Chronic intermittent ethanol produces nociception through endocannabinoid-independent mechanisms in mice

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Abstract

Alcohol use disorder (AUD) affects millions of people and represents a significant health and economic burden. Pain represents a frequently under-studied aspect of hyperkatifeia during alcohol withdrawal, yet to date no drugs have received FDA approval for the treatment of this indication in AUD patients. This study aims to evaluate the potential of targeting bioactive lipid signaling pathways as a therapeutic approach for treating alcohol withdrawal-related pain. We utilized a chronic intermittent ethanol (CIE) vapor exposure model in C57BL/6J mice to establish alcohol dependence, and demonstrated that CIE mice developed robust tactile allodynia and thermal hyperalgesia during withdrawal that was independent of prior blood alcohol levels. Next, we evaluated four drugs for their efficacy in reversing tactile allodynia during abstinence from CIE using a cross-over treatment design that included FDA-approved naltrexone as well as commercially available inhibitors targeting inflammatory lipid signaling enzymes including fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), and 15-Lipoxygenase (LOX). None of these compounds produced significant therapeutic benefit in reversing established CIE-induced tactile allodynia, despite attenuating pain-like behaviors at these doses in other chronic pain models. These findings underscore the need for novel therapeutic approaches to mitigate pain in AUD patients.

Introduction

Alcohol use disorder (AUD) is a persistent public health issue that impacts approximately 400 million people worldwide, with including 28.9 million individuals in the US in 2023[1]. AUD is characterized by a cycle of binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation[1] that perpetuates problematic alcohol use, with the withdrawal/negative affect aspect of this cycle being a powerful driver behind relapse and continued use[2]. Among the patients who were formally diagnosed and sought treatment, 33% experienced additional AUD relapse even after they had been asymptomatic for 12 months or more[3], indicating a necessity for novel therapeutics to treat alcohol use disorder.

Pain is an under-studied aspect of alcohol withdrawal that has been shown to influence relapse and continued use[4,5]. Due in part to the acute antinociceptive effects of alcohol on pain, the misuse of alcohol in an attempt to manage pain as well as disruptions to daily life due to pain are prevalent in problem drinkers[6], with chronic pain serving as a predictor for relapse[7]. AUD patients are more likely to seek care to address health concerns like chronic pain that result from long-term AUD than excessive alcohol use[8,9], and a significant portion of treatment-seeking patients with AUD experience recurring pain[10]. Chronic alcohol consumption can result in peripheral neuropathy[11] and alcohol withdrawal directly leads to an increase in pain hypersensitivity[12]. Preclinical rodent models can recapitulate these symptoms, as chronic intermittent ethanol vapor exposure (CIE) model of ethanol exposure and withdrawal reliably produces increased nociception in both rats and mice, similar to the experience of human AUD patients[13,14]. While there is a clear emerging connection between pain and problematic alcohol use, none of the current FDA approved medications for AUD mitigate pain experienced by patients[15,16,17], and other off-label pain medications often cannot be used long-term[18,19] or carry substantial risk of producing additional dependence[20,21].

Endocannabinoids and related bioactive lipids have emerged as potential targets for treating AUD withdrawal-induced chronic pain by altering inflammatory processes. Patients report

that self-medication with cannabinoid-based products may reduce alcohol intake or even promote abstinence[22,23] as well as minimize use of opioid analgesics[24,25]. However, the use of cannabinoid-based treatments poses additional concerns of dependence for an already vulnerable population[26,27]. In contrast, elevation of endogenous cannabinoids represents an alternative therapeutic approach that could attenuate AUD-induced chronic pain while circumventing safety concerns due to dependence. Inhibition of fatty acid amide hydrolase (FAAH) increases endogenous levels of anandamide (AEA), and has been shown to reduce AUD-induced anxiety-like behaviors and alcohol intake in rat and mouse models of alcohol dependence[28]. In addition, inhibition of monoacylglycerol lipase (MAGL) increases endogenous levels of 2-arachidonoylglycerol (2-AG), and has similar therapeutic benefit on anxiety-like behaviors and alcohol intake in rodent models of alcohol dependence[28]. Both FAAH and MAGL inhibitors also have been shown to reduce inflammatory[29-31] and neuropathic[32,33] pain-like behaviors in rodents, and FAAH inhibitors reverse pain-like behaviors in mice subjected to the two-bottle choice (2BC) model of chronic ethanol exposure[34]. Furthermore, we have demonstrated that in AUD patients, plasma levels of pro-nociceptive 12/15-Lipoxygenase (LOX) metabolites predict alcohol craving during abstinence[35]. However, therapeutics targeting these lipid pathways have not been evaluated for any potential anti-nociceptive effects in a preclinical model of chronic alcohol consumption-induced dependence.

The present study aims to assess the potential for targeting bioactive lipid signaling pathways as a therapeutic approach for treating alcohol withdrawal-related pain. The present study establishes conditions for a reliable mouse model of alcohol dependence with robust and recurring expression of tactile and thermal pain-like behaviors during withdrawal using the chronic intermittent ethanol (CIE) vapor exposure model in mice. Using this approach, FDA-approved naltrexone as well as commercially available inhibitors targeting FAAH, MAGL, or 15-LOX were evaluated as potential treatments for pain hypersensitivity during withdrawal using a cross-over treatment design.

