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Chronic ethanol alters adrenergic receptor gene expression and produces cognitive deficits in male mice

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ABSTRACT

Hyperkateifia and stress-induced alcohol cravings drive relapse in individuals with alcohol use disorder (AUD). The brain stress signal norepinephrine (also known as noradrenaline) tightly controls cognitive and affective behavior and was thought to be broadly dysregulated with AUD. The locus coeruleus (LC) is a major source of forebrain norepinephrine, and it was recently discovered that the LC sends distinct projections to addiction-associated regions suggesting that alcohol-induced noradrenergic changes may be more brain region-specific than originally thought. Here we investigated whether ethanol dependence alters adrenergic receptor gene expression in the medial prefrontal cortex (mPFC) and central amgydala (CeA), as these regions mediate the cognitive impairment and negative affective state of ethanol withdrawal. We exposed male C57BL/6J mice to the chronic intermittent ethanol vapor-2 bottle choice paradigm (CIE-2BC) to induce ethanol dependence, and assessed reference memory, anxiety-like behavior and adrenergic receptor transcript levels during 3–6 days of withdrawal. Dependence bidirectionally altered mouse brain α1 and β receptor mRNA levels, potentially leading to reduced mPFC adrenergic signaling and enhanced noradrenergic influence over the CeA. These brain region-specific gene expression changes were accompanied by long-term retention deficits and a shift in search strategy in a modified Barnes maze task, as well as greater spontaneous digging behavior and hyponeophagia. Current clinical studies are evaluating adrenergic compounds as a treatment for AUD-associated hyperkatefia, and our findings can contribute to the refinement of these therapies by increasing understanding of the specific neural systems and symptoms that may be targeted.

1. Introduction

Alcohol use disorder (AUD) is a chronic, relapsing disease characterized by a compulsion to seek and use alcohol, loss of control in limiting alcohol intake, and the emergence of a negative affective state during alcohol withdrawal. This negative affective state (termed hyperkatefia) can include symptoms of anxiety, dysphoria, pain, irritability, anhedonia, sleep disturbances, and general malaise, that extends beyond the somatic signs of withdrawal (Koob, 2021). Hyperkatefia increases an individual's relapse risk by impairing cognitive function, particularly impulse control and decision making, and by promoting alcohol consumption to alleviate its negative symptoms.

Norepinephrine (NE; also known as noradrenaline) is a major brain stress signal that tightly controls cognitive behavior (Ramos and Arnsten, 2007) and has been implicated in the hyperkatefia associated with alcohol withdrawal (Haass-Koffler et al., 2018; Koob, 2021). It is produced in the locus coeruleus (LC), nucleus tractus solitarius and other deep brain nuclei, and released throughout the brain (Vazey et al., 2018). Withdrawal increased central levels of NE and its metabolites in individuals with AUD, and in the cortex and whole brain of ethanol-exposed rodents (Hawley et al., 1981; Jaime and Gonzales, 2019; Karoum et al., 1976; Lanteri et al., 2008; Patkar et al., 2003).

Abbreviations: AUD, Alcohol use disorder; BM, Barnes maze; CeA, central amygdala; CIE, chronic intermittent ethanol vapor model; CIE-2BC, chronic intermittent ethanol vapor – two bottle choice model; CRF, corticotropin-releasing factor; Dep, ethanol dependent mice; LC, locus coeruleus; mPFC, medial prefrontal cortex; NE, Norepinephrine; Non-dep, non-dependent mice; rt-PCR, Real time polymerase chain reaction; 2BC, two bottle choice ethanol drinking.

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Moreover, LC activation increased NE release in the rodent amygdala and prefrontal cortex, which potentiated ethanol drinking, anxiogenic and aversive behavior (Deal et al., 2020; McCall et al., 2015). NE binds to α1 and β1/2 adrenergic receptors, and their pharmacological blockade decreased ethanol withdrawal symptoms, anxiety-like behavior and relapse-related drinking in rodents (Froehlich et al., 2013, 2015; Gilpin and Koob, 2010; Rasmussen et al., 2009; Verplaetse et al., 2012; Walker et al., 2008). Prazosin (α1 inverse agonist) and propranolol (β1/2 inverse agonist) are both FDA-approved to treat cardiovascular disease, but not currently prescribed for AUD (Haass-Koffler et al., 2018). However, pilot studies with these compounds reported decreased anxiety, withdrawal symptoms and severity, and alcohol craving in individuals with AUD, leading to less alcohol drinking days and lower alcohol consumption on drinking days (Fox et al., 2012; Haass-Koffler et al., 2018; Milivojevic et al., 2020; Simpson et al., 2018; Sinha et al., 2022; Wilcox et al., 2018). Given the promise of these initial clinical results, understanding how chronic ethanol alters noradrenergic signaling in brain regions that contribute to withdrawal-associated negative affect and cognitive impairment is important.

The central amygdala (CeA) and medial prefrontal cortex (mPFC) are key brain regions in the neurobiology of alcohol addiction that are strongly innervated by NE fibers (Vazey et al., 2018). The CeA integrates negative emotional responses associated with anxiety, alcohol and stress (Gilpin et al., 2015), and chronic alcohol dysregulates CeA adrenergic signaling in humans and rodents (Varodayan et al., 2022). Reciprocal connections between the CeA and LC form a corticotropin-releasing factor (CRF)/β1 adrenergic stress circuit that can trigger persistent NE release across the brain (Kravets et al., 2015; Rudoy et al., 2009). The LC also sends a distinct projection to the mPFC (Borodovitsyna et al., 2020; Ramos and Arnsten, 2007), a region involved in stress-induced craving and relapse of abstinent AUD patients (George and Koob, 2013; Haass-Koffler et al., 2018; Koob, 2021; Sinha et al., 2022). Optimal mPFC function requires moderate levels of NE, while stress causes excessive mPFC NE release leading to the engagement of α1 and β1 receptors and impaired cognitive function (Ramos and Arnsten, 2007). Using *post-mortem* brain tissue analysis, we recently reported increased $\alpha1_B$ and a trend for the overexpression of β1 mRNA levels in the amygdala, but not PFC, of individuals with AUD (Varodayan et al., 2022). Thus, here we used a mouse model of AUD to investigate withdrawal-induced cognitive impairment and negative affect, and to evaluate changes in CeA and mPFC adrenergic receptor gene expression.

2. Materials & methods

2.1. Study design

Adult male C57BL/6J mice (n = 67; 30.6 ± 1.9 g; The Jackson Laboratory) were group-housed in a temperature- and humidity-controlled vivarium on a reversed 12 h light/dark cycle (lights off 8:00AM/on 8:00PM) with *ad libitum* food and water (unless noted). All mice were exposed to the chronic intermittent ethanol – two bottle choice (CIE-2BC) model, with separate cohorts used for the behavioral and gene expression studies to ensure matched withdrawal timepoints and eliminate the possibility that the behavioral testing could impact mPFC adrenergic receptor gene expression. All procedures were approved by The Scripps Research Institute (TSRI) Institutional Animal Care and Use Committee, consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental sample sizes were determined using power analyses based on prior studies.

