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Sex differences in ethanol reward seeking under conflict in mice

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Abstract

Background: Alcohol use disorders are characterized by inflexible alcohol seeking that occurs despite adverse consequences. Males and females are differentially sensitive to ethanol reward, but it is unclear whether sex differences in ethanol seeking under reward-aversion conflict are present.

Methods: To investigate sex differences in ethanol seeking under conflict, adult male and female C57BL/6J mice underwent chronic intermittent ethanol (CIE) exposure by vapor inhalation or served as air-exposed controls. After CIE, mice were trained in a modified ethanol conditioned place preference paradigm. During three conditioning sessions, 2g/kg ethanol was administered prior to confinement in the "ethanol-paired" chamber. On alternating days, saline was injected prior to confinement in the "saline-paired" chamber. After conditioning, mice experienced a footshock in the ethanol-paired chamber. Ethanol-seeking behavior was assessed before and after footshock.

Results: Control and CIE-exposed males reduced time in and increased latency to enter the reward-paired chamber following footshock. Control females did not alter ethanol seeking behavior following footshock. CIE-exposed females spent more time in the ethanol-paired chamber at baseline. However, following a footshock, CIE-exposed females significantly reduced time spent in and increased latency to enter the ethanol-paired chamber.

Conclusions: Non-dependent female mice exhibited aversion-resistant alcohol seeking to a greater degree than males. Chronic ethanol exposure did not impact ethanol seeking in males. In females, CIE enhanced ethanol seeking in the absence of conflict, but reduced ethanol seeking after an aversive experience. While these sex-specific effects of CIE are not present when reward seeking is assessed in the absence of an aversive experience, multiple factors may underlie differences in reward seeking despite adverse consequences, including reward- and aversion-related learning and decision-making under conflict. These data highlight the importance of considering sex as a variable influencing ethanol seeking and provide a greater understanding of how sex interacts with ethanol exposure to alter behavior.

Keywords

Sex differences; alcohol; chronic intermittent ethanol; conflict; compulsivity

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Introduction

While men are consistently diagnosed with alcohol use disorders at higher rates than women, this gap has declined in recent years. The rates at which women are diagnosed with alcohol use disorders and report problematic drinking behavior have continued to grow in recent decades (Grant et al., 2017; Keyes, Grant, & Hasin, 2008; White et al., 2015). Despite this, there is a paucity of research characterizing sex differences in inflexible alcohol seeking and the effects of chronic alcohol seeking in women and female animals. While being male is considered a risk factor for the development of alcohol use disorders (Kalaydjian et al., 2009), many studies have indicated that women who participate in high-risk drinking behavior may be at higher risk for the development of alcohol-associated health problems (Erol & Karpyak, 2015). In human populations, it remains difficult to disentangle the contributions of social and biological factors to sex differences in use patterns, but a growing body of literature from animal models indicates that there are sex differences in both use patterns and the outcome of alcohol exposure (Becker & Koob, 2016; Becker, McClellan, & Reed, 2017).

Alcohol use disorders are characterized by the development of inflexible alcohol seeking, including compulsive-like behaviors that persist despite adverse consequences (Everitt & Robbins, 2015; Koob, 2013). Compulsive-like behaviors can develop for a variety of reasons, including a failure to detect the negative consequences of an action. Additionally, compulsive-like behavior may result from either overvaluing a reward or undervaluing potential negative outcomes. Substantial preclinical rodent data indicates that female animals often drink more ethanol than male counterparts in either operant or homecage drinking settings (Anderson & Spear, 2011; Bertholomey, Nagarajan, & Torregrossa, 2016; Cailhol & Mormède, 2001; Morales, McGinnis, & McCool, 2015; Priddy et al., 2017). In addition to elevations in drinking, females may be likely to show an increase in compulsive-like reward seeking as they show heightened sensitivity to the rewarding properties of drugs of abuse (Hilderbrand & Lasek, 2018; Lynch, Roth, & Carroll, 2002).

In contrast to increased self-administration and reward sensitivity in females, we have observed more rapid development of inflexible, habitual ethanol seeking in males (Barker, Torregrossa, Arnold, & Taylor, 2010). In addition to these baseline sex differences, a history of binge-like alcohol exposure facilitates the development of habits in adult male rats, while adult female rats were protected from the effects of alcohol on habit development (Barker, Bryant, Osborne, & Chandler, 2017) though other ethanol exposure paradigms facilitate habit formation in both males and females (Renteria, Baltz, & Gremel, 2018). In addition, female mice are protected from chronic ethanol-induced escalations of drinking (Jury, DiBerto, Kash, & Holmes, 2017), though this may result from baseline elevations in ethanol consumption in females. Together, the existing data on sex differences in ethanol consumption and the consequences of ethanol dependence suggest that males and females are differentially sensitive to both ethanol reward and ethanol-induced alterations in behavioral flexibility.

To our knowledge, minimal research has investigated sex differences in ethanol seeking that occurs despite adverse consequences. Research investigating compulsive-like ethanol

seeking includes a number of models, involving punished operant self-administration and consumption of quinine-adulterated ethanol (Darevsky et al., 2018; Hopf & Lesscher, 2014; Radke et al., 2017). Recent research has identified similar sensitivities to quinine-adulteration in males and females, suggesting that sex differences are not present in this form of inflexible ethanol taking (Sneddon, White, & Radke, 2018). Importantly, in both punished self-administration and quinine adulteration models, the experience of reward and aversion are occurring as the animal makes the decision whether to continue consuming or seeking ethanol. To investigate sex differences in compulsive-like reward seeking in the absence of immediate feedback, we used a modified conditioned place preference paradigm that introduces a conflict between reward and aversion (Barker, Torregrossa, & Taylor, 2013; Barker et al., 2014). In this model, mice first learn that a context is associated with ethanol reward. After conditioning, mice are returned to the reward-paired chamber and receive a footshock. Our previous findings from this model in male mice indicate that males that show increased propensity toward habitual behavior also show increased reward seeking following the experience of footshock (Barker et al., 2014), and further that the neuroanatomical substrates regulating continued ethanol seeking under conflict overlap with those mediating other forms of inflexible behavior (Barker, Torregrossa and Taylor, 2013). Given findings that chronic intermittent ethanol (CIE) exposure reduces sensitivity to punishment in an ethanol self-administration model and promotes habit formation (Radke et al., 2017; Renteria, Baltz, & Gremel, 2018), we further investigated how CIE exposure by vapor inhalation impacted ethanol reward seeking that persists despite an adverse experience.

Materials and Methods

Subjects

Adult male and female C57BL/6J mice (9 weeks) were obtained from Jackson Laboratories and housed at the Drexel University College of Medicine under standard light conditions. A total of 89 mice were included in these studies. A subset of males were removed from the study due to fighting and data has been excluded from all analyses. All behavioral training and testing took place during the light cycle. Mice were group housed in same sex cages. Male and female mice were housed in the same colony rooms on ventilation racks with filter tops. Mice had *ad libitum* access to food and water for the duration of the experiments. All procedures were approved by the Institutional Animal Use and Care Committee at Drexel University and are consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chronic intermittent ethanol exposure

Male and female mice underwent four cycles of chronic intermittent ethanol (CIE) exposure by vapor inhalation. Each cycle consisted of 16 hours/day for 4 consecutive days. Each 4-day cycle was separated by 72 hours of abstinence (See Fig 1a). For mice assigned to the ethanol vapor group (CIE), mice received a loading dose of 1.6g/kg ethanol and 1mmol pyrazole in saline immediately before placement in ethanol vapor chambers. These procedures yield neurobiological and behavioral changes following CIE (Griffin, Ramachandra, Knackstedt, & Becker, 2015; Griffin III, Haun, Hazelbaker, Ramachandra, & Becker, 2013; Lopez, Becker, & Chandler, 2014). Mice assigned to the control group (Air

group) received an injection of 1mmol pyrazole in saline prior to placement in a chamber with pumped room air. Bloods were collected from CIE-exposed mice via submandibular bleed immediately after removal from chambers 1x/week. Seventy-two hours after the final CIE exposure, mice began training in the modified ethanol conditioned place preference (CPP) paradigm described below (See Fig 1b).

