levels in the AcbSh. During the 2nd post-microinjection period, *post-hoc* comparisons revealed significant group differences: 50 mg% EtOH + 500 nM AR and 100 mg% EtOH + 500 nM AR >aCSF, 100 mg% EtOH, and 500 nM AR. During the 3rd post-microinjection period, *post-hoc* comparisons revealed that rats administered 50 mg% EtOH + 500 nM AR has significantly higher levels of dopamine in the AcbSh than all other groups.

Effects of pretreatment with an α7 NAM SB-2770011-A prior to ABAE treatment on adult EtOH consumption

Acquisition

The dependent measure was the weekly average of EtOH intake. Weekly averages were used in the analysis for acquisition of drinking. If daily intake was analyzed, there is a limited time

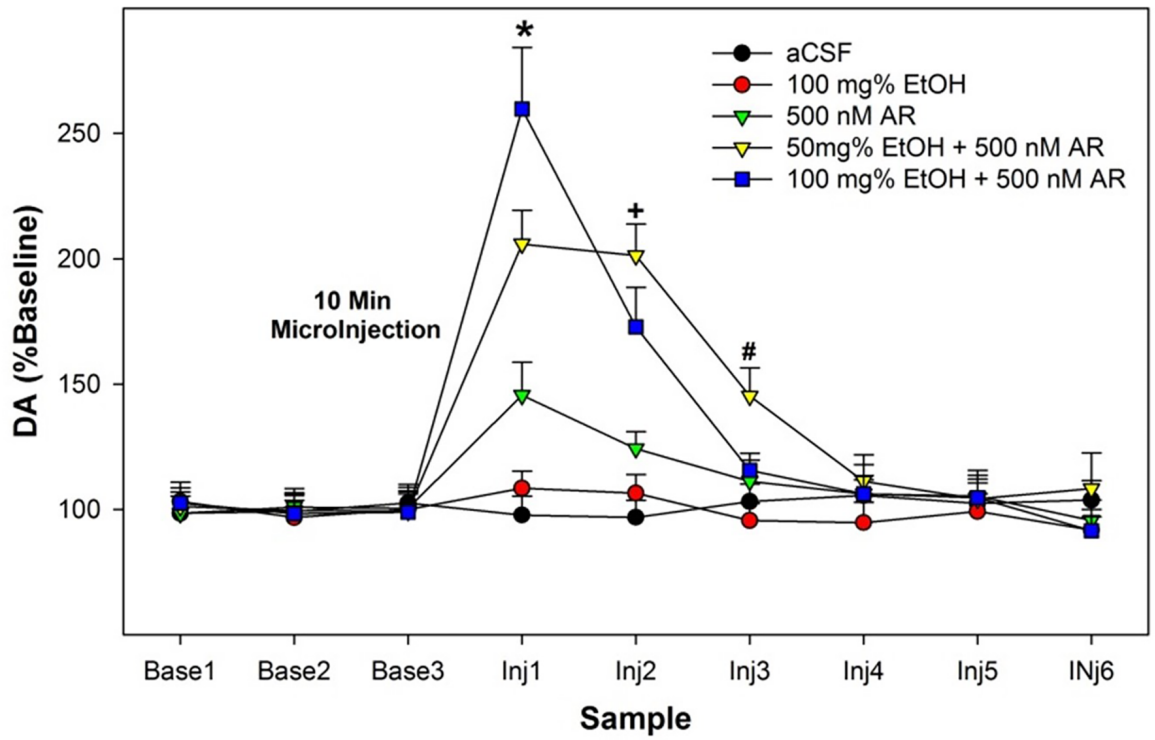

FIGURE 2
Depicts the average extracellular DA levels in the AcbSh in P rats following microinjects of EtOH, AR, or EtOH+AR into the posterior VTA. *indicates in 100 E + 500 AR >50 E + 500 AR >500 AR >aCSF and 100 E . $^{+}$indicates that two E + AR >500 AR >aCSF and 100 E. $^{\#}$indicates 50 E + 500 AR >all other groups.