2.2. Chronic intermittent ethanol – two bottle choice model

The chronic intermittent ethanol-two bottle choice (CIE-2BC) model was used to generate ethanol naive mice, non-dependent mice (Non-dep) that voluntarily consumed ethanol, and ethanol dependent mice (Dep) that were exposed to CIE vapor and escalated their ethanol intake (Fig. 1A) (Becker and Lopez, 2004; Patel et al., 2019, 2021; Varodayan et al., 2023).

First, baseline two bottle choice ethanol drinking (2BC) was established. 5 days per week for 2–4 weeks, mice were individually housed 30 min before lights off for 2 h limited access to two drinking tubes (15% w/v ethanol and tap water). Mice were returned to their group-housed home cages after each session. Naive mice (N = 24) received 2 water bottles. The final week of baseline ethanol consumption was used to assign mice to the Non-dep (N = 21) or Dep (N = 22) group.

Dep mice then underwent 5 cycles of a 2-week protocol: 4 days of CIE (16 h ethanol vapor/8 h air in vapor chambers from La Jolla Alcohol Research, La Jolla, CA), 3 days off, 5 days of 2BC, and 2 days off. Immediately prior to each vapor exposure, Dep mice received an *i.p.* injection of 1.75 g/kg ethanol +68.1 mg/kg pyrazole (an alcohol dehydrogenase inhibitor; Sigma, St Louis, MO). Tail bloods were collected weekly and processed on an Agilent 7820A GC coupled to a 7697A (headspace-flame-ionization) to target blood ethanol levels (BEL) that reliably produces physical dependence (150–250 mg/dL). Non-dep mice were treated similarly with intervening weeks of *i.p.* injections of 68.1 mg/kg pyrazole in saline and air chamber exposure, and weeks of 2BC drinking (Becker and Lopez, 2004; Patel et al., 2019, 2021; Varodayan et al., 2023). Naive mice were treated similarly with intervening weeks of *i.p.* injections of 68.1 mg/kg pyrazole in saline in their home cages, and 2BC with 2 water bottles. Of note, Non-dep and Dep mice have difference routes and time periods of ethanol exposure (2 h drinking vs. 16 h vapor exposure) and reach different average blood ethanol levels (~50 vs. ~150 mg/dL, data not shown). Therefore, the Non-dep mice form a secondary control group that allow for determination of whether changes observed in Dep mice specifically result from the chronic ethanol vapor (which produces the dependent phenotype) or after ethanol drinking alone.

2.3. Barnes maze

We used a modified Barnes maze task with an extended break (13 weeks) between acquisition and retention, during which time this cohort of mice underwent CIE-2BC, to probe spatial reference memory (Fig. 2A) (Gawel et al., 2019; Varodayan et al., 2018). The same experimenter conducted all behavioral testing.

2.3.1. Acquisition

Prior to any ethanol exposure, mice (N = 33) underwent Barnes maze acquisition, with 1 trial per day for 6 consecutive days. The maze was an elevated (60 cm), brightly-lit (400 lux) circular open platform (75 cm diameter) with 20 small holes (5 cm diameter) evenly distributed along the perimeter. A white noise machine (80 dB) and evenly-spaced fans were directed at the platform to slightly increase aversion. The mouse could "escape" the platform by climbing into a recessed chamber below a target hole. The target hole location was consistent for each mouse, but rotated across mice to account for any side biases. Distal visual cues aided the mouse's navigation. On the first day, each mouse was placed in the center of the maze, guided toward the target hole and encouraged to climb in. After spending 1 min in the recessed chamber, the mouse immediately entered its first acquisition trial.

For each acquisition trial, the mouse had 3 min to enter the target hole, where it remained for 1 min before being returned to its home cage. If it failed to complete the trial, it was guided to the target hole. Tracking videos were analyzed with EthoVision software (Noldus, Wageningen, The Netherlands), with latency to approach any first hole, latency to enter the target hole, number of errors (incorrect holes visited prior to entering the target hole), and the mean velocity measured.

Immediately prior to the final acquisition trial, each mouse underwent a 3 min probe test in which the recessed chamber was removed. The percentage of time each mouse spent in the quadrant containing its original target hole was calculated. After the probe test, there was a final