Methods

Animals

C57BL/6J male mice (10 weeks old with an average body weight of 26g at initiation of the experiment) were obtained from Jackson Labs. Mice were housed in individually ventilated cages under sanitary conditions at 3-5 mice per cage, in a 12h reverse dark-light cycle (22:00 on/10:00 off), temperature and humidity-controlled room. Mice have access to food and water *ad libitum*. All protocols and experiments were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) and complied with the ARRIVE guidelines[36].

Drugs

Naltrexone hydrochloride (Sigma-Aldrich N3136, Source BCCF6115) was dissolved in saline (0.9% Sodium Chloride Injection, USP, ICU Medical Inc. Cat. # 798437, NDC 0990-7983-02). ML351 (15-LOX-1 inhibitorMedChemExpress Cat. #HY-111310, Lot # 110818), PF-3845 (FAAH inhibitor, Cayman Chemical Company Cat. # 13279, Batch: 0454664-38, 0454664-50), or MJN110 (MGL inhibitor, MedChemExpress Cat. # HY-117474, lot # 150639) were dissolved in DMSO (Sigma, Cat. # 41640), mixed with Tween-80 (Sigma, Cat. # P9416) at a 1:1 ratio and then diluted in water and followed by PBS for a final ratio of 1:1:9:9 (DMSO : Tween80 : Water : PBS, or 5% DMSO/5%Tween-20).

Chronic Ethanol Exposure (CIE) Model

Mice were assigned to either ethanol vapor group (CIE) or air group (AIR) based on body weight, tactile withdrawal thresholds at baseline, and hot plate response time at baseline to avoid confounding effects. Alcohol exposure was performed following the established chronic ethanol vapor exposure (CIE) paradigm (Fig 1) as previously published[13,14,37]. Briefly, mice were exposed to vaporized ethanol or air for 16 hours overnight using a vacuum-based vapor exposure system (La Jolla Alcohol Research) and returned to housing cages for 8 hours, for a total of four days per

week (one week is considered one vaping cycle) for 13 cycles total. To produce ethanol vapor, an HPLC pump (Waters, 515 HPLC Pump) was used to supply 95% ethanol (Pharmco, ethyl alcohol 95%, 190 proof; Cat. # 111000190) into a vaporizer (Glas-Col, heating mantle, Cat. #: 100B TM106) connected to the vacuum-based system. Mice of the CIE group were injected intraperitoneally (IP, 10 ml/kg) with alcohol (1.75 g/kg, in 0.9% saline solution) and pyrazole (alcohol dehydrogenase inhibitor, Sigma-Aldrich P56607, Source BCCK9523; 68.1 mg/kg, in 0.9% saline solution) before being placed (by cages of 5) into ethanol exposure chambers. Mice of the AIR group were injected with saline (0.9% solution) and pyrazole (68.1 mg/kg) before being placed into air exposure chambers. For all mice, cheek blood was collected immediately after the first and third 16-hour exposure to ethanol vapor or air in each vaping cycle to determine blood alcohol levels (BAL, mg/dL). The flowrate of ethanol was adjusted to maintain a target BAL of 150 – 220 mg/dL during cycles of CIE exposure. Mice were weighed daily prior to the start of vaping sessions during each cycle.

Blood Ethanol Concentration by Gas Chromatography

Levels of ethanol in whole blood samples were assessed using GC-FID with a method adapted from Gonzalez *et. al.*[38] and Doyon *et. al.*[39]. For all CIE and AIR mice, BAL (in mg/dL) was determined in 10 µL of whole blood sample that was added to 90uL of saturated sodium chloride solution (technical, ~26%; Sigma-Aldrich, Cat. # 71392) in a 2 mL glass mass spectrometry sample vial (2mL Clear Glass 12x32mm Flat Base 9-425 Screw Thread Vial; ALWSCI technologies, Cat. # C0000008) immediately following collection. Following collection, the sample was capped and heated at 50°C for 20 minutes, before manual sampling using Solid Phase MicroExtraction (SPME) fiber (d_f 75 µm, carboxen/polydimethylsiloxane; Supelco, Cat. # 57318) for 15 seconds. A HP 6890 GC-FID was used to analyze the ethanol concentrations of the samples, with an Agilent DB-ALC2 column (30m × 0.320mm × 1.20µm) as the stationary phase and Helium as the mobile phase. ChemStation software (v A.10.02) was used to analyze the

resulting ethanol peaks. External standards were assessed for calibration each time (quantitative curve between 25 and 400 mg/dL).

Thermal Escape Latency (hot plate test)

Thermal hyperalgesia was assessed using the hot plate test as previously published[40]. Briefly, mice received room habituation for an hour with cage lids off prior to individual apparatus habituation, where each mouse was placed within an acrylic chamber (IITC, Part #39ME, 9 cm diameter, 30 cm height) on top of the hot plate apparatus (IITC, Part #39) for 10 minutes while it is turned off. Room lighting was maintained between 35 - 40 lux. Thermal escape latency (time in seconds to shaking, lifting, or licking hind paws, and jumping) was recorded using a hand timer for each animal. Baseline measurement was recorded prior to alcohol exposure to determine the paw withdrawal latency (PWL) of each mouse. Recordings were conducted at temperatures of 50˚C, 52.5˚C, and 55˚C with a cutoff time of 20 seconds to prevent tissue damage. Each mouse was tested three times at each temperature on separate days, with each test taking place at 72h after the last alcohol exposure. The average of three measures was taken as the mean thermal PWL of the mouse at a given temperature.