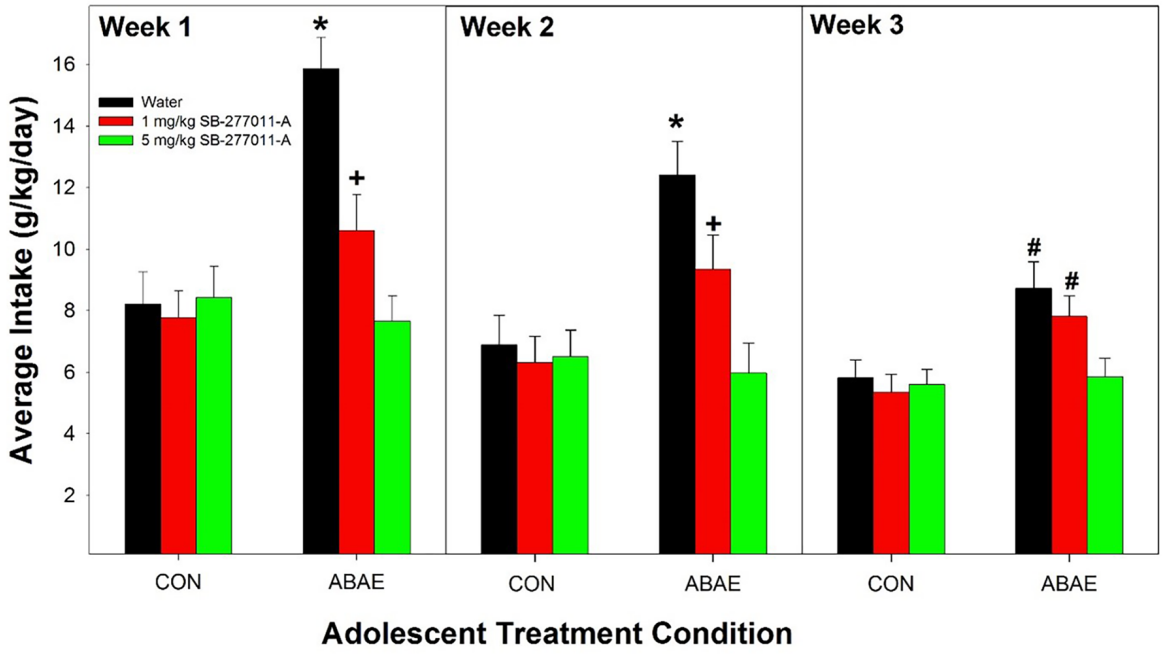

FIGURE 3
Depicts the average alcohol intake in female P rats administered the α7 NAM SB-277011-A 2 h before ABAE treatment during adolescence on the adult consumption of EtOH. *indicates EtOH consumption in water-ABAE rats >1 mg and 5 mg/kg SB rats. $^{+}$indicates 1 mg/kg >5 mg/kg SB rats. $^{\#}$indicates water-ABAE and 1 mg/kg SB >5 mg/kg SB rats.

period to observe drinking behavior because of the elimination of the degrees of freedom. The overall analysis indicated that pretreatment with SB prevented the ABAE-induced increase in EtOH consumption during acquisition (Figure 3). Statistically, there was a significant ABAE × SB Dose × Week interaction term ($F_{4,147}$ = 3.68; p < 0.001). The significant interaction 3-way interaction was decomposed by examining the effects of ABAE × SB Dose on the weekly averages. There were significant 2-way interactions (ABAE × SB Dose) for each average weekly intake values ($F_{4,147}$ values > 2.37; p values < 0.001). Holding ABAE history constant revealed that in the CON groups, there was no effect of SB Dose (p values > 0.72). In ABAE rats, there was a significant effect of SB Dose (p values < 0.003). *Post-hoc* comparisons indicated that during the 1st and 2nd weeks of EtOH access rats given water pretreatment drank more EtOH than rats administered 1 or 5 mg/kg SB (water >1 mg/kg >5 mg/kg). During the 3rd week of EtOH access, rats pretreated with water or 1 mg/kg before ABAE consumed more EtOH during adulthood than rats pretreated with 5 mg/kg SB. To confirm that ABAE enhanced acquisition drinking during adulthood, SB dose was held constant and weekly average EtOH intake was analyzed. The analyses revealed that during the 1st–3rd weeks, the ABAE Water group consumed more EtOH than the CON Water group (p values < 0.0001). In rats pretreated with 1 mg/kg SB prior to adolescent treatment indicated that ABAE—1 mg/kg SB rats consumed more EtOH during the 2nd and 3rd week of acquisition compared to CON— 1 mg/kg SB rats (p values < 0.01). In contrast, there was no significant effect of ABAE history on adult EtOH intake (acquisition) in rats pretreated with 5 mg/kg SB (p values > 0.53).

Relapse

Overall, ABAE increased relapse drinking during adulthood and pretreatment with SB suppressed ABAE-enhancement of relapse EtOH drinking. Statistically, there was a significant ABAE × SB Dose × Day interaction term (Figure 4; $F_{12,244}$ = 2.3; p < 0.01). The 3-way interaction term was decomposed by holding the factor of "Day" constant. There was no significant effect of ABAE × SB Dose on baseline (maintenance) intake (p = 0.78). During the 1st–3rd relapse (ADE; individual ANOVAs performed) drinking days, there were significant ABAE × SB Dose interactions for EtOH consumed ($F_{2,32}$ values > 2.24; p values < 0.024). The interaction term was decomposed by holding both ABAE history and SB Dose constant. During the 1st and 2nd relapse drinking day, in the CON group of rats there was no effect of SB Dose on EtOH intake (p = 0.0.77), but there was an effect of SB Dose in the ABAE group ($F_{2,17}$ values > 11.88; p values < 0.01). *Post-hoc* comparisons for the ABAE group indicated that EtOH intake was significantly higher in the Water and 1 mg/kg SB group compared to the 5 mg/kg group (Tukey's b). Examining the effects of ABAE history during the 1st relapse drinking day, there was significant CON vs. ABAE group differences for the Water and 1 mg/kg SB groups. During the 3rd relapse drinking day, the only statistical change was that in rats with a past history of ABAE the 1 mg/kg SB no longer consumed more EtOH than the 5 mg/kg SB group. To confirm that there was an increase in EtOH intake compared to baseline, within groups analyses were performed (t-tests; p < 0.05). In the CON rats, all three SB Dose groups consumed more EtOH during the 1st and 2nd relapse drinking days. In the ABAE rats, all three SB Dose groups consumed EtOH during the 1st, 2nd, and 3rd relapse drinking days.