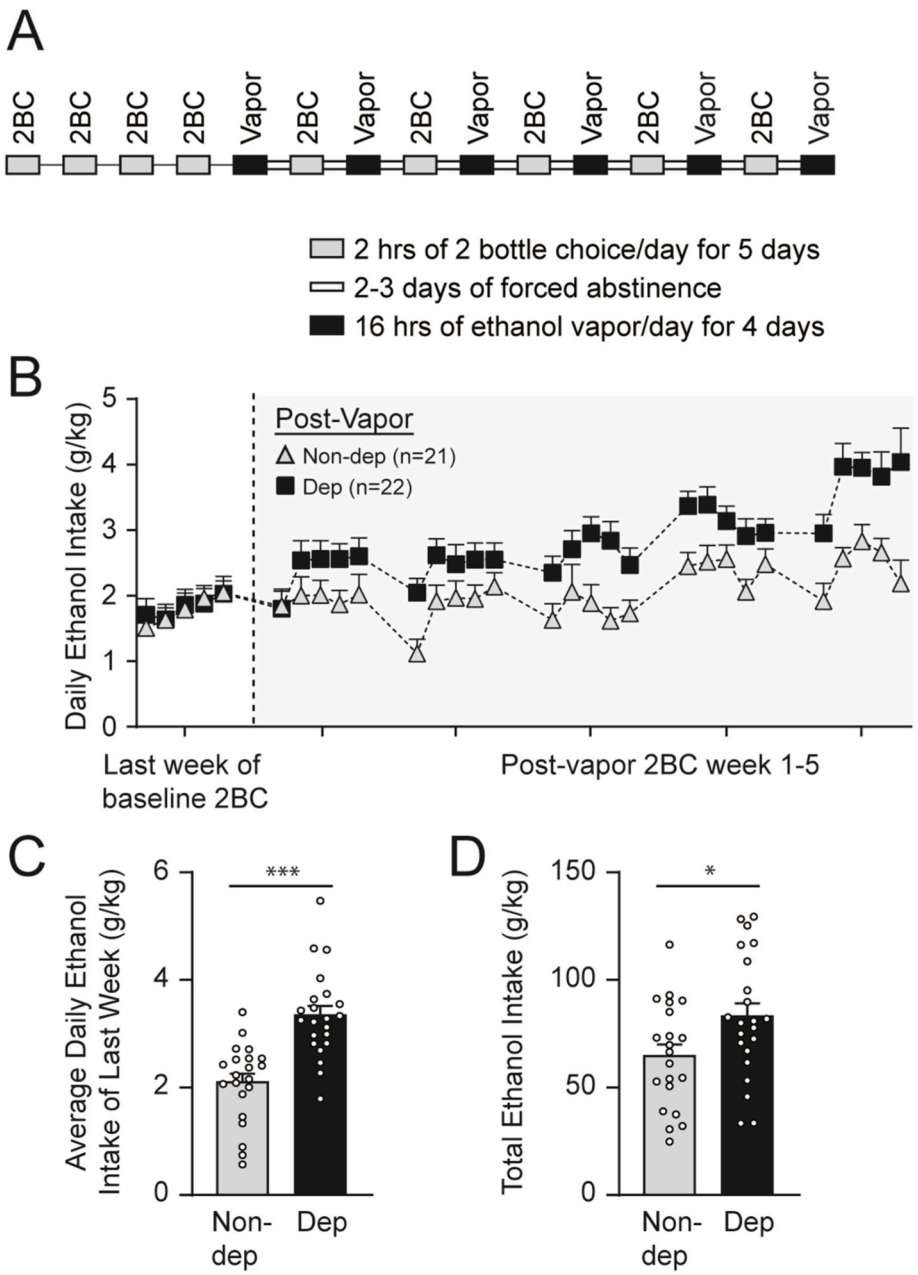

Fig. 1. Chronic-intermittent ethanol (CIE) - two-bottle choice (2BC) paradigm. **A.** Schematic of the CIE-2BC paradigm used to induce ethanol dependence. **B.** Daily ethanol intake during 2BC drinking sessions escalates in dependent vs. non-dependent mice. **C-D**. Dependent mice consume more ethanol (C) during the last week of 2BC, and (D) across all 2BC drinking sessions compared to non-dependent mice. N = 21–22 mice per group. *$p < .05$, ***$p < .001$ by unpaired *t*-test.

acquisition trial, with the strategy to locate the target hole classified as: 1) spatial - mouse enters target hole after no more than 1 error in an adjacent hole, 2) serial - mouse approaches a hole and travels around the platform checking each sequential hole, or 3) random - localized hole searches separated by crossings through the center of the maze.

Mice were then assigned to the Naive, Non-dep or Dep groups based on acquisition performance (Fig. 2B–C) and underwent CIE-2BC. Of note, 3 mice were unexpectedly lost during the 2BC drinking phase of this study, leaving N = 9 in the Non-dep group and N = 12 in the Naïve and Dep groups.

2.3.2. Retention

Three days into withdrawal from CIE-2BC, each mouse underwent a 3 min retention task (same parameters as an acquisition trial).

2.4. Digging task

Spontaneous digging behavior was assessed 5 days into withdrawal (Fig. 2A) (Sidhu et al., 2018). Each mouse was placed in a new cage with 5 cm of bedding under dim red light (20 lux) for 3 min. The number of digging bouts and total time spent digging were recorded.

2.5. Novelty-induced suppression of feeding task

Immediately after the digging test, mice were transferred to new home cages and food-restricted for 24 h (Fig. 2A) (Patel et al., 2021; Sidhu et al., 2018). Just prior to testing, mice were transferred to new holding cages. Testing consisted of two consecutive feeding phases, first in a novel arena and then in the familiar home cage they had been housed in overnight. The open field (50 x 50 × 22 cm) was brightly lit

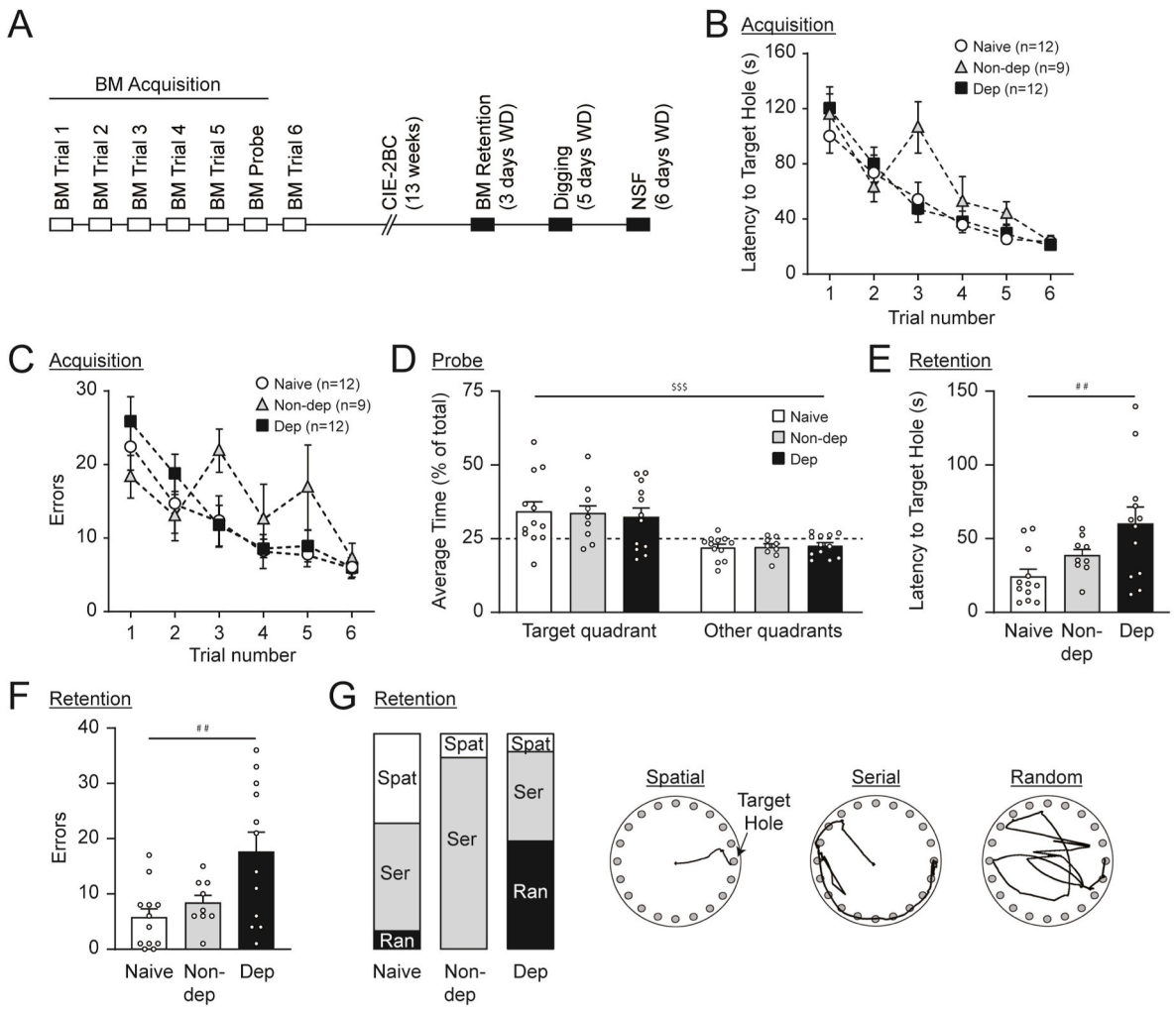

Fig. 2. Ethanol dependence impairs cognitive performance in the Barnes maze task. **A.** Schematic illustrating the behavioral paradigm used to assess reference memory (Barnes maze; BM) and anxiety-like behavior (digging and novelty-suppressed feeding; NSF) after withdrawal from CIE-2BC. **B-C.** Prior to ethanol exposure, mice assigned to all three groups showed similar BM task acquisition based on (B) latency to enter target hole and (C) number of errors. **D.** Prior to ethanol exposure, mice assigned to all three groups spent more time in the target hole quadrant than on average in each of the other quadrants during the BM probe test, indicating successful task acquisition. **E-F.** After CIE-2BC, dependent mice showed a BM retention deficit based on (E) their longer latency to enter the target hole and (F) greater number of errors compared to naive mice. **G.** Naive and non-dependent mice employed mainly spatial or serial strategies to locate the target hole during the BM retention task, while dependent mice mainly employed serial or random strategies. N = 12 naive, 9 non-dependent and 12 dependent mice. $^{\$\$\$}p < .001$ main effect of quadrant by two-way ANOVA; $^{\#\#}p < .01$ by one-way ANOVA and Tukey's post hoc test.