Ethanol seeking under conflict

Mice were conditioned in standard three chamber CPP boxes with retractable doors (Med Associates). Chambers had distinct walls (black, white, or gray) and floors (grid, wire mesh, or solid). The neutral gray chamber separated the black and white conditioning chambers. Photocell beam breaks were used to calculate time spent in each chamber, latency to enter, and locomotor behavior in the boxes by Med-PC V software. During a habituation session, mice were placed in the neutral chamber with doors to the black and white chambers retracted such that mice could freely explore all chambers for 20 minutes. During conditioning, mice received an injection of 2g/kg ethanol in saline immediately prior to placement in the reward-paired chamber, with the door closed. This dose was chosen as it is not expected to yield sex differences in CPP (Cunningham & Shields, 2018). Three conditioning sessions were 20 min in duration. These sessions were alternated with saline-paired sessions in which mice received saline injections immediately prior to placement in the opposite chamber. After six sessions (three ethanol-paired, three saline-paired), mice were placed in the neutral chamber with both doors retracted and allowed to freely explore all chambers for five minutes. Time spent in and latency to enter each chamber was assessed, and these data served as a 'pre-shock' baseline to examine differences in time spent in the reward-paired chamber. On the following day, mice were again confined to the reward-paired chamber. One minute after placement, mice received a single 0.8mA shock, two seconds in duration. Mice remained in the chamber for two minutes after the shock prior to being returned to their home cage. To test the expression of ethanol seeking under conflict, on the following day, mice were returned to the gray chamber with doors retracted and time spent in and latency to enter the neutral and conflict chambers were assessed. Change in time spent in the reward-paired chamber was calculated as a percentage of time during the Day 8 pre-shock test [(time during post-test/time during pre-test) x 100]. Change in latency to enter the reward-paired chamber was calculated by subtracting the pre-shock latency from the post-shock latency (latency post – latency pre).

To confirm that sex differences in ethanol-seeking behavior following a footshock were not mediated primarily by differences in extinction-related behavior, a separate cohort of mice was trained in the task as described above. Ethanol exposure, training, and testing procedures were identical except that mice did not experience a footshock. On day 9, mice were confined to the reward-paired chamber for 3 minutes as in the "shock" conditions, but no footshock was delivered.

Assessment of sensitivity to footshock

To identify differences in sensitivity to footshock, we assessed threshold to retract a single paw ("flinch") and response to 0.8 mA stimulation in shock-naïve male and female mice who had undergone the CIE procedure and behavioral training as described above. Mice

were placed in a novel environment with electrified grid floors. Shocks were presented in increasing fashion starting at 0.1 mA and then increasing at 0.1 mA steps. The intensity at which a single paw was retracted was recorded. Response to 0.8 mA shock was categorized as 1. Single paw retraction, 2. Two paw retraction, or 3. Jump in which 3 or more paws were moved.

Statistical Methods

Data were analyzed using rmANOVA. Significant findings were followed with Sidak's corrected post hoc comparisons as appropriate. Data were analyzed in Graphpad or SPSS for three-way rmANOVA.

Results

Blood ethanol concentrations did not differ between males and females

Males and females were exposed to ethanol vapor under identical conditions. For mice that would undergo footshock conditions, a rmANOVA of blood ethanol concentration indicated no main effect of sex [$F(1,66) = 3.029$, $p = 0.096$] or sex by CIE cycle interaction [$F(3,66) = 0.2355$, $p = 0.871$; Fig 2a]. A main effect of cycle was observed [$F(3,66) = 3.408$, $p = 0.022$]. Post hoc comparisons indicate that blood ethanol concentrations were lower during cycle three than during cycle one ($p = 0.029$), but that no other cycles differed (p's > 0.1).

Mice that did not experience footshock underwent identical CIE procedures. A rmANOVA of blood ethanol concentration indicated no main effect of sex [$F(1,72) = 0.04$, $p = 0.8372$; Fig. 2b] or sex by CIE cycle interaction [$F(3,72) = 1.12$, $p = 0.3473$]. A main effect of cycle was observed [$F(3,72) = 4.822$, $p < 0.01$]. Post hoc comparisons indicate that blood ethanol concentrations were lower during cycle 4 than during cycles one or two (p's < 0.05), but that no other cycles differed (p's > 0.3).

Chronic ethanol, but not sex, impacted activity during conditioning

A rmANOVA of activity counts during ethanol conditioning sessions in which mice received 2g/kg of ethanol indicated that day of conditioning [$F(2,86) = 18.954$, $p < 0.001$] impacted behavior (Fig 3a). Post hoc comparisons indicate that activity was suppressed to a greater degree across conditioning sessions (sessions two and three < session one, $p < 0.05$). CIE exposure impacted response to ethanol such that CIE-exposed mice were more active than air controls during ethanol conditioning sessions [$F(1,43) = 12.837$, $p < 0.01$]. No main effect of sex [$F(1,43) = 1.909$, $p = 0.174$] nor interactions were observed (all p's > 0.1).

A rmANOVA of activity during saline pairings indicated that a history of CIE impacted activity during conditioning sessions in the absence of ethanol exposure [$F(1,43) = 10.909$, $p < 0.01$; Fig 3b] such that Air controls exhibited greater activity than CIE-exposed mice. Day of conditioning also impacted activity [$F(2,86) = 5.917$, $p < 0.01$]. Post hoc comparisons indicate that activity counts were higher on day 1 than day 2 ($p < 0.01$). No main effect of sex [$F(1,43) = 0.519$, $p = 0.475$] nor interactions were observed (all p's > 0.1).

Chronic ethanol exposure did not impact ethanol seeking under conflict in male mice

In order to assess the effect of an aversive experience on ethanol seeking, time spent in the ethanol-paired chamber before and after footshock were compared using rmANOVA. Our data indicate that male mice significantly reduced time spent in a reward-paired chamber after a footshock [$F(1,21) = 22.69$, $p < 0.001$; n = 12 control males, 11 CIE males; Fig 4a]. Chronic ethanol exposure did not impact time spent in the reward-paired chamber independently [$F(1,21) < 0.001$, $p = 0.998$], nor did it interact with shock [$F(1,21) = 0.005$, $p = 0.9419$]. Control and CIE-exposed males exhibited similar changes in time spent in the reward-paired chamber following footshock [$t(21) = 0.1218$, $p = 0.9042$; Fig 4b]. Latency to enter the reward paired chamber was significantly higher after shock [$F(1,21) = 9.886$, $p = 0.005$; Fig 4c], but was not impacted by CIE [$F(1,21) = 2.893$, $p = 0.104$] or CIE by shock interaction [$F(1,21) = 2.666$, $p = 0.117$], indicating that both CIE-exposed and control males increased the latency to enter the ethanol-paired chamber after footshock. Change in latency to enter the reward-paired chamber did not differ significantly between CIE-exposed and Air control males [$t(21) = 1.633$, $p = 0.0587$; Fig 4d].