Tactile Allodynia (von Frey test)

Tactile allodynia was assessed with the up-down method utilizing manual von Frey filaments as previously published[40]. Briefly, filaments with buckling forces ranging from 0.02 and 2 g were used to assess the average 50% paw withdrawal threshold (PWT) of each hindpaw at baseline and at timepoints throughout the experiment as indicated. Mice were habituated to the testing room and chambers for 2 h for up to 5 days prior to baseline and then 1 h on test day. Any mouse with a baseline 50% PWT ≤ 0.79 g was excluded from the study. As alcohol exposure was systemic, PWT for both hindpaws were averaged, and data was presented using the 50% gram threshold vs time, or area under the curve (AUC).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (v 10.3.1). Data are presented as mean ± SEM, with discrete data points included where appropriate. A mixed-effects analysis was applied to body weight data, while a non-linear one-phase decay fit was used for the pharmacokinetic (PK) study. Behavioral test assessments, including baseline and experimental conditions, were analyzed using a two-way ANOVA (tactile pre-test thresholds: weeks × alcohol exposure; hot plate: temperature × alcohol exposure), followed by Tukey's post hoc test. Pearson's R correlation was used for correlation studies, and a paired t-test was applied for the tactile crossover study within each treatment condition and group. Statistical significance was defined as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Results

Chronic intermittent ethanol vapor exposure (CIE) pharmacokinetics

In this study, we used a chronic intermittent ethanol (CIE) vapor paradigm of alcohol dependence in C57BL/6J male mice (Fig 1) as previously described[37]. Mice were exposed to 13 cycles of CIE, and compared with control mice exposed to air without alcohol (AIR) throughout the study. The CIE mice displayed average BALs between 150-220 mg/dl at each CIE cycle starting at week 2 (Fig 2A), a paradigm previously shown to establish alcohol dependence in mice[14,37]. Additionally, CIE mice exhibited slower body weight gain than AIR control mice over 13 cycles of CIE (Fig 2B), in line with prior studies using this model in rodents[41]. In order to produce dependence, the CIE model incorporates daily pyrazole administration which can influence BAL and alcohol-induced pain-like responding in mice[13,14,37]. Thus, we conducted a post-vapor exposure BAL time-course to evaluate the impact of pyrazole on ethanol pharmacokinetics in CIE mice. Following a vapor exposure session (Fig 2C,D), CIE mice ($T_{1/2}$ = 1.9 h) metabolize alcohol similar to naïve mice in the absence of pyrazole ($T_{1/2}$ = 1.9 h) and faster than naïve mice immediately after pyrazole treatment ($T_{1/2}$ = 3.4 h). Collectively, these data confirm that CIE mice exhibit near complete

metabolism of alcohol between vaping sessions, and indicate that nociceptive behaviors emergent during withdrawal can be evaluated without the confounding anti-nociceptive influence of alcohol starting at 24 h of abstinence.

CIE induces tactile and thermal pain hypersensitivity during abstinence in male mice

Next, we validated that mice develop robust and persistent tactile allodynia during abstinence following at least 3 cycles of CIE[13,14]. To this end, we measured baseline tactile paw withdrawal thresholds (PWT) prior to initiating the CIE vapor exposure paradigm and subsequently tested mice 24h after the end of each cycle to determine alcohol withdrawal-induced changes to PWT. We found a significant effect of group and time whereby CIE withdrawal precipitated a reduction in tactile thresholds starting at the third cycle, achieving maximal effect from the 4th cycle until the end of the study (Fig 3A). Since alcohol itself exhibits acute analgesic effects, we hypothesized that the immediately prior BAL may correlate with the subsequent pain levels that emerge during withdrawal. For this reason, we performed a Pearson correlation between each mouse's tactile threshold during abstinence with the prior BAL during CIE (Fig 3B). We found no significant correlation between the tactile allodynia during abstinence and their prior BAL ($R^2 = 0.00064$, $p = 0.3968$), suggesting that the tactile allodynia observed during withdrawal is independent of the acute analgesic effects of alcohol.

Previous studies reported mixed findings regarding the effect of alcohol exposure on thermal hyperalgesia in mice[14,42]. These discrepancies may result from the thermal stimulus intensity required to unmask a hyperalgesic response during alcohol withdrawal, and thus we performed an assessment of thermal paw withdrawal latencies (PWL) using the hot plate at three different calibrated temperatures (50°C, 52.5°C, 55°C). As expected, both naïve and CIE mice display a temperature-response relationship, with higher temperatures leading to shorter PWL (Fig 4A). Importantly, at 55°C we identified that the expression of thermal hyperalgesia in CIE mice at 72h of withdrawal that persists for at least 7 days (Fig 4B). In contrast, no difference

between the CIE and the AIR groups were detected at either 50°C or 52.5°C at both time points (Fig 4C) in agreement with previous reports[14]. To confirm that prior single day BALs do not strongly influence the subsequent expression of thermal hyperalgesia that emerges during withdrawal, we performed a Pearson correlation between each mouse's thermal response latency (at 55°C) during abstinence with the prior day BAL during CIE and found no significant effect (R^2 = 0.06774, p = 0.1310) (Fig. 4D). Collectively, these data demonstrate the development of post-CIE tactile and thermal pain hypersensitivity in male mice using this paradigm.