(400 lux) and filled with 1 cm of fresh bedding, with a food pellet was placed in the center. Each mouse was released in a corner and the latency to feed recorded, with a cutoff time of 10 min. The mouse was removed as soon as it began to feed and immediately transferred to its own dimly red-lit (20 lux) home cage where a single food pellet was placed at one end. The latency to feed was recorded, with a cutoff time of 5 min. The mouse was then returned to a holding cage with free access to food and water.

2.6. Adrenergic receptor gene expression

Adrenergic receptor (α1 and β) gene expression was assessed in the CeA and mPFC of a separate cohort of Naive (N = 12), Non-dep (N = 12) and Dep mice (N = 10). 3 days after their last CIE-2BC ethanol exposure, mice were anesthetized with 3–5% isoflurane and their extracted brains immediately flash frozen and stored at −80 °C. Brains were shipped on dry ice from The Scripps Research Institute to Binghamton University, where they were sliced using a cryostat and micropunches (0.75 mm) enriched for the CeA and mPFC were collected. Samples were homogenized in Trizol reagent (Sigma-Aldrich, St. Louis, MO) with 5 mm stainless steel beads (Qiagen, Hilden, Germany) and a TissueLyser (Qiagen, Valencia, CA). Total RNA was extracted using RNeasy columns (Qiagen) according to manufacturer instructions. Total RNA concentration and purity were measured with a Nanodrop spectrophotometer (Themoscientific, Waltham, MA). cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (Cat. No. 205313, Qiagen). Real time polymerase chain reaction (rt-PCR) was performed using the CFX384 real-time PCR detection system, the IQ SYBER Green Supermix (Biorad, Hercules, CA), cDNA template, and primers for the following genes encoding adrenergic receptor subtypes: *Adra1a_v1-3, Adra1a_v1,2,4, Adra1b, Adra1d, Adrb1* and *Adrb2* (Table 1). Of note, alternative splicing of the gene encoding $\alpha 1_A$ (*Adra1a*) leads to four transcript variants, and we designed two separate primer pairs that amplified either variants 1–3 or variants 1,2,4 to measure the mRNA levels of all four *Adra1a* variants.

Each gene was normalized to the reference gene TATA-box binding protein (*Tbp;* Suppl. Fig. 1) using the $\Delta\Delta C_q$ method, and the percent change from control calculated with the naive group selected as the ultimate control. All data points falling in the outlier range of ±2 standard deviations were dropped from that specific region/gene analysis.

Table 1
Primers pairs used for gene expression study.

Gene name	Gene symbol	Accession number	Forward primer	Reverse primer
TATA-box binding protein	*Tbp*	NM_013684.3	TTCTGCGGTCGCGTCATTT	GTGGAAGGCTGTTGTTCTGGT
Adrenergic receptor, $\alpha1_A$ variants 1-3	*Adra1a v1-3*	NM_001271761.1	CCGTGAGGCTGCTCAAGTTT	AAATTCGGGAAGAAGGACCCAAT
Adrenergic receptor, $\alpha1_A$ variants 1,2,4	*Adra1a v1,2,4*	NM_001271761.1	GACTGGGTCTTGGTCTTTGGA	GGCCCTGGAGCTTCGTTT
Adrenergic receptor, $\alpha1_B$	*Adra1b*	NM_001284381.1	ACCTTGGGCATTGTAGTCGG	GGAGAACAGGGAGCCAAGTG
Adrenergic receptor, $\alpha1_D$	*Adra1d*	NM_013460.5	TCTCCGTAAGGCTGCTCAAG	GAGGGAACAGAGAACCCAGAG
Adrenergic receptor, β1	*Adrb1*	NM_007419.3	CTGCTACAACGACCCCAAGT	CACGTAGAAGGAGACGACGG
Adrenergic receptor, β2	*Adrb2*	NM_007420.3	AATAGCAACGGCAGAACGGA	TCAACGCTAAGGCTAGGCAC

For all primer pairs, a single peak in the melt curve was used to confirm specificity for the target gene.

2.7. Statistics

Statistical analyses were performed using one-sample and unpaired t-tests, Pearson correlations, one-way ANOVAs with *post hoc* Tukey's multiple comparisons tests where appropriate, and two-way ANOVAs, with differences significant at $p < .05$ (Prism v.9, GraphPad, San Diego, CA). Data are represented as mean ± SEM.

3. Results

3.1. Ethanol vapor exposure increases voluntary ethanol consumption in dependent mice

Three animal groups with varying levels of ethanol exposure were used in this study: 1) ethanol naive mice, 2) non-dependent (Non-dep) mice that drank a moderate amount of ethanol in their 2BC sessions, and 3) ethanol dependent (Dep) mice that escalated their ethanol consumption after CIE vapor exposure (Fig. 1A). Specifically, Dep mice consumed more ethanol daily during their last week of 2BC drinking and consumed a greater overall amount of ethanol compared to Non-dep mice (Daily: $t(41) = 5.23$, $p < .001$; Total: $t(21) = 2.24$, $p < .05$ by unpaired *t*-test; Fig. 1B–D).

3.2. Dependence impairs cognitive performance during withdrawal

To probe the impact of dependence on reference memory we used a modified version of the Barnes maze task with an extended break between acquisition and retention (Fig. 2A) (Auger and Floresco, 2014; Gawel et al., 2019; Negrón-Oyarzo et al., 2018; Varodayan et al., 2018). Prior to any ethanol exposure, mice assigned to the Naive, Non-dep and Dep groups showed similar acquisition performance (Fig. 2B–C). Specifically, two-way repeated measures ANOVA revealed a significant main effect of trial number on latency to enter the target hole ($F(3.74, 112.2) = 28.72$, $p < .001$), but no main effect of animal group ($F(2,30) = 2.19$, $p = .13$) and no interaction ($F(10, 150) = 1.66$, $p = .095$). Likewise, there was a significant main effect of trial number on the errors made ($F(3.93, 118.0) = 10.39$, $p < .001$), but no main effect of animal group ($F(2,30) = 1.47$, $p = .25$) and no interaction ($F(10, 150) = 1.50$, $p = .14$). Importantly, the probe test is designed to assess spatial memory retrieval based on whether the mice spend more than 25% of their time in the target quadrant despite the fact that they cannot locate the escape box there (Gawel et al., 2019; Paul et al., 2009). During the probe test, mice from all three groups spent more time in the target hole quadrant than on average in each of the other quadrants, indicating successful task acquisition (Fig. 2D). Specifically, two-way ANOVA revealed a main effect of percent time spent in the target vs. other quadrants ($F(1, 60) = 28.23$, $p < .001$), but no main effect of animal group ($F(2, 60) = 0.036$, $p=.97$) and no interaction ($F(2, 60) = 0.14$, $p=.87$). A similar analysis of the percent time spent in the quadrants during early acquisition revealed no main effects or interaction (Suppl. Fig. 2). Finally, a similar proportion of mice in each group used spatial (Naive: 41%, Non-dep: 33%, Dep: 41%) or serial (Naive: 59%, Non-dep: 67%, Dep: 59%) strategies to locate the target hole. No mice used a random strategy.