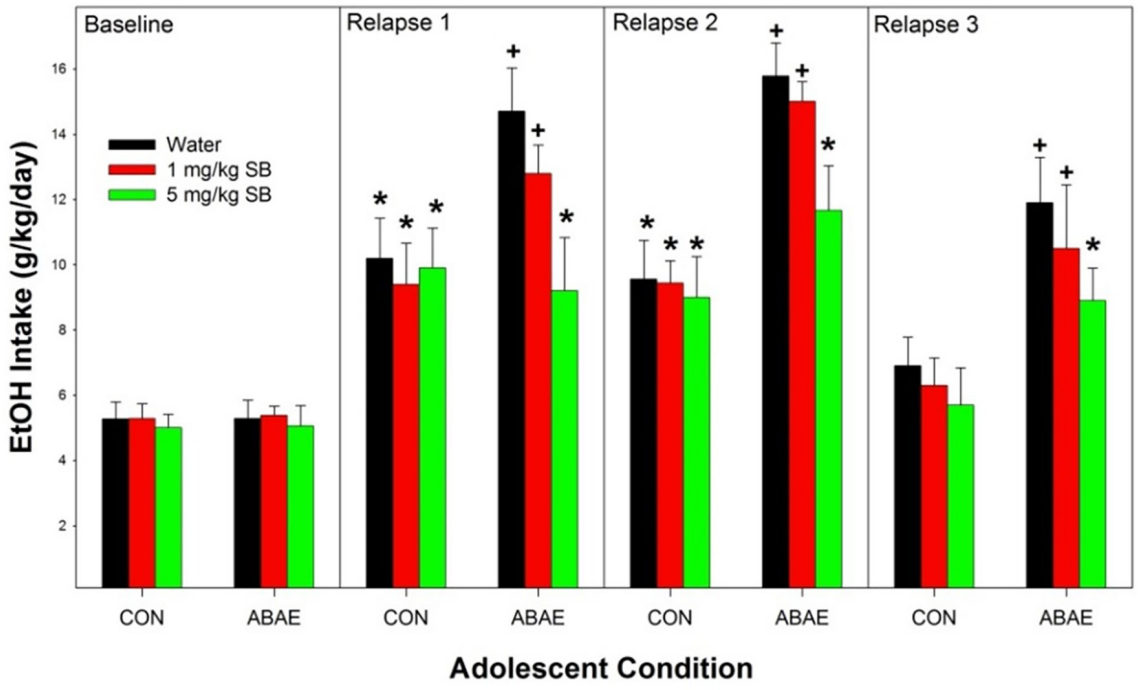

FIGURE 4
Depicts the mean (+ SEM) for female alcohol-preferring (P) rats during relapse EtOH drinking. *indicates EtOH consumption exceeds baseline intake. $^{+}$indicates that rat pretreated with saline prior to ABAE exposure (water and 1 mg/kg SB) consumed more alcohol than corresponding CON group.

Effects of co-administration of the α7 PAM PNU simultaneously with low-dose ABAE treatment on adult EtOH consumption

Males—24-h free-choice drinking

Males consumed a large amount of beer. In general, all rats consumed a large amount of beer which increased over the time of access. During the first week of access, male rats consumed on average 7.1 g/kg/day. The level of intake was significantly higher during the 6th week of access (average intake 13.8 g/kg/day. Statistically, there were no significant effects of ABAE, PNU, or ABAE × PNU interactions on beer consumption during the first 6 weeks of access (p values > 0.37). There was a main effect of Week (Figure 5; $F_{5,32} = 12.6$; $p < 0.01$).

There was a significant increase in beer drinking following the 2-week deprivation period ($F_{2,34} = 10.8$; $p < 0.01$). Statistically, there were no significant effects of ABAE, PNU, or ABAE × PNU interactions on beer consumption during the relapse beer drinking (p values > 0.46). On average, male Wistar rats consumed 125.2 g of beer per day during the first week of beer re-exposure. Given that the rats weighed on average 457.8 g during this time, rats were consuming 27.3% of their body weight in beer each day. For comparison, this would be similar to an 85 kg human consuming 23.5 kg of 7.7% beer per day.

Females—1-h daily operant beer self-administration

Examining the acquisition of beer self-administration in female Wistar rats revealed a significant Session × ABAE × PNU interaction term (Figure 6; $F_{12,368} = 3.4$; $p < 0.01$). For ease of decomposing the significant three-way interaction term, ANOVAs were performed on all groups of ABAE and PNU testing (six total). Individual ANOVAs revealed that sessions 4–13 there was a significant effect of Group ($F_{5,37}$ values > 23.8; p values < 0.01). *Post-hoc* analyses (Tukey's b) revealed that female rats treated with 2.0 g/kg EtOH during adolescence (ABAE) and simultaneously administered 5.0 mg/kg PNU responded more for the 7.7% beer than all other groups. After the 14th session, all groups responded equivalently. At each FR schedule of reinforcement, all rats self-administered about 60 reinforcers per session (Figure 6; middle panel). During Extinction Training, there was a Session × ABAE × PNU interaction term ($F_{12,368} = 2.0$; $p < 0.01$). ANOVAs indicated that there was a significant Group difference during Extinction Sessions 1–4 (p values < 0.02). *Post-hoc* comparisons (Tukey's b) indicated that female rats treated with 2.0 g/kg EtOH during adolescence (ABAE) and simultaneously administered 5.0 mg/kg PNU responded more on the lever previously associated with the delivery of 7.7% beer than all other groups.