Targeting pro-inflammatory signaling did not reverse CIE tactile allodynia

While currently there are 3 FDA-approved drugs for treating AUD, none of these has been shown to attenuate hyperkatifeia by reversing withdrawal-induced pain hypersensitivity. Using our established chronic vapor AUD model, we conducted a series of 4 cross-over therapeutic studies (Fig 5) where both AIR and CIE mice received either vehicle or drug treatment on alternating testing cycles at 48h from the last alcohol vapor exposure. First, we evaluated the efficacy of naltrexone in reducing hyperkatifeia because it is an FDA-approved treatment for AUD, reduces ethanol intake in both preclinical AUD models and clinical studies of AUD patients[43-48], and has been suggested as a potential therapeutic approach for treating inflammation and chronic pain[49]. Following systemic treatment with naltrexone at a dose that effectively reduces alcohol consumption (3 mg/kg, IP, 2h prior to testing)[43,44], tactile response thresholds in AIR (p=0.61, Fig 5A) or CIE mice (p=0.28, Fig 5B) were not significantly different, indicating no effect of opioid receptor antagonism on hyperkatifeia in this model.

Second, we chose to examine the effects of a selective inhibitor of 15-LOX (ML351) on hyperkatifeia. 15-LOX synthesizes pro-inflammatory lipid metabolites including 15-HETE which we found to be associated with alcohol craving in humans[35]. Additionally, we have shown that 15-LOX inhibition reverses tactile allodynia in a preclinical model of neuropathic pain-like behavior in rats and mice[50,51]. Following systemic treatment with an anti-hyperalgesic dose of ML351 (30

mg/kg, IP, 1h prior to testing), tactile response thresholds in AIR ($p=0.91$, Fig 5A) or CIE mice ($p=0.98$, Fig 5B) were not significantly different, indicating no effect of 15-LOX inhibition on hyperkatifeia in this model. These results show that two treatments with therapeutic benefit in other chronic pain models do not influence the AUD pain state that develops following CIE.

Targeting anti-inflammatory lipid signaling did not reverse CIE tactile allodynia

Given the established preclinical success in targeting endocannabinoid signaling for treating other aspects of AUD as well as other models of chronic pain, we interrogated these pathways as a potential treatment option for the CIE-induced pain state in mice. Pro-resolving endocannabinoids such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) may affect AUD-induced chronic pain as these bioactive lipids reduce alcohol intake in rodents[52,53] and elicit antinociceptive effects in other preclinical models of chronic pain[31,34,54,55]. First, we evaluated the effects of elevating endogenous AEA levels using the selective fatty-acid amide hydrolase (FAAH) inhibitor PF3845 because it reduces ethanol intake in rodent AUD dependence models[28], decreases withdrawal signs including anxiety-like and depression-like behaviors[28], reverses pain-like behaviors in models of inflammatory pain[54,55], and an optimized clinical candidate derived from this compound successfully advanced through Phase 1 Clinical Trials[56]. Following systemic treatment with PF3845 at a dose that effectively reduces alcohol consumption (10 mg/kg, IP, 2h prior to testing), tactile response thresholds were unchanged in the AIR group ($p=0.82$, Fig 5A) and pain-like behaviors were instead exacerbated in the CIE mice ($p < 0.05$, Fig 5B) , indicating no effect of FAAH inhibition on hyperkatifeia in this model.

Finally, we examined the effects of elevating endogenous 2-AG levels using the selective monoacylglycerol lipase inhibitor (MAGL) MJN110 as it also reduces ethanol intake in rodent AUD dependence models[28,57], decreases withdrawal signs including anxiety-like and depression-like behaviors[28,57], reverses pain-like behaviors during alcohol withdrawal[34,41] and during chemotherapy-induced peripheral neuropathy[33], and a humanized MAGL inhibitor has advanced

successfully through Phase 1 Clinical Trials[58,59]. Following systemic treatment with MJN110 at a dose that effectively reduces alcohol consumption (10 mg/kg, IP, 2h prior to testing), tactile response thresholds in CIE mice ($p=0.17$, Fig 5B) were not altered significantly. While MJN110 treatment did produce a significant reduction on tactile thresholds in AIR controls ($p < 0.01$, Fig 5A), the effect is driven largely by an acute increase in the vehicle group when compared to previous vehicle treatments (likely an artifact). Collectively, these findings suggest that elevating endogenous anti-inflammatory endocannabinoids with FAAH or MAGL inhibitors does not reverse the CIE-induced chronic pain state in mice.

Discussion

Pain represents an important component of alcohol dependence and withdrawal that motivates relapse and continued use[4,5], yet to date no therapeutic options have been identified for addressing this aspect of hyperkatifeia. In this study, we implemented the CIE vapor model of alcohol dependence in C57BL/6J mice, demonstrating that it elicited both tactile allodynia and thermal hyperalgesia during acute abstinence. These withdrawal symptoms emerged at 24 hours, lasted for at least 7 days, and persisted throughout 11 CIE cycles. Using this model of AUD-induced hyperkatifeia, we performed a multi-drug crossover treatment study using several compounds targeting either pro-inflammatory or pro-resolving signaling pathways. Despite showing therapeutic effects on other models of chronic pain and other output measures of AUD, these drug treatments did not produce a significant reversal of AUD-induced tactile allodynia. Taken together, these findings suggest that CIE may induce a chronic pain state distinguishable from injury-induced pain models.