Long-term spatial memory was assessed 3 days after CIE-2BC withdrawal using the retention test. Naive mice performed similarly in the retention test as in their final (6th) acquisition trial (Latency to target hole: $t(11) = 0.11$, $p > .05$; Errors: $t(11) = 0.48$, $p > .05$ by paired *t*-test). However, Dep mice showed a retention deficit with increased latency to enter target hole and greater errors made compared to the Naive mice (Latency to target hole: $F(2,30) = 5.25$, $p < .05$; Errors: $F(2,30) = 6.27$, $p < .01$ by one-way ANOVA and *post hoc* Tukey's test; Fig. 2E–F). These retention measures did not correlate with average daily ethanol intake during the last week of drinking (Suppl. Fig. 3). There were no group differences in the latency to approach the first hole or mean velocity, indicating that ethanol exposure did not impact retention of the task goal to find the target hole, motivation to complete the task or locomotor ability (Latency: $F(2,30) = 0.54$, $p > .05$; Velocity: $F(2,30) = 2.24$, $p > .05$ by one-way ANOVA). Critically, the retention deficits were accompanied by a shift in the search strategy with Naive and Non-dep mice employing mainly spatial or serial strategies, and Dep mice employing mainly serial or random strategies (Fig. 2G).

3.3. Ethanol dependence produces a negative affective state during withdrawal

Five days into withdrawal the mice were assessed for spontaneous digging behavior, a naturalistic rodent behavior that may capture some aspects of anxiety-like and compulsivity-like behavior (Pond et al., 2021; Thomas et al., 2009). Dep mice spent more time digging and had more digging bouts compared to other groups (Time digging: $F(2,30) = 5.45$, $p < .01$; Bouts: $F(2,30) = 6.11$, $p < .01$ by one-way ANOVA and *post hoc* Tukey's test; Fig. 3A–B). These digging measures did not correlate with average daily ethanol intake during the last week of drinking (Suppl. Fig. 3).

The next day the novelty-suppressed feeding task was used to more directly measure anxiety-like behavior (Dulawa and Hen, 2005; Patel et al., 2021). Dep mice had a higher latency to feed in the novel arena (brightly-lit novel open field) compared to both Naive and Non-dep mice ($F(2,30) = 5.96$, $p < .01$ by one-way ANOVA and *post hoc* Tukey's test; Fig. 3C). Interestingly, latency to feed in the novel arena was positively correlated with average daily ethanol intake during the last week of drinking, suggesting a possible link between the two ($p < .05$; Suppl. Fig. 3). There were no group differences when the task was repeated in the dimly-lit home cage, indicating that ethanol exposure did not alter their feeding behavior or motivation to feed ($F(2,30) = 2.33$, $p > .05$ by one-way ANOVA; Fig. 3D).

3.4. Withdrawal from ethanol dependence brain region-specifically alters adrenergic gene expression

In a separate cohort of mice, we used rt-PCR to probe the effects of 3 days of withdrawal on adrenergic receptor gene expression in the mPFC and CeA. There was a significant decrease in transcript levels of the genes encoding adrenergic receptor subtypes $\alpha1_A$ (*Adra1a v1-3* and *Adra1a v1,2,4*) and β2 (*Adrb2*) in the mPFC of dependent mice (Fig. 4; see Table 2 for all gene expression statistical analyses; see Suppl. Fig. 4 for non-significant correlation analyses). There was also a dependence-

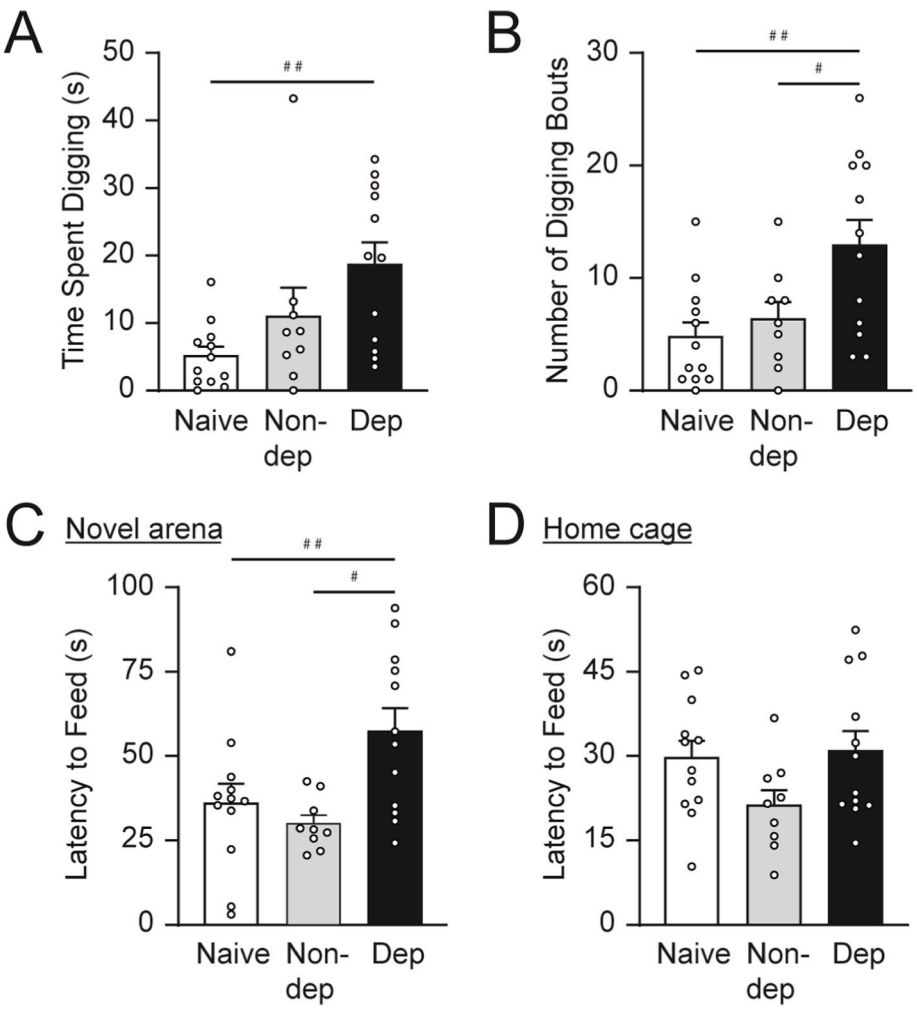

Fig. 3. Ethanol dependence produces a negative affective state. **A-B.** Dependent mice (A) spent more time digging and (B) had more digging bouts than naive and non-dependent mice. **C-D.** In the novelty-suppressed feeding task, (C) dependent mice had a higher latency to feed in the novel arena compared to naive and non-dependent mice, (D) with no group differences in the familiar home cage. N = 12 naive, 9 non-dependent and 12 dependent mice. $^{\#}p < .05$, $^{\#\#}p < .01$ by one-way ANOVA and Tukey's post hoc test.