During context-induced alcohol-seeking testing (Figure 6; right panel), there was a significant Session × ABAE × PNU interaction term (Figure 6; $F_{8,312} = 1.9$; $p < 0.01$). The was a significant Group difference during PSR test sessions 1–3 ($F_{5,37}$ values > 17.5; $p < 0.01$). Similar to acquisition and extinction training, *post-hoc* analyses revealed that female rats treated with 2.0 g/kg EtOH during adolescence (ABAE) and simultaneously administered 5.0 mg/kg PNU responded more on the lever previously associated with the delivery of 7.7% beer than all other groups. Alcohol craving

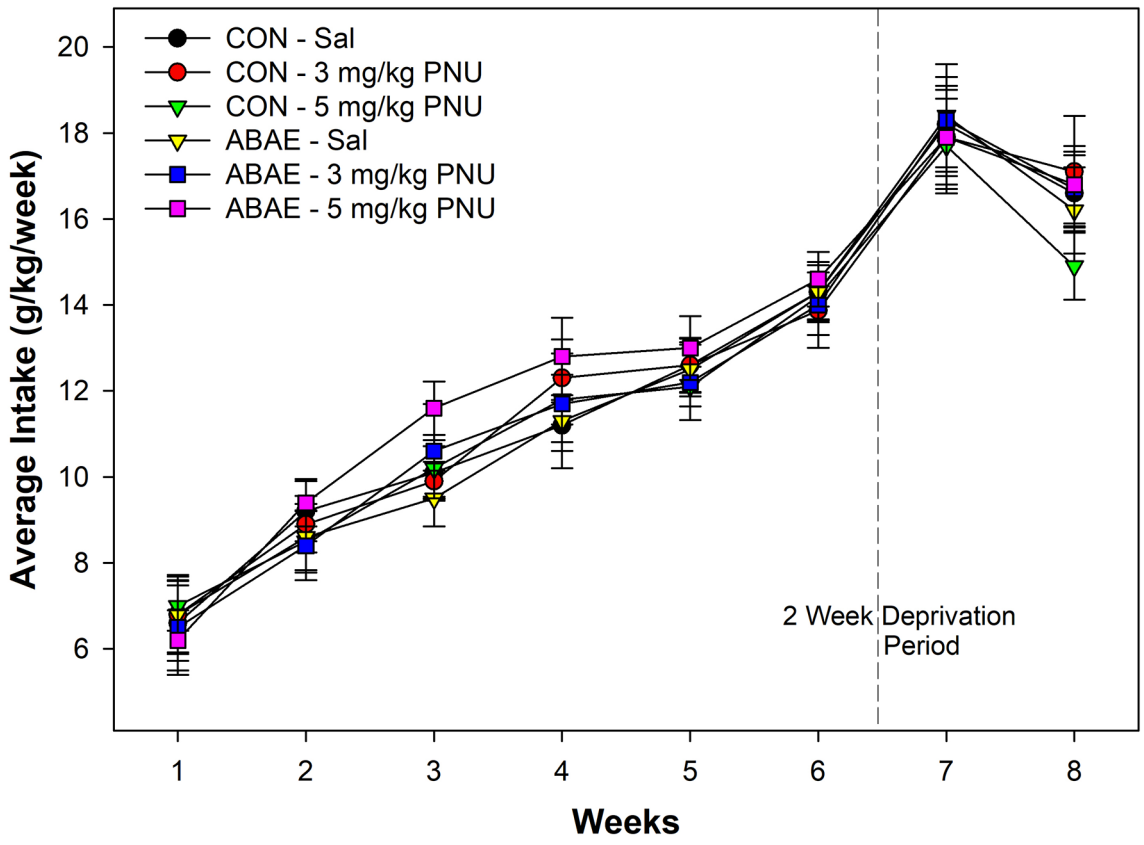

FIGURE 5
Depicts the mean (+ SEM) weekly average intake (g/kg/day) of alcohol (beer) in male Wistar rats administered a subthreshold dose of EtOH during adolescence and the α7 PAM PNU. There was a significant increase in alcohol consumption in all groups during the experiment, but not effects of ABAE or PNU treatment.

is indicated in the PSR model by an increase in the level of responding during the PSR test sessions compared to responding during the last three sessions of extinction training. This within subject analysis is determined by *t*-tests. All rat groups responded more during the 1st PSR test session than during extinction (p values < 0.032). There were no significant effects during relapse testing.

Discussion

The current results indicate that co-administration of EtOH enhances the activation of posterior VTA dopamine neurons (the source of increased DA in the AcbSh) by an α7 receptor agonist (**Figure 2**). Electrophysiological studies of dissociated neurons and oocyte research previously indicated that EtOH acts on the α7 receptor with equivocal effects (Doyon et al., 2013a). Stimulation of the α7 receptor in the posterior VTA activates DA neurons (Schilström et al., 2003). On VTA DA neurons, the D2 receptor is linked-expressed with the α7 receptor (Garzón et al., 2013). Activation of the D2 autoreceptors inhibits activation of DA neurons, while activation of the α7 receptor stimulates VTA DA neurons (Garzón et al., 2013). Classic antipsychotic drugs (inhibitors of D2 receptors) are thought to be efficacious through disinhibition of the stimulatory actions of the α7 receptor on VTA DA neurons (Garzón et al., 2013). Reduced ABAE exposure (2.0 g/kg/day) failed to alter adult EtOH consumption (**Figures 5** and **6**). Co-administration of the α7 PAM PNU with low-dose ABAE did result in enhanced beer self-administration in Wistar female rats (**Figure 6**). These data indicate that enhancing the likelihood that the α7 receptor will be activated if stimulated by another ligand (EtOH) or activator (actions of a PAM) resulted in a leftward shift in the dose response curve for EtOH ABAE consequences on adult alcohol consumption. The data generated in rats given exposure to a subthreshold dose of EtOH (2 g/kg) and the α7 PAM PNU, parallels the findings (increased acquisition, reduced rate of responding, and enhanced Alcohol-Seeking behaviors) observed in *p* and Wistar rats administered the typical (4 g/kg) ABAE (Rodd-Henricks et al., 2002a; Gass et al., 2014). These data indicate the importance of the α7 receptor in mediating the effects of EtOH in the adolescent brain.