While multiple preclinical AUD studies have all identified tactile pain hypersensitivity in rodents during abstinence, similar studies measuring thermal responses in mice have been equivocal. Our study found that CIE led to a small but significant decrease in thermal response latency during abstinence when mice were tested at a higher hotplate temperature (55°C) typically utilized for examination of analgesic effects of drugs, thereby aligning with published findings that evaluated thermal thresholds in alcohol-exposed mice by Hargreaves test using similar testing conditions with the glass maintained at 32°C[60]. Alternatively, results from our lab and others[14] using lower hotplate temperatures (50°C to 52.5°C) show no differences in thermal response latency in male mice. By contrast, abstinence results in much more robust tactile allodynia across multiple studies despite difference species, sex, and model of alcohol exposure[13,14,34,41,60-68]. Taken together, these findings suggest a possible role for activation of thinly myelinated A-delta afferent fibers from the dorsal root ganglia in AUD-induced hyperkatifeia as they are sensitive to tactile stimuli, exhibit high temperature thresholds, and are recruited in alcohol-induced

neuropathy[69]. Sensory information from nociceptors in the dorsal root ganglia is transmitted through the spinal cord dorsal horn and communicated to the brain along several ascending pathways, where it then projects to multiple supraspinal brain areas including the amygdala, prefrontal and anterior cingulate cortices, ventral tegmental area, and nucleus accumbens responsible for the composite interpretation of sensory/discriminative, affective, cognitive and motivational aspects of nociceptive information known as pain. The experience of pain also is regulated by descending excitatory and inhibitory pathways to the spinal cord from brain regions such as the anterior cingulate cortex and the periaqueductal gray[79]. In rats, alcohol dependence alters supraspinal circuits responsible for interpretation and response to high intensity thermal stimuli, including exacerbation of pronociceptive central amygdalar projections to the ventrolateral periaqueductal gray[62] as well as signaling within the lateral habenula[41].

Despite strong evidence in support of elevating endogenous 2-AG as a therapeutic strategy for reversing the CIE-induced pain state, acute treatment with a systemic therapeutic dose of the selective MAGL inhibitor MJN110 did not attenuate tactile allodynia. This observation was surprising as JZL184 (another selective MAGL inhibitor) mitigates other affective behaviors during alcohol withdrawal[28], and reduced tactile allodynia during abstinence in male mice using the two-bottle choice model of alcohol exposure[34]. In addition, Marchigian Sardinian alcohol-preferring rats of both sexes subjected to alcohol two-bottle choice exhibit tactile allodynia during protracted withdrawal that correlates with decreased 2-AG levels within the lumbar dorsal root ganglion[65], suggesting a potential for MAGL inhibition to address tactile allodynia that emerges during abstinence. Given that multiple studies indicate that our treatment dose of MJN110 (10 mg/kg, IP) results in near complete inactivation of MAGL[70] and reduces pain-like behavior in chemotherapy induced neuropathy[33], the absence of an antihyperalgesic effect is not due to insufficient dosing. It has been suggested that elevated 2-AG signaling may counteract withdrawal-associated tactile allodynia in moderate alcohol exposure models like two-bottle choice, but that alcohol exposure models achieving higher BALs like CIE may override this

buffering system[34]. Additionally, other genetic factors may influence the impact of 2-AG on tactile hypersensitivity in AUD, as 2-AG levels in the dorsal root ganglion do not predict tactile allodynia in Wistar rats undergoing alcohol two-bottle choice exposure[65]. It is also possible alcohol leads to circuit-specific changes in different preclinical models of AUD that may not respond to systemic interventions, as bilateral infusions of JZL184 into the lateral habenula (LHb) attenuated CIE-induced pain-like responding Long Evans rats[41]. For these reasons, the role of 2-AG in alcohol-induced hyperkatifeia across multiple domains and alcohol exposures models should be further explored to fully capture the specific influence of pain.

It also was somewhat unexpected that treatment with a selective FAAH inhibitor did not reverse tactile allodynia during CIE-induced abstinence. Bilateral infusions of the FAAH inhibitor URB597 into the lateral habenula (LHb) attenuated CIE-induced pain-like responding Long Evans rats[41], and systemic administration of the selective inhibitor PF-3845 reduces alcohol intake and anxiety-like behavior in mice and rats[28]. In our study, we observed enhanced tactile allodynia in male PF-3845-treated CIE versus vehicle-treated CIE mice, suggesting that anandamide and other ethanolamides may signal through non-cannabinergic pathways during CIE. For example, anandamide can also signal through the pro-nociceptive TRPV1 receptor under certain physiological conditions[71]. Accordingly, alcohol sensitizes TRPV1 in the lateral habenula during alcohol withdrawal to facilitate alcohol-seeking behaviors using the intermittent access two-bottle choice in Long Evans rats[72]. Alternatively, some chronic pain states may be unresponsive to FAAH inhibition. FAAH inhibition did not reverse functional deficits in the monosodium iodoacetate model of osteoarthritis[73], and a Phase 2 clinical trial using a well-controlled crossover design showed no beneficial effect of FAAH inhibitor PF-04457845 on pain measures despite producing elevated levels of anandamide in these patients. Many of the preclinical pain models where FAAH inhibitors effectively reduce or reverse pain-like behaviors such as carrageenan-induced inflammation[54] and chronic constriction injury[74] develop through TLR4-dependent mechanisms[75-77], and recent work suggests that many symptoms of AUD may develop through TLR4-

independent mechanisms[78]. These findings would suggest that alcohol-induced hyperalgesia may represent a distinct mechanism of chronic pain that requires unique therapeutic approaches for treatment.