induced decrease in $\alpha1_D$ (*Adra1d*) mRNA that approached significance ($p = .0501$). In contrast, the CeA of dependent mice showed increased expression of the genes encoding $\alpha1_A$ (*Adra1a v1-3*), $\alpha1_B$ (A*dra1b*) and β1 (*Adrb1*) (Fig. 5; Table 2). Interestingly, CeA *Adra1a v1-3* transcript levels positively correlated with average daily ethanol intake during the last week of drinking, suggesting a possible link between the two ($p < .05$; Suppl. Fig. 5).

4. Discussion

Hyperkateifia and alcohol cravings during abstinence are significant motivating factors for the escalation of alcohol consumption and/or relapse in individuals with AUD (Koob, 2021). Similar mechanisms of negative reinforcement are hypothesized to drive the escalation of ethanol intake in rodents, including in our CIE-2BC model of ethanol dependence (Becker and Lopez, 2004; Sidhu et al., 2018). Thus, here we investigated the affective and cognitive behavior of ethanol dependent mice after 3–6 days of withdrawal from CIE-2BC. Dependence produced long-term retention deficits and a shift in search strategy in a modified Barnes maze task, as well as greater spontaneous digging behavior and hyponeophagia. These behavioral changes were accompanied by brain region-specific alterations in gene expression of multiple adrenergic receptor subtypes, with decreases generally observed in the mPFC and increases in the CeA. Since NE is released widely, withdrawal may also alter its regulation of other addiction-related brain regions including the basolateral amygdala, bed nucleus of the stria terminalis, anterior insula, rostromedial tegmental nucleus and lateral hypothalamus (Burnham et al., 2021; Chesworth and Corbit, 2018; De Oliveira Sergio et al., 2021; Dornellas et al., 2021; Fetterly et al., 2019; McElligott et al., 2013; Silberman et al., 2012; Snyder et al., 2019). Current clinical studies are evaluating adrenergic compounds as a treatment for AUD-associated hyperkatefia, and our findings can help support the refinement of these therapies by increasing understanding of the specific neural systems and symptoms that may be targeted.

4.1. Dependence produces cognitive impairment and decreases mPFC adrenergic gene expression

We found that only the ethanol dependent mice showed impaired reference memory. These long-term retention deficits were accompanied by a loss of search strategy in over half the dependent mice. Our findings are inconsistent with previous studies examining ethanol's effects on Barnes maze retention (Marszalek-Grabska et al., 2018; Varodayan et al., 2018). However, key experimental differences may have increased cognitive load in our task; we used fewer acquisition trials (6 vs. 10–24), higher ethanol exposure (13 weeks of CIE-2BC vs. either 5 days of intragastric gavage or 3–4 weeks of CIE) and a longer break before retention testing (13 weeks vs. 2–4 weeks). Similarly, individuals with AUD only showed cognitive deficits when the working memory load was increased, suggesting that they have lower neurophysiological capacity (Wesley et al., 2017). Another possibility is that withdrawal associated anxiety-like behavior may have increased motivation to "escape" the platform, confounding ethanol-induced retention deficits. This is unlikely in our study as our dependent mice showed increased

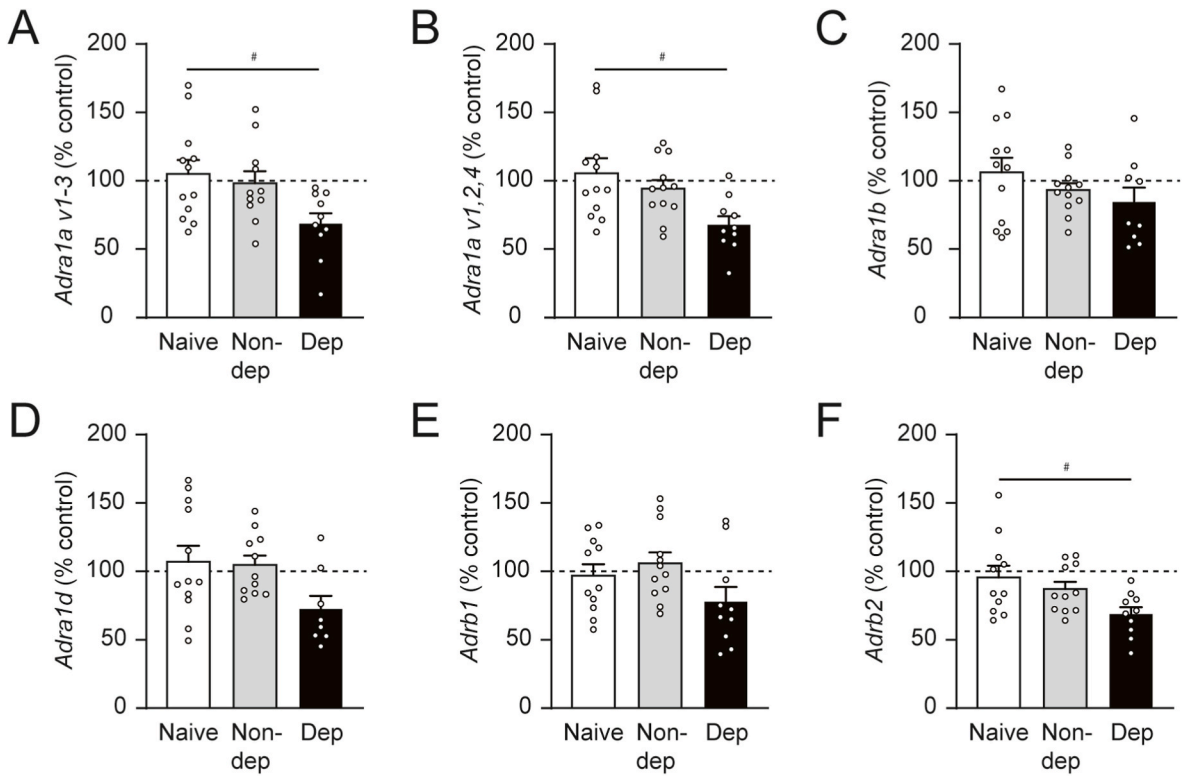

Fig. 4. Ethanol dependence decreases the gene expression of adrenergic receptor subtypes in the mPFC. **A-F.** mPFC mRNA levels for (A–B) $\alpha 1_A$ (*Adra1a v1-3* and *Adra1a v1,2,4*) and (F) β2 (*Adrb2*) were lower in dependent mice compared to naive mice, with no significant differences across groups in (C) $\alpha 1_B$ (*Adra1b*), (D) $\alpha 1_D$ (*Adra1d*) and (E) β1 (*Adrb1*). N = 10–12 mice per group. $^{\#}p < .05$ by one-way ANOVA and Tukey's post hoc test.