Furthermore, reducing activation of the α7 receptor (e.g., an α7 NAM) prevents the development and expression of social stress-induced neuroadaptations (Morel et al., 2018). Similar to our findings that administration of an α7 receptor agonist during adolescence enhances adult EtOH consumption, activating the α7 receptor promotes stress-induced cellular neuroadaptations and predisposes organisms to express anxiety-like behaviors (Morel et al., 2018). Thus, ABAE-induced upregulations of the *Chrna7*

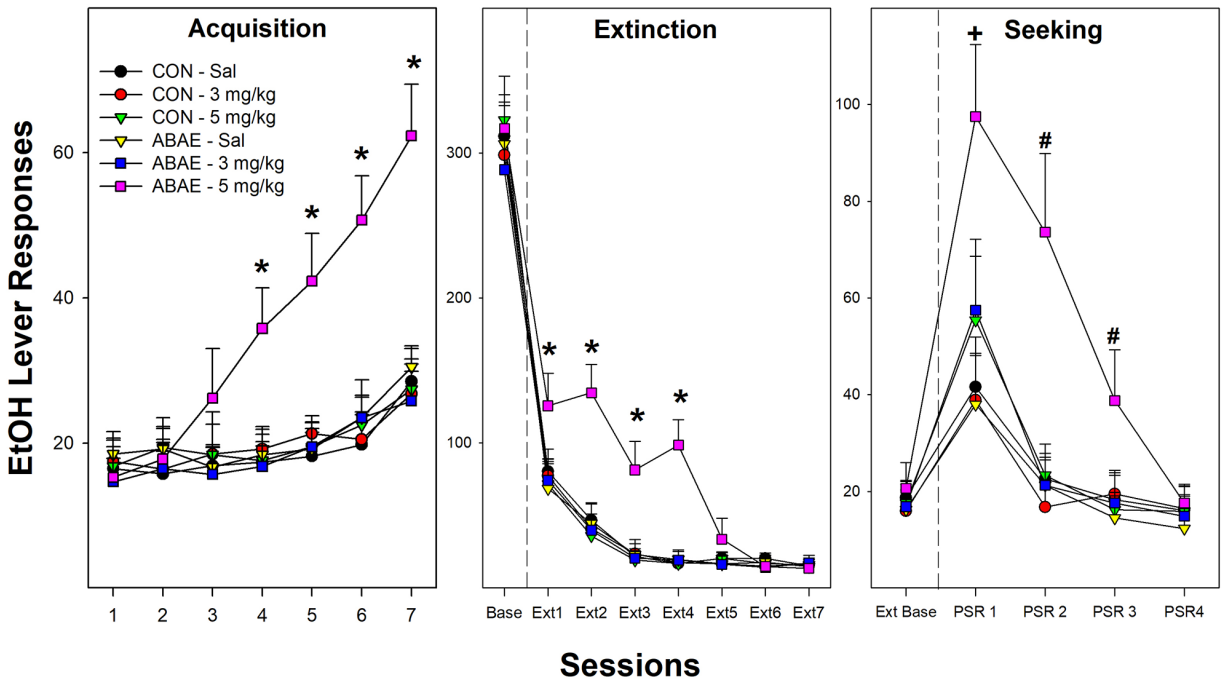

FIGURE 6
Depicts the mean (+ SEM) number of EtOH (beer) lever responses in female Wistar rats as a product of exposure to low-dose ABAE (2 g/kg) and treatment with the α7 PAM PNU. During Acquisition of responding (left panel), female rats treated with 2 g/kg ABAE and 5 mg/kg PNU during adolescence responded more on the lever associated with the delivery of beer (7.7% West Coast IPA; *indicates significant difference from all other groups). Rats treated with 2 g/kg ABAE and 5 mg/kg PNU during adolescence responded more on the lever previously associated with the delivery of beer during the first four Extinction training sessions (middle panel; *indicates significant difference from all other groups). During context-induced EtOH-seeking (right panel), female Wistar rats treated with 2 g/kg ABAE and 5 mg/kg PNU during adolescence responded more on the lever previously associated with the delivery of beer than all other groups during sessions 1–3. $^{+}$sign indicates that all groups displayed an increase in responding compared to extinction baseline, and 2 g/kg ABAE and 5 mg/kg PNU are significantly higher than all other groups. $^{\#}$sign indicates that 2 g/kg ABAE and 5 mg/kg PNU are significantly higher than all other groups and higher than extinction baseline.

gene throughout the brain (Hauser et al., 2019a, 2021b) could be a critical biological basis for the replicated finding that ABAE results in increased anxiety levels during adulthood (Sánchez-Marín et al., 2022).