Taken together, our findings show that targeting endocannabinoid signaling in a prolonged CIE model does not impact the severe withdrawal-induced tactile allodynia. However, our results do not preclude the involvement of endocannabinoids in chronic pain experienced by moderate drinkers, nor do they suggest that these pathways do not contribute to other symptoms of AUD. Furthermore, despite showing efficacy in reducing drinking in both preclinical models and clinical studies[43-48], the FDA-approved drug naltrexone did not reverse tactile allodynia following CIE. Finally, treatment with a 15-LOX inhibitor also did not attenuate CIE-induced tactile allodynia in mice despite our observation that one of its pro-nociceptive metabolites predicts craving during abstinence in patients with severe AUD[35]. Given the lack of available and effective treatments for AUD-induced pain, future studies should explore new therapeutic approaches using the CIE model.

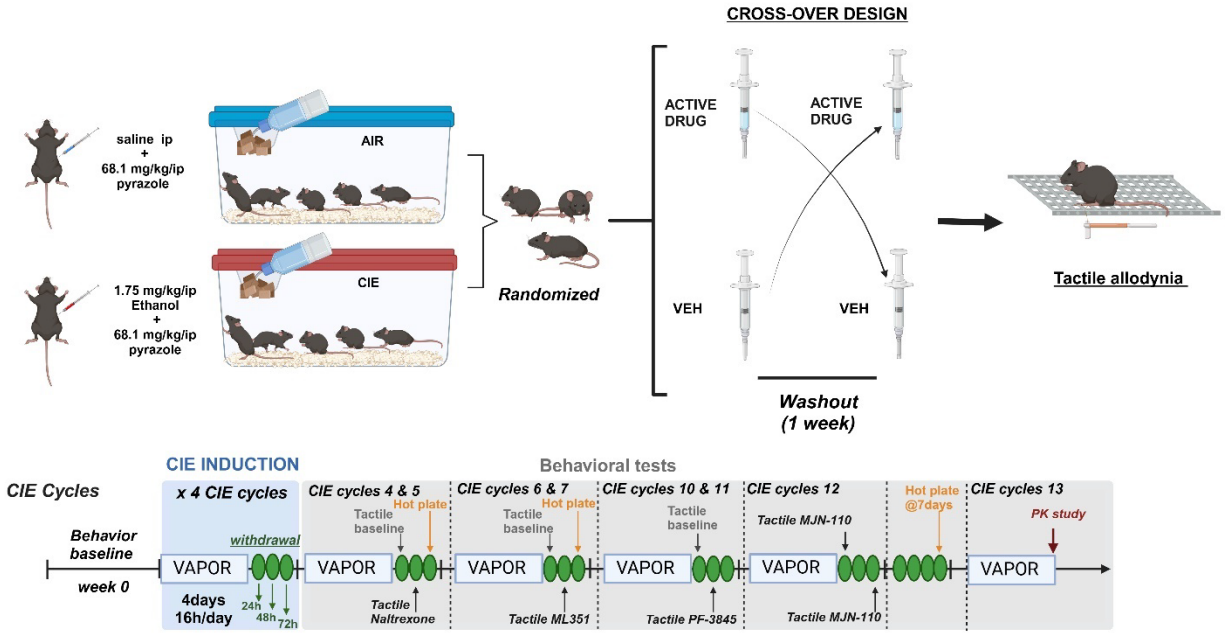

Figure 1. CIE Timeline and experimental design. Alcohol exposure was performed following the established chronic ethanol vapor exposure (CIE) model where mice were exposed to either room air (AIR) or vaporized ethanol (CIE) for 13 cycles. Each cycle consisted of 4 consecutive days of exposure (16 hours overnight) with preexposure administration of pyrazole (68.1 mg/kg) and alcohol (1.75 g/kg, IP), followed by 3 days of abstinence. Therapeutic treatments were performed using a crossover design where all mice received either vehicle of drug treatment on separate testing days. This figure was created using BioRender.com.

Figure 2.