Chronic ethanol reduces ethanol seeking under conflict in female mice

In female mice, time spent in the ethanol-paired chamber was determined by an interaction between shock and chronic ethanol exposure [$F(1,22) = 15.02$, $p < 0.001$; Fig 5a; n = 12 females/group]. Post-hoc comparisons indicate that in Air controls, time spent in the reward-paired chamber was not modulated by footshock ($p > 0.9$), indicative of compulsive-like ethanol seeking that persisted despite adverse consequences. In contrast, time spent in the reward-paired chamber was significantly reduced following footshock in CIE-exposed females ($p < 0.001$), consistent with sensitivity to an adverse consequence. This interaction may be in part driven by an increase in CPP in CIE-exposed females, as CIE-exposed females spent more time in the reward-paired chamber than Air controls prior to footshock ($p < 0.05$). No effect of CIE was observed in time spent in the reward-paired chamber after footshock ($p > 0.7$). Time spent in the reward paired chamber by CIE-exposed females following footshock was also significantly lower than pre-footshock time by Air-exposed females ($p < 0.05$). There was no main effect of CIE on time spent in the ethanol-paired chamber [$F(1,22) = 0.2179$, $p = 0.6453$], but a main effect of footshock was observed [$F(1,22) = 29.54$, $p < 0.001$]. A similar pattern of results was observed when investigating latency to enter the reward-paired chamber. Female mice exposed to CIE exhibited a greater reduction in time spent in the reward-paired chamber than Air controls [$t(22) = 2.57$, $p < 0.05$; Fig. 5b].

A rmANOVA revealed an interaction between footshock and CIE on latency to enter the reward-paired chamber [$F(1,21) = 5.798$, $p = 0.025$; data from one air exposed female were excluded as it was an outlier, > 3 standard deviations from the mean; Fig 5c]. Main effects of CIE and shock were also observed [$F(1,21) = 9.347$, $p = 0.006$ and $F(1,21) = 8.523$, $p = 0.008$, respectively]. Post hoc analyses indicate that latency to enter the reward-paired chamber was not altered after footshock in control females ($p > 0.9$). However, latency to enter was significantly increased after footshock in CIE-exposed females ($p < 0.001$). Latency to enter the reward-paired chamber following footshock was also higher for CIE-exposed females than control females under either testing condition (p's < 0.01). Latency to

enter the reward-paired chamber was increased to a greater degree in CIE-exposed females than in Air controls [$t(21) = 2.408$, $p < 0.05$; Fig. 5d).

Male and female mice do not alter ethanol seeking in the absence of footshock

To confirm that sex differences in and/or CIE effects on extinction learning or repeated testing did not underlie the observed differences in ethanol-seeking after a footshock, a separate cohort of mice underwent CIE and behavioral training as described above, but no footshock was administered. A rmANOVA indicated that males did not alter the time spent in the reward-paired chamber in the absence of a footshock [$F(1,16) = 3.428$, $p = 0.0826$; Fig 6a; n = 10 Air control males, 8 CIE males]. There was no effect of CIE on time spent in the reward-paired chamber [$F(1,16) = 0.0135$, $p = 0.9087$] nor was there a ethanol exposure x extinction interaction [$F(1,16) = 1.62$, $p = 0.2213$]. Air control and CIE-exposed males did not differ in change in time spent in the reward-paired chamber in the absence of footshock [$t(16) = 0.665$, $p = 0.5154$; Fig 6b]. Similarly, a rmANOVA indicated that in the absence of footshock, both CIE-exposed and Air control males did not alter their latency to enter the reward-paired chamber after repeated testing [no main effect of extinction session: $F(1,16) = 2.863$, $p = 0.11$; Fig 6c]. No main effect of CIE [$F(1,16) = 0.1287$, $p = 0.7245$] nor a CIE x testing interaction were observed [$F(1,16) = 0.5097$, $p = 0.4856$]. Change in latency did not differ between Air controls and CIE-exposed males [$t(16) = 0.7139$, $p = 0.4856$; Fig. 6d].

Similar patterns of results were observed in females. A rmANOVA of time spent in the reward-paired chamber “pre” and “post” a no shock control exposure indicates that the female mice did not alter time spent in the reward-paired chamber in the absence of footshock [$F(1,22) = 3.581$, $p = 0.0717$; Fig 7a; n = 12 females/group]. No main effect of CIE exposure [$F(1,22) = 2.322$, $p = 0.1418$] or CIE x exposure interaction were observed [$F(1,22) = 0.0351$, $p = 0.8531$]. Air controls and CIE-exposed females did not differ in change in time spent in the reward-paired chamber in the absence of footshock [$t(22) = 0.03568$, $p = 0.9719$; Fig 7b]. A rmANOVA indicated that latency to enter the reward-paired chamber did not increase in the absence of a footshock [no main effect of exposure: $F(1,22) = 0.1488$, $p = 0.7034$; Fig 7c]. A main effect of CIE was observed such that CIE-exposed females exhibited lower latencies to enter the reward-paired chamber overall than Air control females [$F(1,22) = 5.282$, $p < 0.05$]. No CIE x testing interaction was observed [$F(1,22) = 0.5131$, $p = 0.4813$]. Change in latency following repeated testing did not differ between CIE-exposed and Air control females in the absence of a footshock [$t(22) = 0.7163$, $p = 0.4813$; Fig 7d].

Neither sex nor CIE impacted sensitivity to footshock

To confirm that differences in reward seeking following a footshock were not mediated by sensitivity to footshock, we assessed paw retraction threshold and response to the footshock intensity used in these experiments (0.8 mA). A chi-square test indicated that there was no relationship between sex or CIE exposure and the shock intensity threshold at which mice retracted one paw ($\chi^2 = 6.399$, $p = 0.38$; Fig 8a). A between-subjects ANOVA indicated that the mean one paw retraction threshold did not differ by sex [$F(1,38) = 1.481$, $p = 0.2312$; Fig 8b] or CIE exposure [$F(1,38) = 0.04113$, $p = 0.8404$], nor was there a CIE x sex interaction [$F(1,38) = 0.5039$, $p = 0.4821$]. A chi-square test indicates that the distribution of responses

to 0.8 mA footshock did not differ based on CIE exposure or sex ($\chi^2 = 1.418$, $p = 0.7013$; Fig 8c).

Discussion

Our findings indicate that male and female mice show different patterns of reward seeking under conflict. Further, we find that CIE exposure may have unanticipated, sex-specific effects on ethanol seeking. In a model of compulsive-like reward seeking, both Air control and CIE-exposed males were sensitive to the ability of aversive experiences to reduce ethanol seeking. Male mice increased latency to enter and spent less time in the ethanol-paired chamber following a footshock. A different pattern of results was observed in female mice. Control female mice did not reduce time spent in or latency to enter the ethanol reward-paired chamber following footshock, suggesting that non-dependent female mice exhibit more compulsive-like ethanol seeking and are less sensitive to the ability of an aversive experience to reduce ethanol seeking. Prior to footshock, CIE-exposed female mice spent more time in the reward-paired chamber than control females, indicating that CIE may increase ethanol reward or reward-context learning in females. CIE-exposed females also showed increased sensitivity to the ability of footshock to reduce ethanol seeking. CIE-exposed females reduced time spent in the reward-paired chamber following footshock compared to the pre-footshock test session. Of note, CIE-exposed females also spent less time in the reward-paired chamber following footshock than air control females. Thus, CIE had a bidirectional effect in females, increasing time spent in the reward-paired chamber prior to footshock, and reducing time spent in the chamber after conflict between reward and aversion was introduced.