Table 2
Statistical values for gene expression study.

Gene name	Gene symbol	Statistical value
mPFC	*Tbp*	$F(2,31) = 0.31$, p=.73
	Adra1a_v1-3	$F(2,30) = 4.37$, $p < .05$
	Adra1a_v1,2,4	$F(2,31) = 4.96$, $p < .05$
	Adra1b	$F(2,30) = 1.44$, p=.25
	Adra1d	$F(2,28) = 3.34$, p=.0501
	Adrb1	$F(2,30) = 2.50$, p=.10
	Adrb2	$F(2,29) = 4.34$, $p < .05$
CeA	*Tbp*	$F(2,30) = 2.54$, p=.10
	Adra1a_v1-3	$F(2,30) = 7.85$, $p < .01$
	Adra1a_v1,2,4	$F(2,30) = 3.10$, p=.06
	Adra1b	$F(2,30) = 4.15$, $p < .05$
	Adra1d	$F(2,31) = 1.07$, p=.36
	Adrb1	$F(2,30) = 4.24$, $p < .05$
	Adrb2	$F(2,31) = 2.55$, p=.10

anxiety-like behavior compared to their naive/non-dependent counterparts (see Section 4.2 below), but a similar level of motivation based on the lack of group differences in the latency to approach the first hole. Similar to our study, Pereira et al. observed reference memory deficits when testing pre-trained ethanol-exposed rats in an eight-arm radial maze after 1 year (Pereira et al., 1998).

The mPFC plays a critical role in reference memory, with mPFC/PFC lesions causing impairment in both humans and rodents (Auger and Floresco, 2014; Ciaramelli, 2008; Ethier et al., 2001; Kolb et al., 1994). Moreover, the Barnes maze target hole is specifically encoded by mPFC neurons when mice employ an optimal navigation strategy (Negrón-Oyarzo et al., 2018). Optimal mPFC function requires moderate levels of NE (Ramos and Arnsten, 2007), and it was recently observed that 24 h abstinence after a week of ethanol self-administration reduced mPFC NE tone in awake behaving rodents (Jaime et al., 2020). Here we found that 3 days of withdrawal decreased mPFC transcript levels of the $\alpha 1_A$ and β2 adrenergic receptor subtypes in dependent mice. It is difficult to speculate how these changes in mRNA levels impact receptor expression and function, particularly since the mPFC has varying patterns of adrenergic receptor expression and each receptor has a different affinity for NE (Goldman-Rakic et al., 1990; Nicholas et al., 1993; Ramos and Arnsten, 2007; Santana et al., 2013). In general, α1 and β receptor activation increase pyramidal cell excitability to facilitate tasks that require a higher cognitive load (Barth et al., 2007; Berridge and Spencer, 2016; Lapiz and Morilak, 2006; Mueller et al., 2008; Zhang et al., 2013), suggesting that ethanol dependence may reduce mPFC function. This interpretation is complicated by the fact that each mPFC subregion has distinct control over various aspects of behavior; the prelimbic cortex integrates contextual information to mediate conflicting motivational drives (e.g. reward-seeking vs. escaping to safety), whereas the infralimbic cortex plays a more generalized role in active avoidance (Capuzzo and Floresco, 2020; Moorman et al., 2015). Our molecular study did not distinguish between the prelimbic vs. infralimbic subregions of the mPFC, and future studies should evaluate these potential differences. Regardless, our data suggest that under conditions of high cognitive load, mPFC noradrenergic dysregulation may contribute to ethanol dependence-associated cognitive impairment.

4.2. Dependence produces negative affect and increases CeA adrenergic gene expression

To probe whether ethanol dependence generated a negative affective state, we used the novelty-suppressed feeding task where mice experience innate conflict between fear of the novel environment and motivation to eat (Dulawa and Hen, 2005). It is important to note that the naïve mice in our study showed similar latencies to feed in the novel arena and home cage. Only the dependent mice displayed hyponeophagia (increased latency to feed in a novel environment) after 5 days of withdrawal, indicating a heightened anxiety-like state. This is in

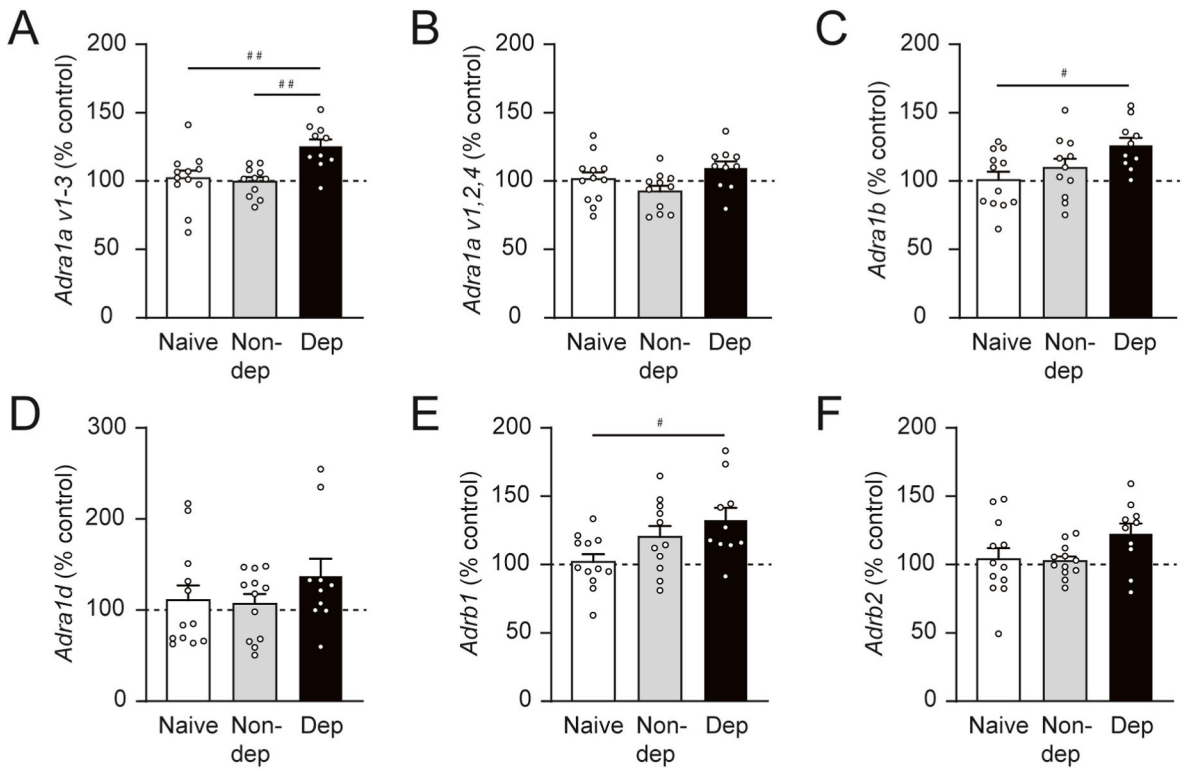

Fig. 5. Ethanol dependence increases the gene expression of adrenergic receptor subtypes in the CeA. **A-F.** CeA mRNA levels for (A) $\alpha1_A$ (*Adra1a v1-3*), (C) $\alpha1_B$ (*Adra1b*) and (E) β1 (*Adrb1*) were higher in dependent mice compared to naive mice, with no significant differences across groups in (B) $\alpha1_A$ (*Adra1a v1,2,4*), (D) $\alpha1_D$ (*Adra1d*) and (F) β2 (*Adrb2*). N = 10–12 mice per group. $^{\#}p < .05$, $^{\#\#}p < .01$ by one-way ANOVA and Tukey's post hoc test.