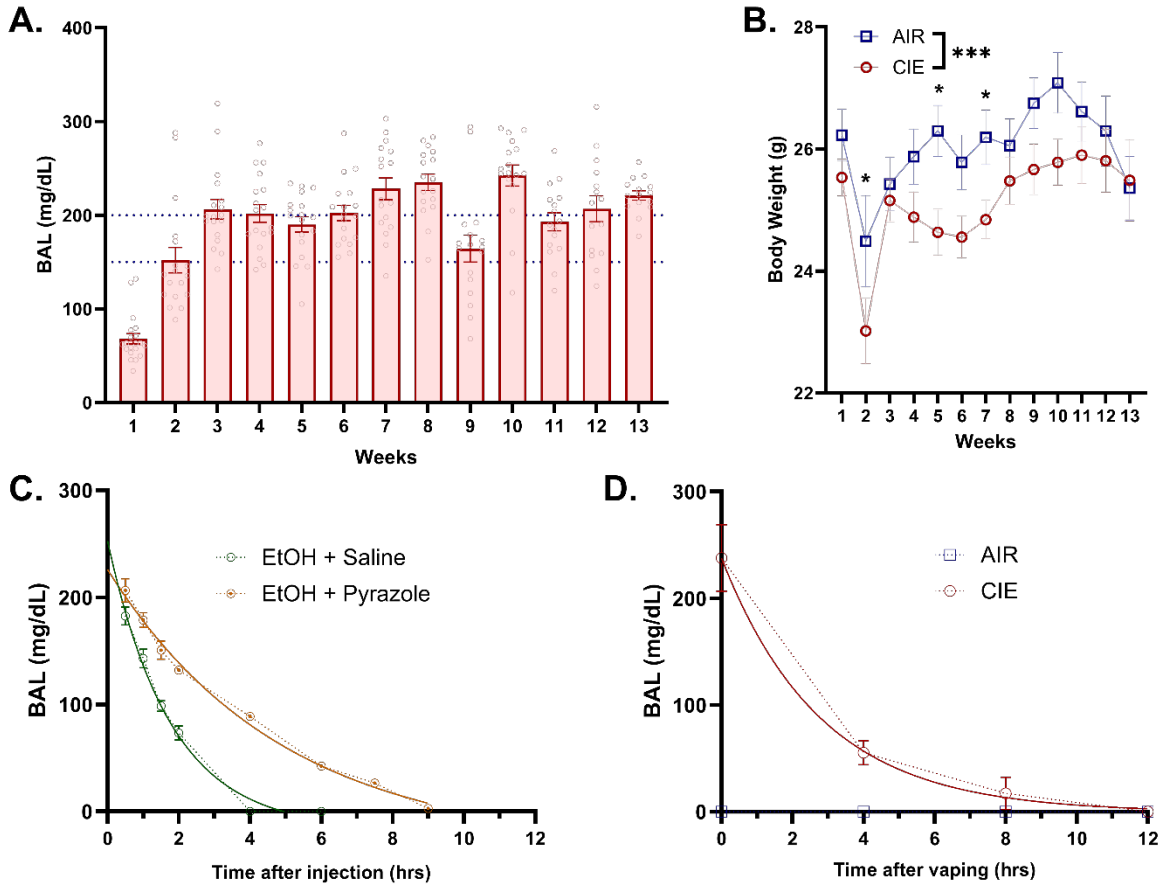

Figure 2. Establishment of the CIE paradigm. (A) BAL time course of mice exposed to vaporized ethanol for 16 hours overnight following an IP injection of ethanol and pyrazole (n = 15 - 20 mice/group). **(B)** Body weight of CIE and AIR mice through the course of the study (n = 15 - 20 mice/group). **(C)** Time Course following IP EtOH injection paired with saline or IP EtOH paired with pyrazole for determination of half-life (n = 6 mice/group). **(D)** Time Course following overnight 16 hour ethanol vaping session paired IP injection of EtOH and pyrazole (n= 4 - 5 mice/group). CIE, chronic ethanol vapor exposure; BAL, blood alcohol level; IP, intraperitoneal; EtOH, ethanol. Data presented as mean ± SEM. Significance indicated by *P < 0.05, **P < 0.01.

Figure 3.

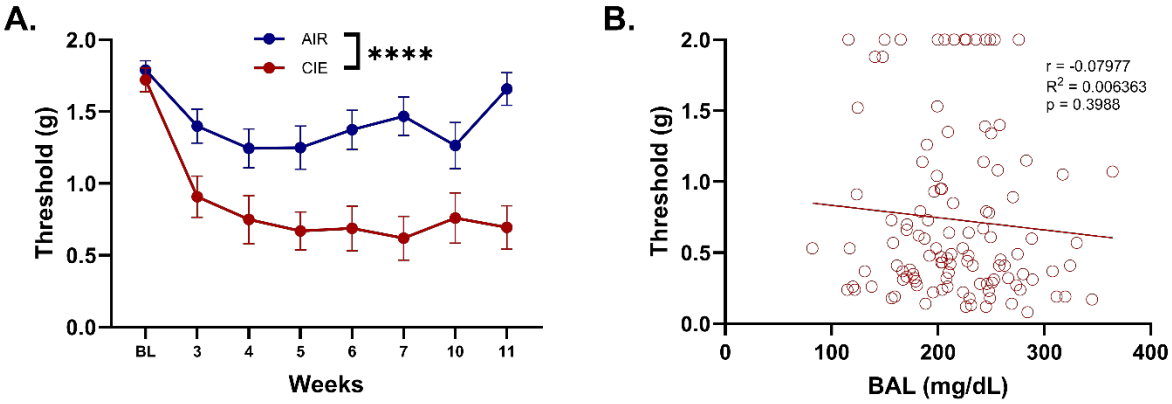

Figure 3. Measurement of tactile allodynia during abstinence using the CIE paradigm. (A) Tactile allodynia time course. **(B)** Correlation graph comparing individual mice BAL to its corresponding tactile allodynia results. Data presented as mean ± SEM. Significance indicated by ****P<0.0001. n = 15-20 mice/group.

Figure 4.