Male and female mice undergoing CIE achieved similar BEC's and were similarly sensitive to ethanol effects on activity during both saline and ethanol conditioning sessions, suggesting that these factors did not underlie the differential sensitivity of mice to ethanol reward or reward seeking following footshock. To control for differences in sensitivity to extinction learning or repeated testing, a separate cohort of animals underwent identical training and testing procedures, except that footshock was not administered. Differences in time spent in or latency to enter the reward-paired chamber were not observed in the absence of the footshock, suggesting that the aversive experience underlies the sex differences reported here. Sex differences in footshock sensitivity were not observed, and both CIE-exposed and Air control males and females exhibit similar responses to the shock intensity used in these experiments suggesting that differences in response to footshock are not mediated by differences in sensitivity.

Compulsive-like reward seeking may result from a number of factors which influence behavior, including increased sensitivity to reward, decreased sensitivity to aversion, or a failure to flexibly regulate behavior under conditions of conflict. In male mice, CIE did not appear to impact any of these factors. Time spent in and latency to enter the reward-paired chamber either before or after footshock was not modulated by CIE exposure. It is possible, however, that an increase in sensitivity to footshock or aversion-related learning following CIE would not be detected in males in this paradigm and should be investigated using other conditioning and testing parameters in future work.

CIE-exposed females appeared to be more sensitive to both the rewarding effects of ethanol and the aversive experience of a footshock. It is possible that CIE-induced alterations in fear learning or recall in females drove the reduction in time spent in a reward-paired chamber under reward-aversion conflict. The effects of CIE and other models of chronic ethanol exposure and withdrawal on fear conditioning and expression are inconsistent, with some reporting increased fear learning in males intermittently exposed to ethanol (Bertotto, Bustos, Molina, & Martijena, 2006), while others observe no effect (Borlikova, Elbers, & Stephens, 2006). Sex differences in CIE outcomes have been reported (Jury, DiBerto, Kash, & Holmes, 2017), though to our knowledge, sex differences in CIE effects on fear learning and expression have not been characterized. Our findings here do not differentiate between an increase in sensitivity to shock, an increase in fear learning, or reduction in willingness to seek ethanol under conflict in CIE-exposed female mice, which are important questions for further study. Our current findings did not consider estrous cycle effects on behavior, but this important question will be of interest in future studies as estrous stage and ovarian hormone signaling contribute to ethanol reward, learning, and ethanol-seeking behavior (Becker, McClellan, & Reed, 2017; Bertholomey, Nagarajan, & Torregrossa, 2016; Bertholomey & Torregrossa, 2017; Hilderbrand & Lasek, 2018; Young & Becker, 2009).

A substantial literature suggests that a history of alcohol exposure in males promotes the development of alcohol habits (Barker, Bryant, Osborne, & Chandler, 2017; Corbit, Nie, & Janak, 2012; Renteria, Baltz, & Gremel, 2018) and impairs behavioral and cognitive flexibility (Trantham-Davidson et al., 2014). Of particular relevance to these studies, others have reported that a similar CIE exposure protocol resulted in insensitivity to punished alcohol seeking in an operant self-administration paradigm (Radke et al., 2017). In addition, a history of exposure to cocaine promotes reward seeking under conflict in males (Nguyen, Schumacher, Erb, & Ito, 2015). Thus, we had anticipated that CIE would promote compulsivity in males. Unexpectedly, after four weeks of CIE, we observed no effect on time spent in an ethanol reward- and aversion-paired context in male mice. One substantial difference in these models is that in the punished self-administration model, mice were trained to self-administer ethanol over a prolonged period of time prior to CIE and mice voluntarily consumed ethanol. In the current studies, mice were ethanol-naïve prior to CIE, and were exposed only to ethanol by vapor or injection. No voluntary self-administration either in an operant setting or home cage drinking occurred. As self-administration is known to induce distinct patterns of behavior and neurobiological alterations when compared to experimenter administered drugs and alcohol, (McFarland, Davidge, Lapish, & Kalivas, 2004; McFarland, Lapish, & Kalivas, 2003; Moolten & Kornetsky, 1990; Robinson, Gorny, Savage, & Kolb, 2002), this may explain some of the discrepancy observed between these studies. Relatedly, findings indicating a lack of sex differences in consumption of quinine-adulterated ethanol were in mice that exclusively self-administered alcohol (Sneddon, White, & Radke, 2018). It is possible that the sex differences observed in our findings in part result from experimenter administration of ethanol and/or restriction to the rewarding effects of post-ingestive ethanol. Additionally, the model used for these experiments requires acquisition and recall of both the reward- and aversion-context associations, while quinine adulteration and punished self-administration models involve online experiences of reward and aversive outcomes. We expect that sex differences and the effects of CIE observed in

these studies may be in part due to differences in outcome learning and the use of this information to guide behavior, suggesting that multiple models of compulsive-like ethanol seeking or ethanol seeking under conflict should be considered when investigating neurobiological substrates and risk factors.

Together, the findings in this study indicate that non-dependent females were less sensitive to the effects of adverse experiences on ethanol seeking and exhibited greater compulsive-like ethanol seeking than males. Under conditions in which males reduced ethanol seeking, non-dependent female mice continued to seek ethanol despite adverse experiences. Exposure to CIE resulted in a bidirectional shift in ethanol seeking behavior in females. Dependent females showed increased ethanol seeking in the absence of conflict. However, CIE-exposed females reduced ethanol seeking in the presence of reward-aversion conflict, indicative of less compulsive-like ethanol seeking than non-dependent females. Chronic ethanol exposure did not impact ethanol seeking in males in this model, either in the absence or presence of conflict. These data argue for the investigation of sex differences in ethanol reward, and inclusion of females in studies investigating the neurobiological factors driving the development and expression of compulsive-like inflexible ethanol seeking.