The current data indicate that the co-administration of EtOH and an α7 receptor agonist into the posterior VTA synergistically increases dopamine release in the AcbSh (**Figure 2**). Past research has indicated that selective breeding for high alcohol preference (P, HAD-1, and HAD-2 rats) is associated with a posterior VTA–AcbSh neurocircuitry that is more sensitive (leftward and upward shift in the dose-response curve) to the stimulatory effects of nicotine in the posterior VTA (Deehan et al., 2018). The activation of the mesolimbic dopamine system is considered critical for the reinforcing properties of drugs of abuse and the regulation of alcohol consumption (Rodd et al., 2004). The hyperdopaminergic consequence of ABAE is indicated by basal neurochemical differences (Sahr et al., 2004), the increase in the genetic expression of dopamine reuptake system (Brancato et al., 2021, 2022), and the increase in sensitivity and response to stimulation during adulthood (Hauser et al., 2019a, 2021b).

EtOH and nicotine microinjected into the posterior VTA (but not nicotine or EtOH microinjected alone) results in an increase in glutamate release in the AcbSh and an increase in the expression of BDNF in the AcbSh (Waeiss et al., 2020). In addition, microinjection pretreatment of EtOH and nicotine into the posterior VTA (but not nicotine or EtOH microinjections alone) enhanced the rewarding properties of EtOH within the AcbSh. The current study suggests that the activation of the α7 receptor in conjunction with EtOH administration into the posterior VTA could be the biological basis for these observed effects (**Figure 2**). A limitation of the current study is that the neurochemical experiment occurred in adult and not adolescent rats. Performing research involving dual placement surgery in adolescent rats has concerns including: (1) similar aged rats will have marked differences in brain structure arrangements (failure to get consistent placement); (2) increased mortality rates and neurological events (seizures) since the cannulas are within a developing and expanding brain; (3) reduced mobility (adolescent rats tend to keep their head lower in response to the weight of the head cap), and (4) justified rejection of approval of experiments by review agencies (Drzewiecki and Juraska, 2020).

The current data replicate the findings reported by multiple laboratories that ABAE enhances EtOH consumption during adulthood (**Figures 2**, **3**, and **6**; McKinzie et al., 1996; Rodd-Henricks et al., 2002a,b; Schramm-Sapyta et al., 2008; Toalston et al., 2015; Amodeo et al., 2017, 2018; Spear, 2018; Rodd et al., 2020). The current findings replicate a previous report that pretreatment with an α7 NAM can prevent the ABAE-induced enhancement of adult EtOH consumption (Rodd et al., 2020; **Figures 3** and **4**). Thus, repeated datasets

have now indicated that pretreatment with an α7 NAM can prophylactically prevent ABAE-induced enhancement of adult EtOH consumption presumably through regulation of the α7 receptor during ABAE exposure. Conversely, the data also indicates that modulation of the α7 receptor during ABAE can have bi-directional effects. Co-administration of a α7 NAM blocks ABAE-induced enhancements of adult alcohol consumption, while co-administration of a α7 PAM promotes the deleterious consequences of ABAE.

SB-277011-A is a D_3 receptor antagonist and an α7 NAM (Zheng et al., 2016). SB has been tested as a potential pharmacotherapeutic for cocaine, nicotine, morphine, and alcohol. SB significantly decreases binge-like consumption of EtOH in C57B6 mice (Rice et al., 2015). Nicotine-seeking and self-administration can be inhibited by SB (Sabioni et al., 2016). Similarly, SB reduces cocaine and morphine self-administration and seeking (Rice et al., 2013). The SB compound reduces EtOH consumption in alcohol-preferring (P) rats (Thanos et al., 2005). Despite the extensive use of SB for the D_3 receptor antagonism, there is no evidence reported that a D_3 antagonist administered during adolescence has persistent effects inhibitory on behaviors during adulthood. The possibility that the effects of the SB compound on EtOH drinking in reported studies could be mediated in part by the α7 NAM actions of SB was not considered.

There are both endogenous α7 PAMs and a NAM. Secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptides (SLURP-1 and SLURP-2) are endogenous α7 PAMs that are expressed in all organs of the mammalian body (Chimienti et al., 2003). Kynurenic acid (KYNA) is an endogenous α7 NAM (KYNA active at glycine and ionotropic glutamate receptors; Kiss et al., 2003). Enhancing KYNA levels in the brain prevents nicotine-induced increases in dopamine (Erhardt et al., 2001) and glutamate (Konradsson-Geuken et al., 2009), reduces the reinforcing properties of Tetrahydrocannabinol (THC) (Justinova et al., 2013), and reduces nicotine craving and relapse (Secci et al., 2017). Although α7 PAMs have been reported to reduce the reinforcing properties of nicotine (Jackson et al., 2019), complete dose-response analyses have indicated that α7 PAMs produce leftward-shifts (increase sensitivity) in the dose-response curve for nicotine reward behaviors (Perkins et al., 2018). Recent clinical data have indicated that treatment with an α7 PAM increased nicotine craving and nicotine use (Perkins et al., 2018). Activation of the KYNA pathway reduces EtOH consumption and EtOH-induced dopamine release in the Acb (Giménez-Gómez et al., 2018).

A NAM does not reduce the activity of a neuron. Instead, NAMs prevent the increase or decrease activity of a receptor produced by ligands (Lopes et al., 2007). NAMs bind at allosteric sites on a receptor which are different from the binding sites for agonists and antagonists. For example, KYNA prevented choline-induced alterations of hippocampus neurons but did not affect the basal activity of these neurons (Smelt et al., 2018). The ability of α7 NAMs to prevent ligand alterations in receptor activity, without affecting basal activity, of neurons was replicate in six other α7 NAMs (Pocivavsek et al., 2014). Drugs that inhibit the α7 receptor during adolescence are associated with learning deficits during adulthood. Since α7 NAMs do not inhibit the basal activity of neurons containing the α7 receptor, there is no evidence that α7 NAMs administered during adolescence have this effect (Smelt et al., 2018). KYNA prenatally administered impairs adult learning, primarily through the glutamatergic and glycine actions of KYNA. However, KYNA administered during adolescence does not affect adult learning (Pocivavsek et al., 2014). Although the window of ABAE is fully unexplored, one would be tempted to find parallelism with effects of early-life exposure to environmental toxicants on the development of neurological disorders later in life *via* the LEARn pathway as well as across generations (*t*-LEARn; Lahiri et al., 2009, 2016).