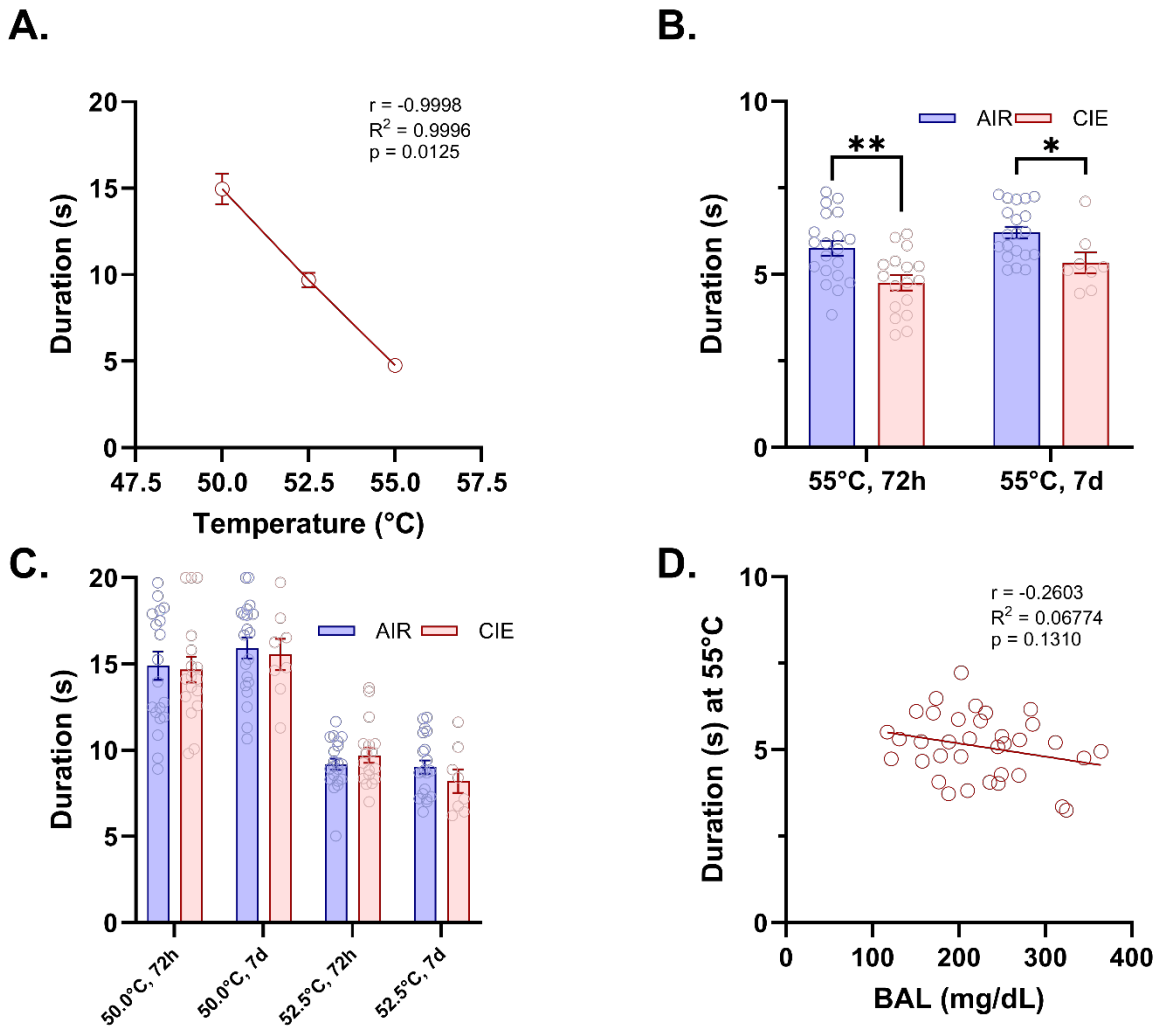

Figure 4. Measurement of thermal allodynia during abstinence using the CIE paradigm. (A) Comparison of varying hot plate temperatures in Air VS CIE (n= 17-20 mice/group). **(B)** Correlation graph comparing individual mice BAL to its corresponding thermal allodynia results (n = 17-18). **(C)** Correlation graph comparing latency to hot plate temperature setting (n= 17-18). **(D)** Post 7-day withdrawal from CIE paradigm displays persisting thermal allodynia (10-18 mice/group). Significance indicated by *P < 0.05, **P < 0.01.

Figure 5.

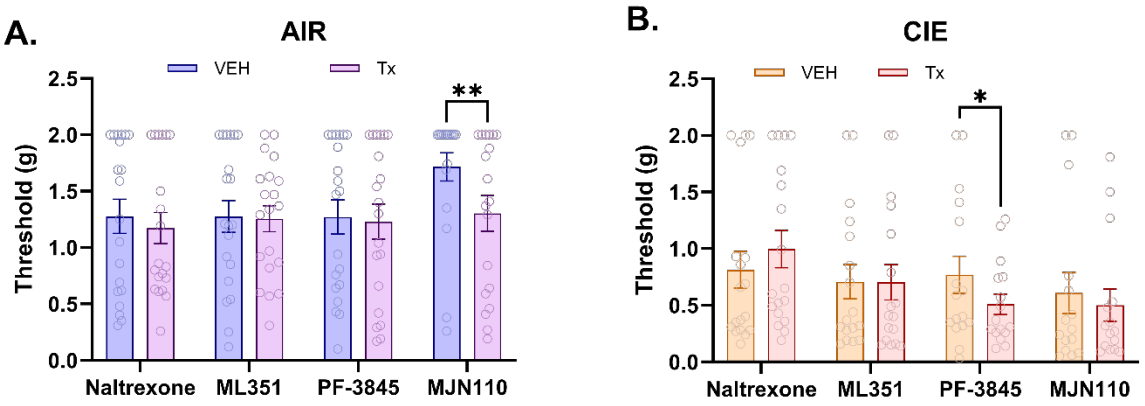

Figure 5. Drug treatments do not reverse CIE produced tactile allodynia. (A) Threshold comparison of vehicle air to treated air with each drug in a double-blinded crossover study (n = 19 - 20). **(B)** Threshold comparison of vehicle CIE to treated CIE with each drug in a double-blinded crossover study (n = 15 -18). Naltrexone: 3 mg/kg. ML351: 30 mg/kg, PF-3845: 10 mg/kg, MJN110: 10 mg/kg. Data presented as mean ± SEM. Significance indicated by *P < 0.05, **P < 0.01.

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