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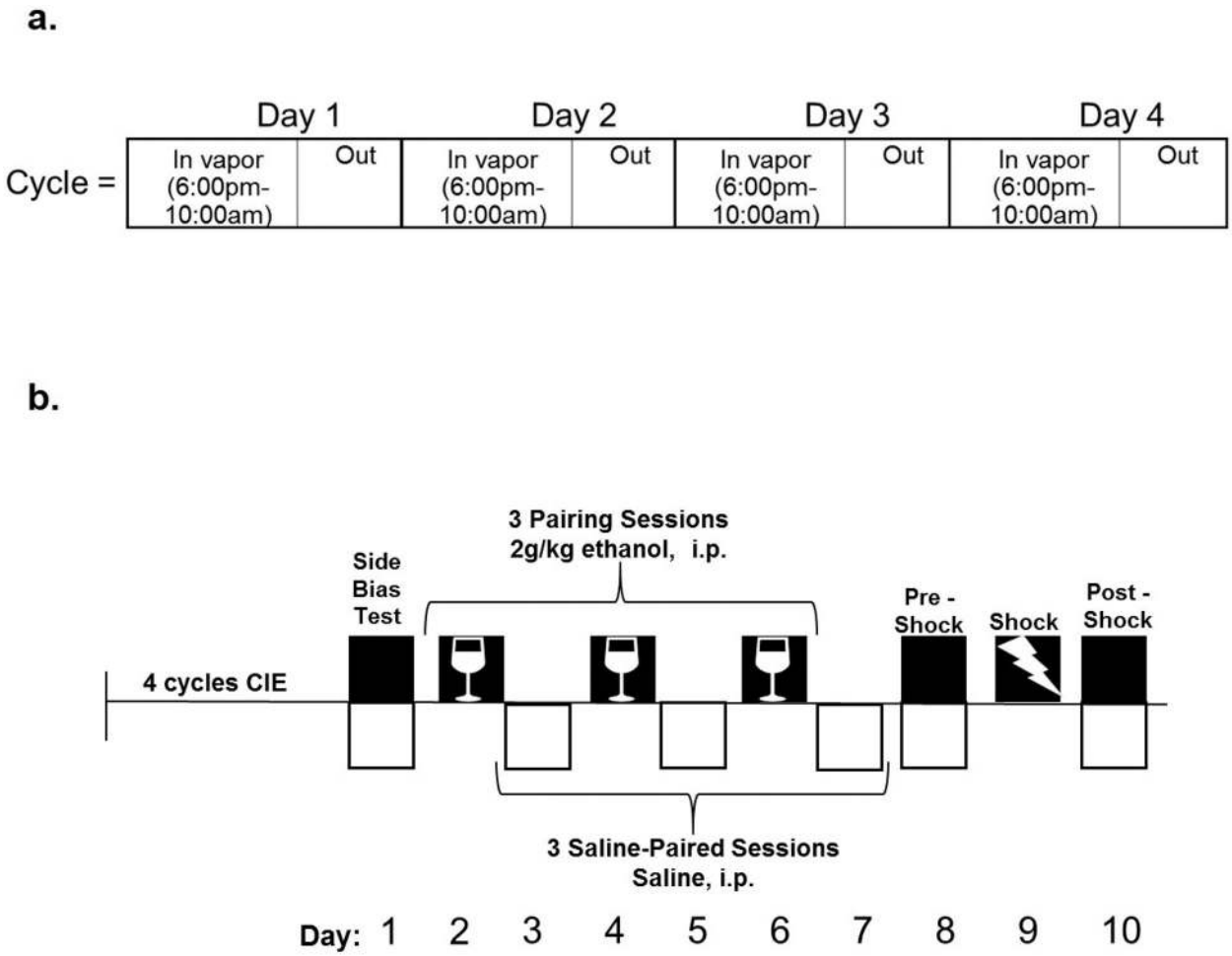

Figure 1. Experimental design and timeline.

(a) Adult male and female mice were exposed to ethanol by vapor inhalation over four cycles consisting of four, 16-hour exposures per cycle. **(b)** Seventy-two hours after the final ethanol exposure, mice were trained in a modified conditioned place preference paradigm. Mice received 3 conditioning sessions in which they received 2g/kg ethanol immediately prior to placement in the paired chamber. These sessions were alternated with control sessions in which mice received saline injections prior to placement in the saline-paired chamber. After ethanol conditioning, mice experienced a single footshock in the reward-paired context. Time spent and latency to enter the reward-paired chamber was assessed before and after footshock. Control mice underwent identical ethanol exposure procedures and conditioning except that on Day 9, no footshock was delivered.

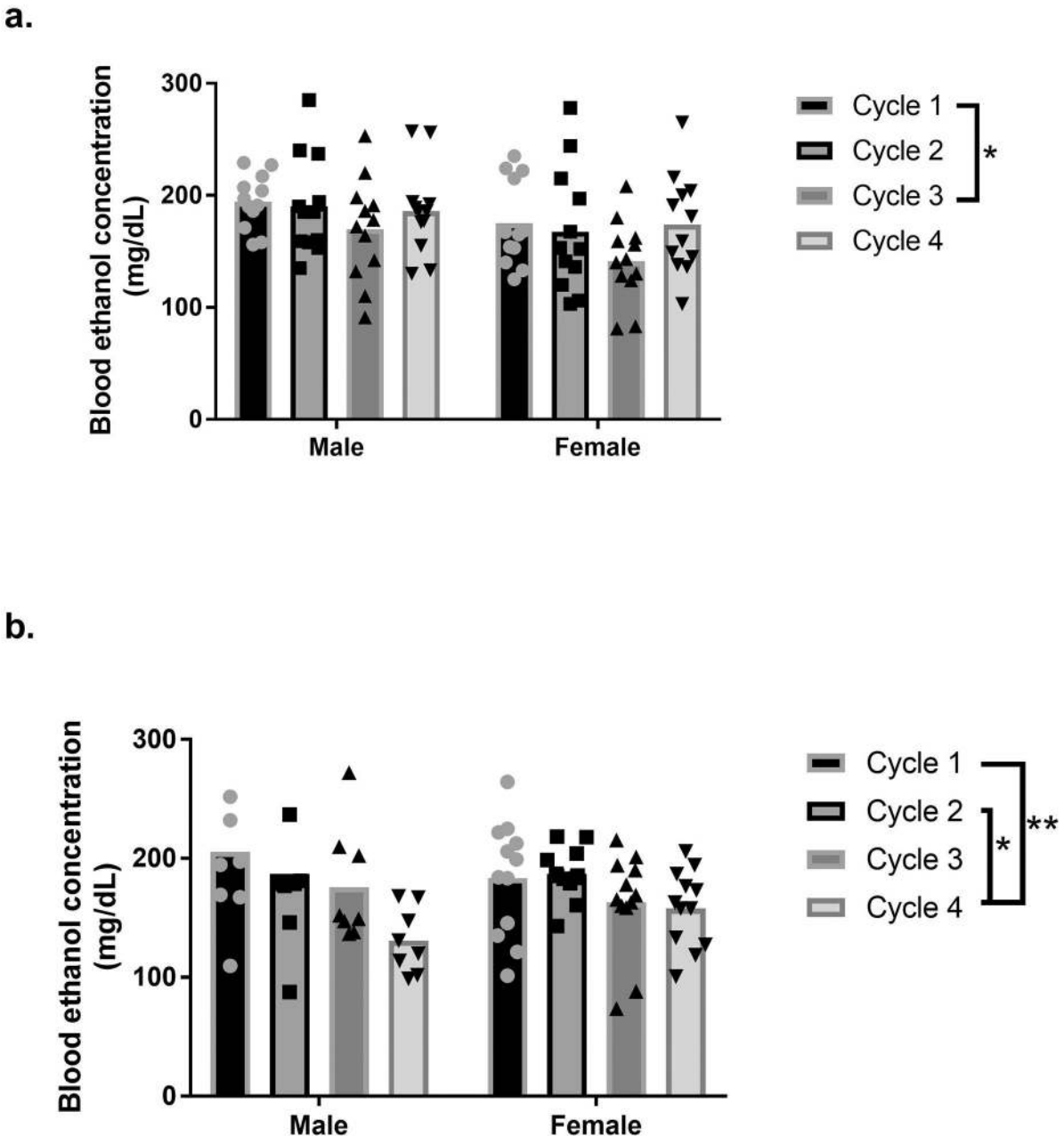

Figure 2. Blood ethanol concentrations.

(a) Male and female mice achieved similar blood ethanol concentrations across CIE. **(b)** This pattern was also observed for mice to be trained in the no shock control paradigm. *p < 0.05, **p < 0.01.