Recent critical reviews of clinical interventions to treat adolescent problematic alcohol consumption have indicated no or small benefits of the treatments (Foxcroft and Tsertsvadze, 2011; Carney et al., 2016). Preclinical adolescent alcohol research should identify functional targets that could lead to the development of pharmacotherapeutics to counter the deleterious consequences of ABAE. The current data adds to the growing literature that cholinergic agents (in particular α7 NAMs) may be viable targets to treat ABAE and more extensive research with the goal of developing efficacious treatment is justified. Prevention is no longer a speculative concept. The findings reported here and in (Rodd et al., 2020) joined with the findings that galantamine blocked ABAE-induced increases in the expression of genes associated with the innate immune system (*TLR4* and *pNF-κB*) and histones/chromatin related genes (*RGE* and *HMGB1*) and during adulthood (Crews et al., 2021) to highlight replicated findings of preventative interventions against the persistent effects of ABAE.

Prevention has numerous benefits (prevention of harm/reduction of damage) compared to attempting to "reconstruct" a brain in a person who has suffered the etiological process to establish the AUD disorder. It is possible that novel targets will be more efficacious than α7 NAMs at preventing ABAE-induced neuroadaptations that persist throughout adulthood (or a combination treatment that includes α7 NAMs and other agents.

The development of preventative pharmacological treatment of other neuropsychological disorders is rapidly occurring. Prophylactic administration of ketamine (and ketamine metabolites) has been shown to block/prevent the development of fear-associated memories and post-traumatic stress disorder-like behaviors (PTSD; Henter et al., 2021; Zoladz et al., 2022). In addition, ketamine administered hours after exposure to the PTSD-inducing stimuli can similarly block/prevent the development of PTSD-like behaviors (Sala et al., 2022). Research attempting to develop efficacious treatments for Alzheimer's Disease (AD) has produced very limited results. In contrast, a serendipitous finding may change the trajectory of AD in societies. Briefly, Cheng and colleagues performed a predictive functional structural analysis of FDA approved compounds that could interact at AD target sites and a lead target compound was determined to be sildenafil (Viagra; Fang et al., 2021). Examining over 7 million users of sildenafil, the researchers report that the common "lifestyle" cGMP-specific phosphodiesterase type 5 (PDE5) inhibitor reduced the likelihood of individuals to develop symptoms of AD in a 6-year period by 69% after controlling for a plethora of factors (Fang et al., 2021). Preventing neuropsychological disorders such as PTSD, AD, AUD, and addiction has such an overwhelming benefit to society that efforts should be focused on developing these treatments. Another possibility is that individuals will benefit

from a combined approach in which prophylactic treatment against ABAE is used in conjunction with reversal treatments during adulthood (if needed). Gabapentin can normalize the alterations in sleep parameters induced by ABAE (Ehlers et al., 2018a,b), reverse ABAE-induced increases of glutamatergic activity in the hippocampus (Swartzwelder et al., 2017), and reverse ABAE-induced reductions of astrocyte-synaptic proximity (Healey et al., 2020). Donepezil can reverse other ABAE-induced alterations in the hippocampus (reduced dendritic spine density and expression of the *Fmr1*, Mulholland et al., 2018). The a7 NAChR system mediates many actions of Donepezil including neuroprotection, regulation of neuroimmune system, modulation of genetic expression (Takada-Takatori et al., 2008; Russo et al., 2017). These data suggest that the a7 NAChR system may also have utility to reverse the effects of ABAE during adulthood.

The effects of ABAE on adult behaviors are not limited to adult alcohol consumption. ABAE increases the risk for psychological disorders (depression, anxiety disorders, and schizophrenia), neurodegenerative disorders (AD, PD, and other dementia-related illnesses), and auto-immune diseases during adulthood (Harwood et al., 2010; Langballe et al., 2015; Schwarzinger et al., 2018; Coleman et al., 2019; Barnett et al., 2022; Tucker et al., 2022). ABAE also affects the reproductive system (hypogonadism and reduced sperm viability (Duca et al., 2019). The α7 receptor is expressed in the testes and on sperm cells. Cellular loss in the testes (like that observed following ABAE) is thought to occur through activation of the α7 receptor, and α7 receptors mediate sperm viability (Duca et al., 2019). Individuals who experience ABAE but do not develop AUD may still have these other, deleterious effects of ABAE. Therefore, any neuroprotection due to treatment with an α7 NAM could prevent such dysregulation in the adult α7 receptor system produced by excessive alcohol consumption, which would increase the risk of these disorders.

Nevertheless, the present work is the significant first step as it might cover several studies that suggest the effects of early-life exposures to chemicals and environmental and psycho-social factors on the later-life development of cognitive disorders, including AD (Maloney and Lahiri, 2016). Notably, the initial perturbation is maintained and later triggered via epigenetic mechanisms as conceptualized in the LEARn pathway, with evidence from animal studies and a small cohort of human autopsied brain tissue samples (Maloney and Lahiri, 2016). The present studies add to the epigenetic role on the effects of ABAE as described herein. For example, intermittent adolescent ethanol caused a persistent increase in adult H3K9me2, close to the NTRK1 gene and DNA methylation in ChAT promoter regions.