contrast to other ethanol studies that have shown increased inhibition in the novel arena across all treatment groups ((Kreifeldt et al., 2022; Patel et al., 2021), but see (Warden et al., 2020)). While there can be large variability in this assay due to individual differences in hunger levels after a fixed deprivation period, size of novel arena, lighting conditions, etc. (Deacon, 2011; Dulawa and Hen, 2005), we suspect that repeated handling by the same experimental tester and exposure to the same testing room during the eight prior behavioral training/testing days, as well as our use of standard lab chow instead of a novel highly palatable bait, may have reduced feeding inhibition in our naïve group. Importantly, there were no group differences in home cage responses suggesting that chronic ethanol exposure did not alter general feeding behavior or motivation to feed. Hyponeophagia can persist for up to 5 weeks of ethanol withdrawal (Holleran et al., 2016; Jury et al., 2017; Pang et al., 2013; Patel et al., 2021; Sidhu et al., 2018), and LC-NE signaling is necessary and sufficient for hyponeophagia expression (Lustberg et al., 2020). CeA activation exacerbates hyponeophagia (Kreifeldt et al., 2022), suggesting that dependence may increase the CeA's sensitivity to NE (see also (Varodayan et al., 2022)).

Here we found that 3 days of withdrawal increased CeA transcript levels of the $\alpha1_A$, $\alpha1_B$ and β1 adrenergic receptor subtypes in dependent mice. This matches our recent human *post-mortem* brain analysis where we found an increase in $\alpha1_B$ mRNA levels and a trend for the overexpression of β1 mRNA levels in the amygdala of individuals with AUD (Varodayan et al., 2022). In that same study, ethanol dependence reduced the number of rat CeA cells that contain $\alpha1_A$ and β1 mRNA. We also found that intra-CeA infusion of the β antagonist propranolol decreased ethanol intake in dependent rats, but had no effect in non-dependent rats. Assuming there are no species-specific differences in how ethanol regulates the CeA noradrenergic system, collectively our work suggests that chronic ethanol exposure causes adrenergic receptor mRNA levels to become enriched in a subpopulation of CeA cells that mediate withdrawal-induced ethanol intake. Future studies should characterize this CeA subpopulation, and determine how its regulation by the noradrenergic system contributes to CeA output. This is particularly important as β1 adrenergic receptors are expressed on CRF + neurons in the CeA that project back to the LC, and so their increased activity can trigger persistent NE release across the brain (Kravets et al., 2015; Rudoy et al., 2009). Interestingly, this CeA-LC feed-forward loop is sex-dependently regulated; female LC neurons do not show activity-dependent CRF1 receptor internalization making them particularly vulnerable to chronic ethanol exposure (Retson et al., 2015). As a result, one major limitation of the current study is that only male subjects were used. Future studies should determine whether ethanol dependence produces similar brain region-specific alterations in noradrenergic control of cognitive and affective behavior in male and female mice.

Our dependent mice also displayed increased spontaneous digging, consistent with previous chronic ethanol studies that have assessed digging or the related marble burying test (den Hartog et al., 2020; Jury et al., 2017; Lee et al., 2015; Pleil et al., 2015; Sidhu et al., 2018; Vazey et al., 2018). While it is difficult to determine the exact motivation underlying digging behavior, it is generally considered to be a repetitive and perseverative behavior that may reflect both anxiety-like and compulsive-like tendencies (Pond et al., 2021; Thomas et al., 2009). Interestingly, den Hartog et al. reported that systemic administration of an α1 inverse antagonist (prazosin) alleviated CIE vapor-induced increases in marble burying in male and female mice (den Hartog et al., 2020), highlighting once again the importance of studying the noradrenergic system in the development of negative affect and ethanol dependence in both sexes.

4.3. Conclusions

Historically, synchronous activation of the LC was thought to cause brain-wide NE release; however, recent studies have identified LC

neuronal subpopulations with distinct projection targets and activity patterns that bidirectionally regulate ethanol consumption, anxiogenic and aversive behavioral responses, and cognitive function (Chandler et al., 2019; Cope et al., 2019; Deal et al., 2020; McCall et al., 2015; Uematsu et al., 2017). Likewise, here we found that ethanol dependence bidirectionally alters mouse brain α1 and β receptor mRNA levels, potentially leading to reduced mPFC adrenergic signaling and enhanced noradrenergic influence over the CeA. These brain region-specific gene expression changes are accompanied by cognitive and affective disturbances. Hyperkatefia increases AUD relapse risk by impairing cognitive function and promoting alcohol consumption to alleviate negative emotional symptoms, and ongoing clinical studies are evaluating the potential of several adrenergic compounds as treatments for AUD-associated hyperkatefia (Fox et al., 2012; Haass-Koffler et al., 2018; Koob, 2021; Milivojevic et al., 2020; Simpson et al., 2018; Sinha et al., 2022; Wilcox et al., 2018). As mentioned earlier, one major limitation of the current study is that we only used male subjects, since female C57BL/6J mice do not always escalate their ethanol intake in this CIE-2BC model possibly due to their higher baseline ethanol consumption ((Jury et al., 2017; Macedo et al., 2023; Okhuarobo et al., 2020), but see (Blednov et al., 2019; Ferguson et al., 2022; Huitron-Resendiz et al., 2018; Schweitzer et al., 2016) (Borgonetti et al., 2023). The female LC is larger than the male LC in both humans and rodents, and the rodent female LC is more sensitive to repeated ethanol exposure (Bangasser et al., 2016; Retson et al., 2015). This suggests that the chronic ethanol-induced changes in adrenergic receptor gene expression that we observed in the male mouse brain could potentially be greater in females. For these reasons, it is particularly important that future studies determine the neurobiological and mechanistic effects of chronic ethanol/withdrawal on the noradrenergic system in both males and females to support the refinement of adrenergic-based therapies to alleviate AUD.

Authorship contributions

Conceptualization (AJR, MR, FPV); Formal analysis (FPV); Funding acquisition (AJR, MR, FPV); Investigation (AA, TN, C-CG, FPV); Methodology (AJR, FPV); Resources (AJR, MR, FPV); Supervision (AJR, MR, FPV); Verification (AJR, FPV); Visualization (FPV); Roles/Writing - original draft (FPV); Writing - review & editing (AA, TN, C-CG, AJR, MR, FPV).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2023.100542.

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