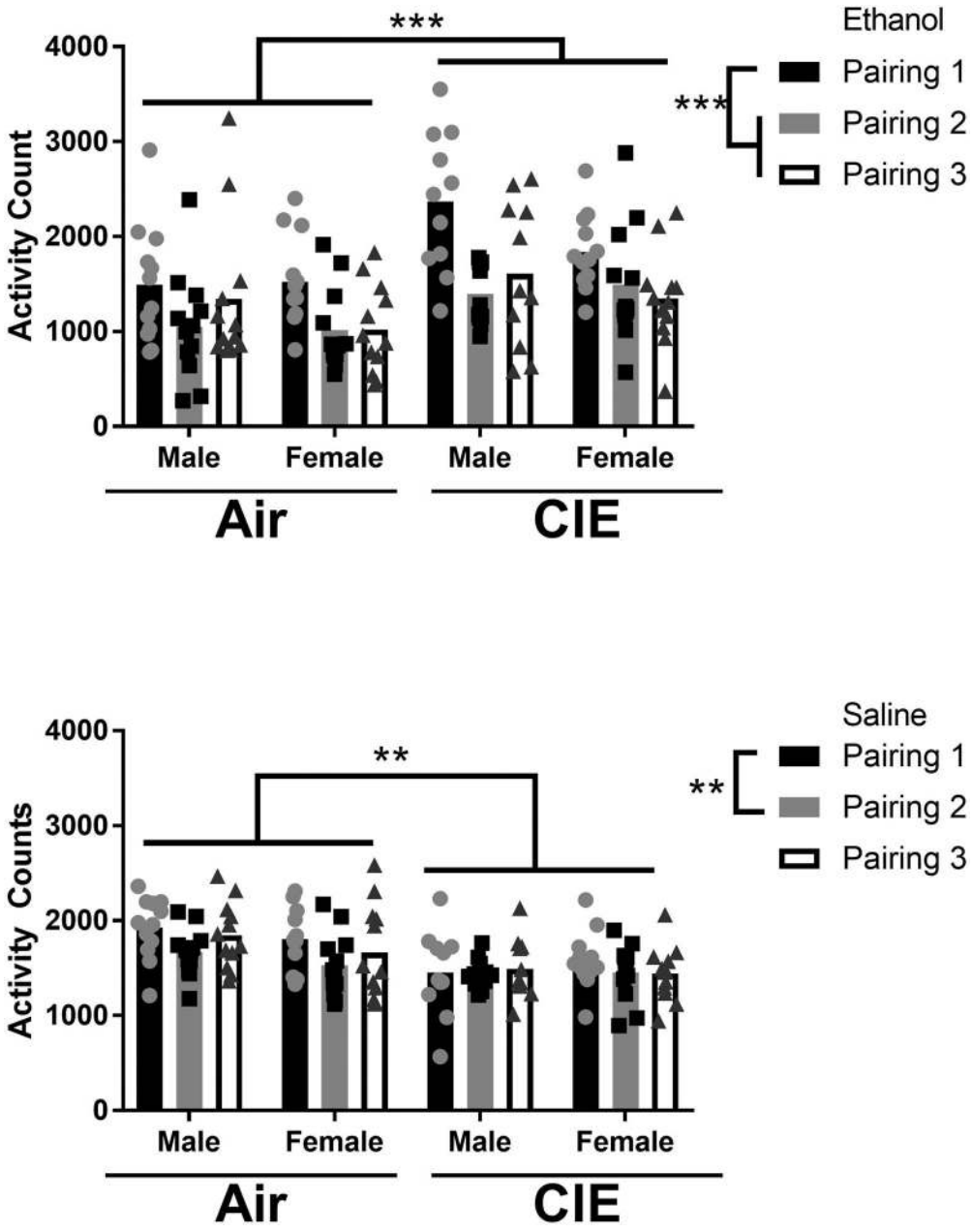

Figure 3. Activity counts during conditioning.

(a) Beam breaks following ethanol injection were similar in male and female mice during ethanol reward conditioning. CIE-exposed mice showed greater activity than Air controls, but this was not moderated by sex. **(b)** Activity during saline pairing sessions were not different between males and females. However, during saline session, CIE-exposed mice showed lower activity than Air controls. **p < 0.01, ***p < 0.001.

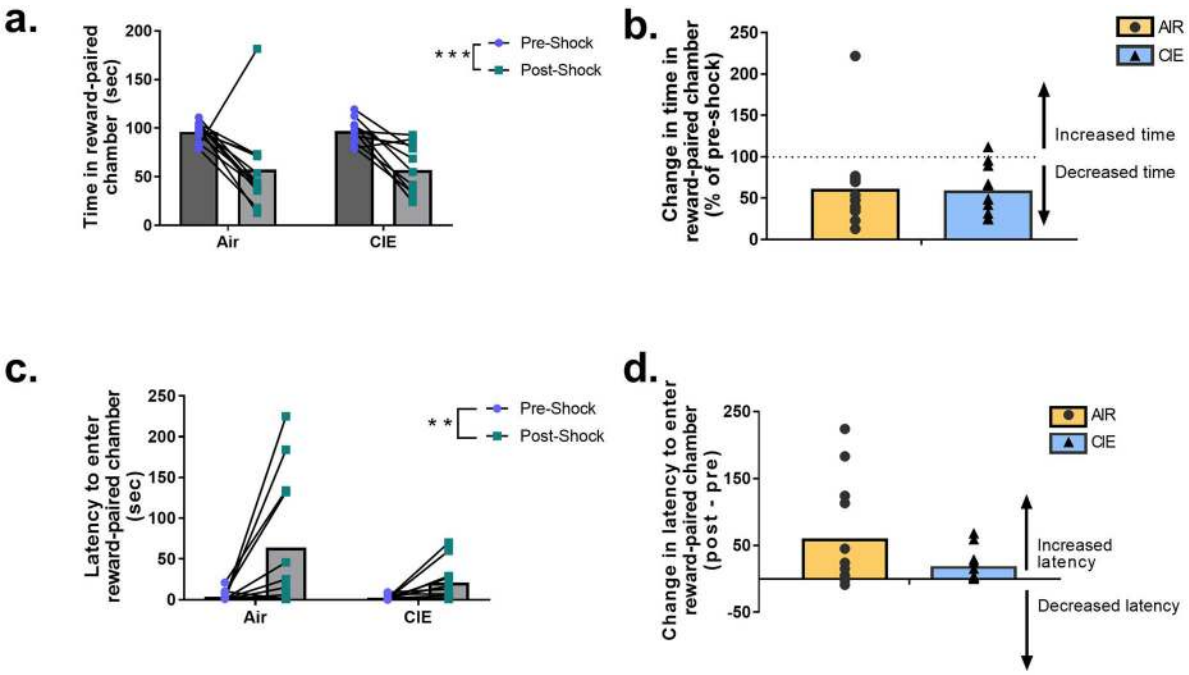

Figure 4. Ethanol seeking following footshock in male mice.

(a) Male mice reduced time spent in a reward-paired chamber following a footshock. CIE-exposed males did not differ from Air controls. **(b)** Air control and CIE-exposed males reduced time spent in the ethanol-paired chamber to a similar degree under reward/aversion conflict. **(c)** Latency to enter the ethanol-paired chambers was increased following a footshock in CIE-exposed and Air control males. **(d)** Change in latency to enter the ethanol-paired chamber after footshock was not significantly different between CIE-exposed and Air control males. $^{**}p < 0.01$, $^{***}p < 0.001$.