A novel α7 NAM has been created, BNC210. This compound has been approved for testing in human patients and has been shown to have little side-effects (Wise et al., 2020). BNC210 has been shown to reduce the expression of Seasonal Affect Disorder (SAD) and the altered neurocircuit activity profile associated with this disorder (Wise et al., 2020). BNC210 has also been shown to be efficacious at reducing the symptoms of General Anxiety Disorder (GAxD) and may have anxiolytic properties (Perkins et al., 2021). BNC210 has been given FastTrack Designation by the US FDA for the treatment of SAD and GAxD (Perkins et al., 2021). BNC210 is currently being assessed for treatment of PTSD. It is possible that BNC210 may have a similar prophylactic effect on preventing the development of PTSD as ketamine (another α7 NAM). Testing BNC210 on addictive behaviors could greatly advance the development of pharmacotherpeutics.

Overall, independent laboratories have reported that pretreatment with cholinergic agents (α7 NAMs and cholinesterase inhibitor) can preventatively block persistent effects of ABAE (Rodd et al., 2020; Crews et al., 2021). To briefly review, agonism of the α7 receptor synergistically activates posterior VTA DA neurons with EtOH (**Figure 2**), potentiating the actions of EtOH on the α7 receptor (PAM) during subthreshold exposure to ABAE that can produce enhanced intake of alcohol during adulthood (**Figure 6**), and ABAE induces persistent upregulation of the expression of *Chrna7* in the posterior VTA and AcbSh (Hauser et al., 2019a, 2021b) which is associated with an increase in protein expression of the α7 receptor in the posterior VTA (Waeiss et al., 2020), agonism of the α7 receptor results in a downregulation of ChAT (similar to ABAE; Dineley et al., 2015), and manipulations of the α7 receptor prevents the persistent effects of ABAE. Future studies need to determine the effects of pretreatment with α7 NAMs on the biological effect of exposure to ABAE in the adolescent brain and body. Currently, the actual biological effects of ABAE in adolescents are understudied. Extensive research needs to be conducted (and independently replicated) to advance the use of α7 NAMs as a preventative treatment against ABAE.

Data availability statement

All relevant data is contained within the article: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Animals care facilities at IUSM are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Research performed in the current experiments were approved by the IUSM Institutional Animal Care and Use Committee (IUSM IACUC) and were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, the NIH, and the Guide for the Care and Use of Laboratory Animals (2011).

Author contributions

The experiments were conceived by ZR, DL, SH, HS, and RB. The microdialysis experiment was performed by RW, SS, EE, SH, and ZR. The EtOH drinking experiment was performed by RW, SS, SH, RB, WT, and ZR. HPLC analyses were performed by EE, RW, and SH. Statistical analyses were performed by RB, DL, SS, and ZR. The manuscript was written by ZR, SH, DL, and RB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ZR, SH, RW, and RB have used the current dataset to apply for a patent titled "The use of alpha-7 nicotinic receptor negative allosteric modulators for the prevention of adolescent alcohol consumption producing alcohol use disorder (AUD) and drug addiction during adulthood" with the US and EU patent office. All have no direct influence on the research presented here. DL is a member of the advisory boards for Entia Biosciences, Drug Discovery and Therapy World Congress, and Provaidya LLC. He also has stock options from QR Pharma for patents or patents pending on AIT-082, Memantine, Acamprosate, and GILZ analogues. DL is the Editor-in-Chief of the journal "Current Alzheimer Research". DL also had prior funding from Baxter and Forest Research Labs. All have no direct influence on the research presented here. Finally, DL declares no other actual or potential competing interests in the subject matter of this article.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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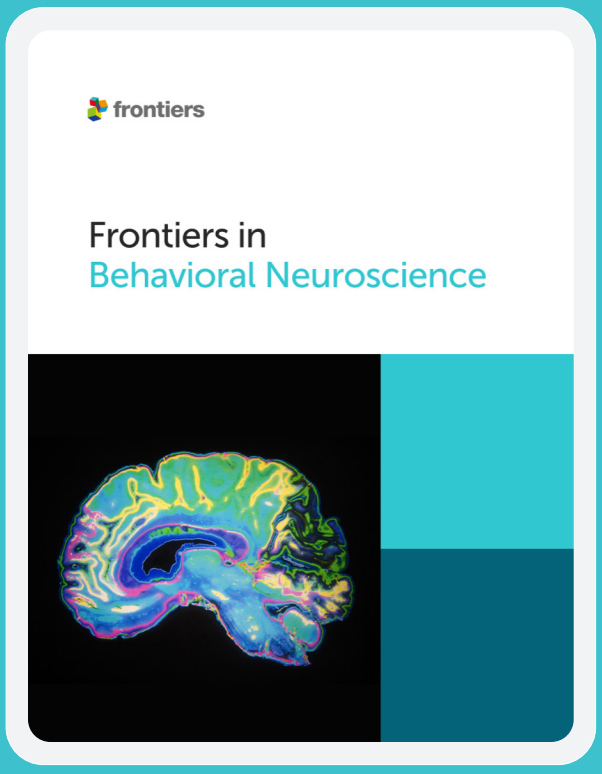

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