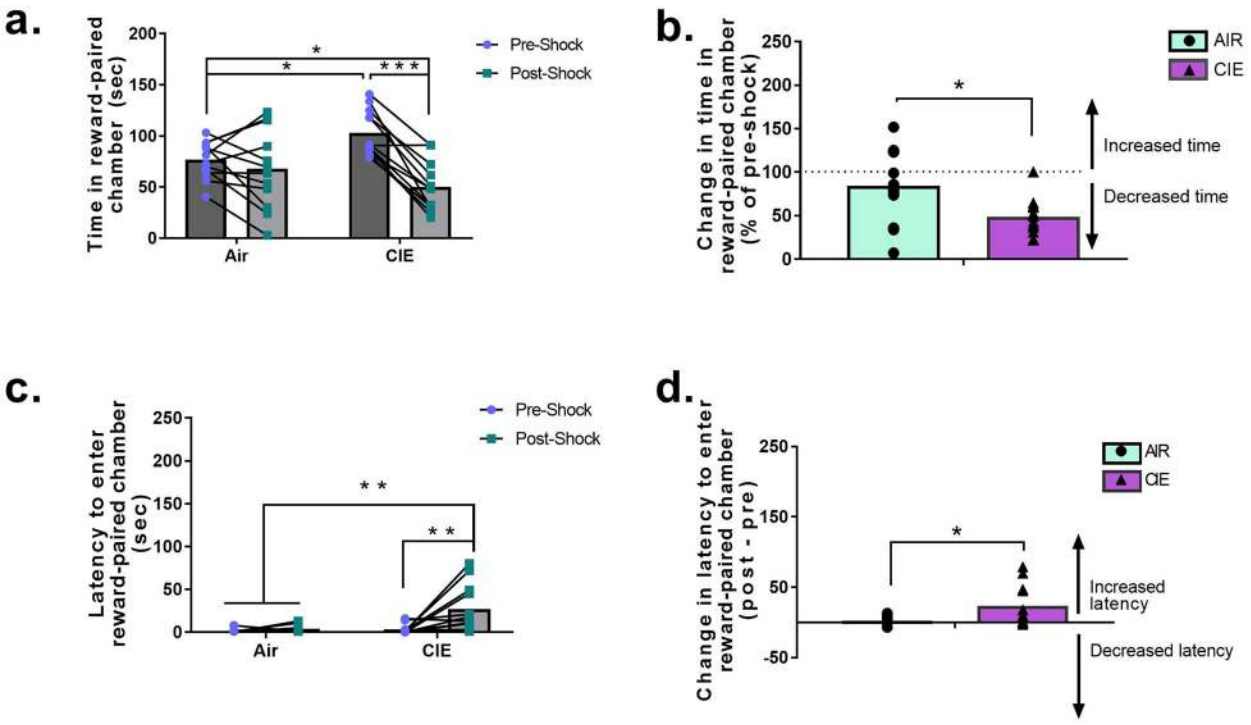

Figure 5. Ethanol seeking following footshock in female mice.

(a) Air control female mice were insensitive to an aversive experience and spent similar amounts of time in a reward-paired chamber following a footshock. CIE-exposed females exhibited increased time spent in the ethanol reward-paired chamber prior to footshock compared to control females. However, CIE females reduced time spent in the reward-paired chamber following a footshock as compared to their own baseline and to air controls. **(b)** CIE-exposed females reduced time spent in the ethanol-paired chamber to a greater degree than Air control females. **(c)** Control females did not increase their latency to enter the ethanol-paired chamber following footshock. However, CIE-exposed females showed significantly increased latencies to enter the ethanol-paired chamber following footshock. Latencies to enter the reward-paired chambers following footshock in CIE-exposed females were also significantly higher than control females under both conditions. **(d)** CIE-exposed females exhibit a greater increase in latency than Air control females. *p < 0.05, **p < 0.01, ***p < 0.001.

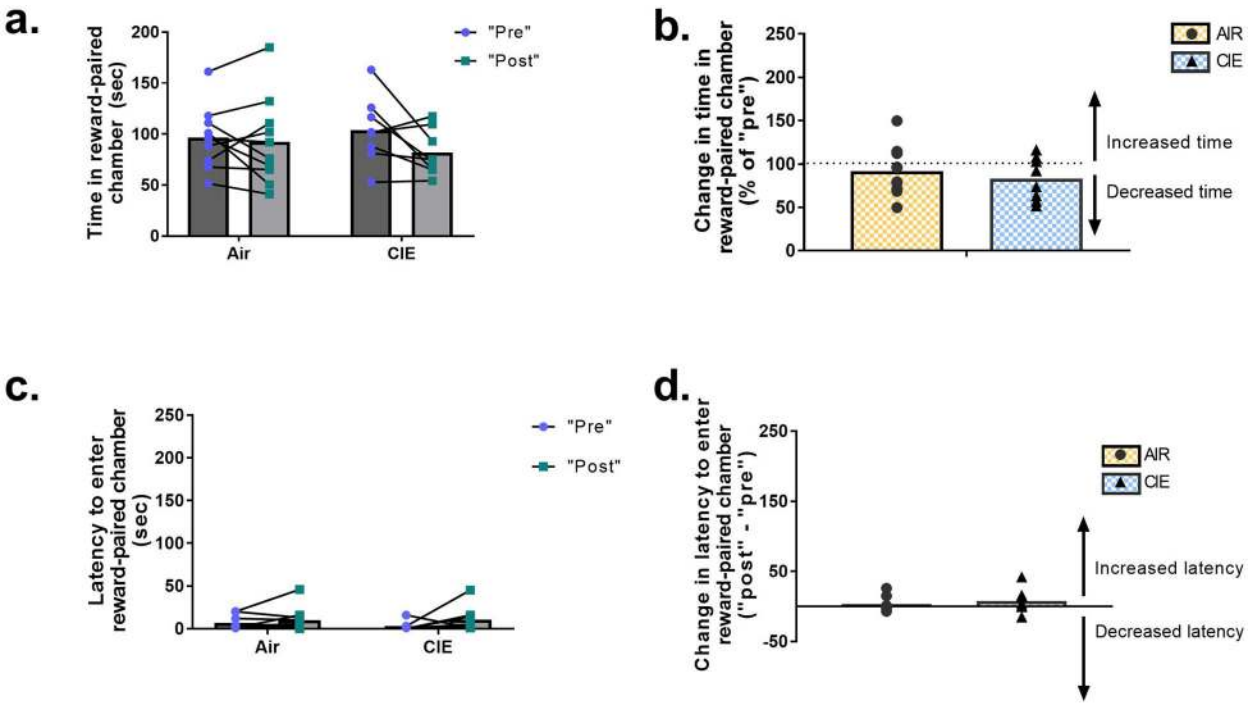

Figure 6. Persistent ethanol seeking in the absence of footshock in males.

(a) Neither Air controls and CIE-exposed male mice altered time spent in the reward-paired chamber following no-shock control sessions. **(b)** Change in time spent in the reward-paired chamber in the absence of footshock did not differ between CIE-exposed and Air control males. **(c)** Male mice did not alter their latency to enter a reward-paired chamber before and after a no-shock control session. **(d)** No difference was observed in change in latency between CIE-exposed and Air control males.

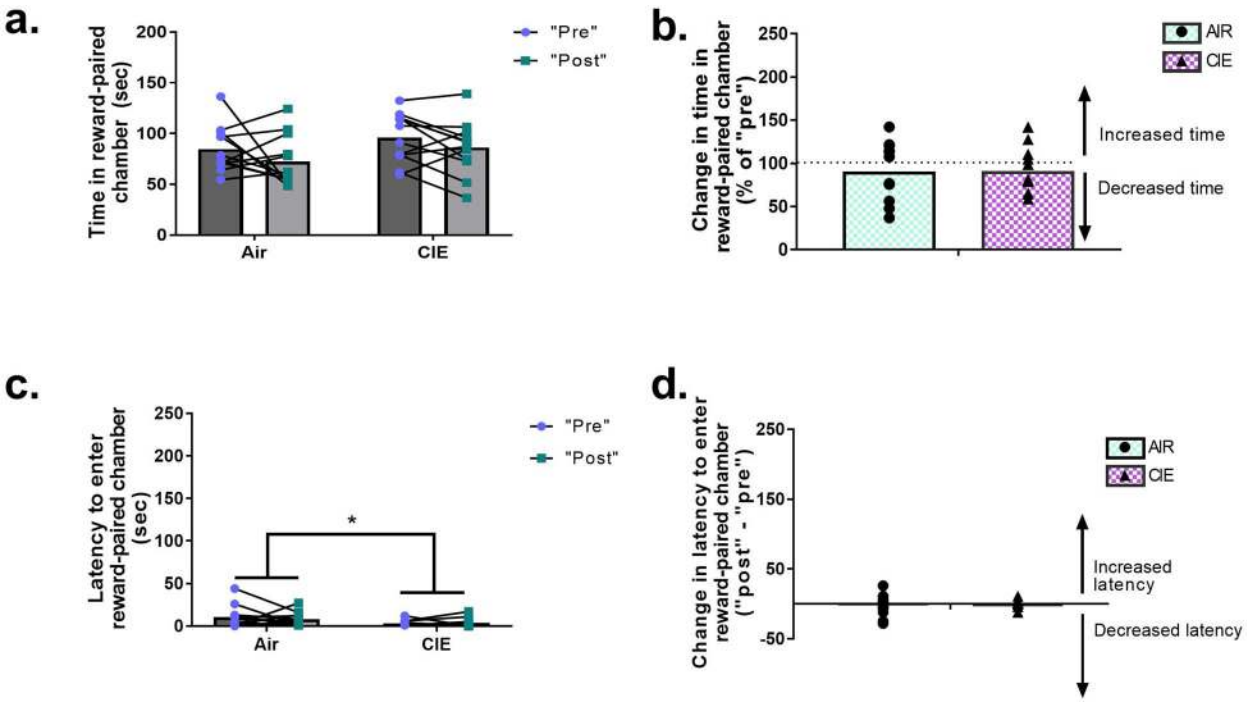

Figure 7. Persistent ethanol seeking in the absence of footshock in females.

(a) In female mice, time spent in the reward-paired chamber was not different before and after a no-shock control session. **(b)** Change in time spent in the reward-paired chamber in the absence of footshock did not differ between CIE-exposed and Air control females. **(c)** Female mice did not exhibit increased latency to enter a reward-paired chamber before and after a no-shock controls session. CIE-exposed females entered the reward-paired chamber at a lower latency than Air controls. **(d)** No difference was observed in change in latency between CIE-exposed and Air control female mice. *$p < 0.05$.

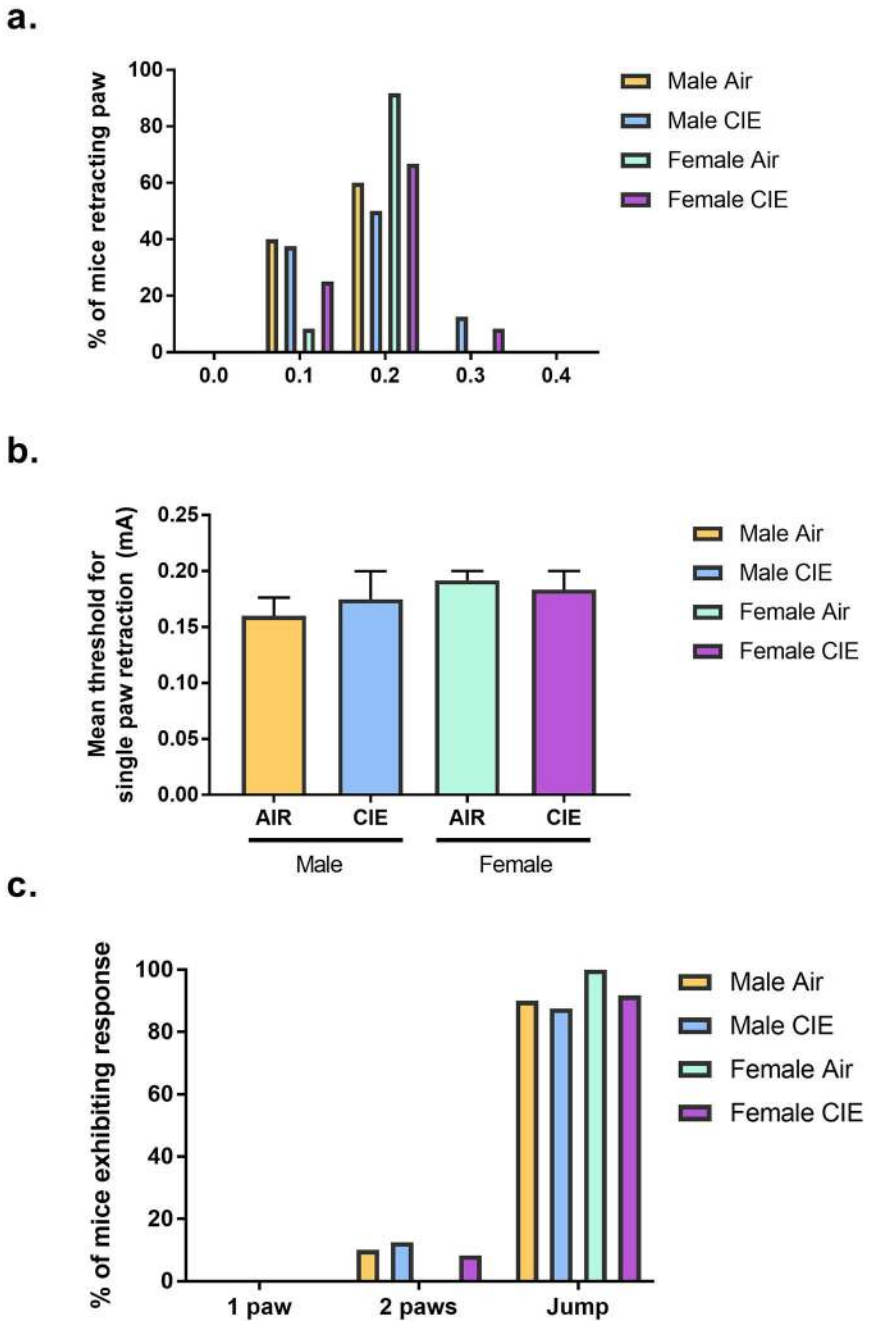

Figure 8. Footshock sensitivity in male and female mice.

(a) Distribution of footshock intensity threshold at which mice retracted one paw. **(b)** Mean footshock intensity threshold for one-paw retraction. **(c)** Distribution of responses to 0.8 mA footshock in male and female mice.