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Chronic ethanol exposure increases voluntary home cage intake in adult male, but not female, Long-Evans rats

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Abstract

The current experiment examined the effects of 10 days of chronic intermittent ethanol (CIE) exposure on anxiety-like behavior and home cage ethanol intake using a 20% intermittent access (M, W, F) paradigm in male and female Long-Evans rats. Withdrawal from alcohol dependence contributes to relapse in humans and increases in anxiety-like behavior and voluntary ethanol consumption in preclinical models. Our laboratory has shown that 10 days of CIE exposure produces both behavioral and neurophysiological alterations associated with withdrawal in male rats; however, we have yet to examine the effects of this exposure regime on ethanol intake in females. During baseline, females consumed more ethanol than males but, unlike males, did not show escalations in intake. Rats were then exposed to CIE and were again given intermittent access to 20% ethanol. CIE males increased their intake compared to baseline, whereas air-exposed males did not. Ethanol intake in females was unaffected by CIE exposure. Notably, both sexes expressed significantly elevated withdrawal-associated anxiety-like behavior in the plus maze. Finally, rats were injected with the cannabinoid CB1 receptor antagonist, SR141716A (0, 1, 3, 10 mg/kg, i.p.) which reduced ethanol intake in both sexes. However, females appear to be more sensitive to lower doses of this CB1 receptor antagonist. Our results show that females consume more ethanol than males; however, they did not escalate their intake using the intermittent access paradigm. Unlike males, CIE exposure had no effect on drinking in females. It is possible that females may be less sensitive than males to ethanol-induced increases in drinking after a short CIE exposure. Lastly, our results demonstrate that males and females may have different pharmacological sensitivities to CB1 receptor blockade on ethanol intake, at least under the current conditions.

Keywords

home cage self-administration; dependence; ethanol vapor; anxiety; sex differences; SR141716A

I. Introduction

Drinking alcohol to alleviate the withdrawal symptoms (e.g., anxiety) that emerge when long-term alcohol exposure is terminated appears to be a strong contributing factor to

relapse in alcohol-dependent individuals (Driessen *et al.*, 2001, Koob and Le Moal, 2001, Schellekens *et al.*, 2015, Wise, 1988). As a result, research has focused on examining the factors contributing to alcohol dependence and withdrawal, which may then lead to increased drinking and susceptibility of developing an alcohol use disorder (AUD; alcohol abuse and dependence). Given that researchers cannot systematically control for environmental/genetic factors that may influence the severity and manifestation of the withdrawal syndrome in humans, animal models of alcohol dependence have been developed (Becker, 2000). These preclinical models have proven useful for studying the factors contributing to alcohol withdrawal, its symptoms, and, more importantly, its effects on drinking. For instance, several laboratories have shown that alcohol-dependent rats also demonstrate withdrawal-related symptoms upon cessation of alcohol exposure (Holter *et al.*, 2000, Lack *et al.*, 2007, Valdez *et al.*, 2002), and that alcohol intake is typically increased in these animals as well (Criado and Ehlers, 2013, Finn *et al.*, 2007, O'Dell *et al.*, 2004, Rimondini *et al.*, 2002, Roberts *et al.*, 2000, Sommer *et al.*, 2008, Valdez *et al.*, 2002). However, the factors influencing this relationship are unknown; therefore, research utilizing preclinical models that examine the relationship among ethanol dependence, withdrawal, and intake is necessary.

Sex influences the development of drug abuse and dependence (Carroll and Anker, 2010, Carroll *et al.*, 2004, Devaud *et al.*, 2006). In humans, clear drinking patterns exist between men and women. For instance, women tend to drink alcohol less often, have fewer drinks per occasion, and have lower rates of heavy drinking than men (Hiller-Sturmhofel *et al.*, 1994, Wilsnack *et al.*, 2000). Despite this, men are almost twice as likely to develop an AUD compared to females (SAMHSA, 2013). Clinical research on the transition to alcohol dependence is mixed, with some reporting that once women begin drinking regularly they progress faster than men to drinking-related problems (Randall *et al.*, 1999), whereas others found no effect (Alvanzo *et al.*, 2011). Animal models have also proven to be useful for determining sex-differences in alcohol intake, dependence, and withdrawal. The directionality of sex differences in adult ethanol intake is opposite in humans and animal models. For instance, adult female rodents generally consume more ethanol than their male counterparts (Lancaster *et al.*, 1996, Lancaster and Spiegel, 1992, Vetter *et al.*, 2007); however, this discrepancy is not always observed. For example, individual patterns in drinking that mimic human consumption (e.g., males drinking more alcohol than females, greater percentage of males being heavy drinkers) have been demonstrated in cynomolgous monkeys (Vivian *et al.*, 2001). Overall, these data suggest that sex differences in patterns of alcohol consumption likely reflect the drinking access paradigm and species used (Lynch *et al.*, 2002, Schramm-Sapota *et al.*, 2014). Sex differences also emerge for the transition to and severity of alcohol dependence with male rats becoming ethanol dependent faster and taking longer to recover from withdrawal seizures than females (Devaud and Chadda, 2001). Interestingly, the sex differences in ethanol withdrawal severity observed in preclinical models is paralleled with that of humans, with men typically demonstrating greater and more severe withdrawal symptoms than women (Deshmukh *et al.*, 2003; however see, Schuckit *et al.*, 1998). The reported sex-related differences in ethanol consumption, progression to ethanol dependence and withdrawal severity support the use of preclinical

models as well as the need for the inclusion of males and females in drug and alcohol studies, with the ultimate goal of finding unique treatment strategies for men and women.

Several drinking paradigms have been developed to produce high ethanol intake in rodents (Mello, 1973). Intermittent ethanol exposure using modifications of the 2-bottle-choice drinking paradigm (Wise, 1973) has become a favorable approach to measure drinking in rodents (Hwa *et al.*, 2011, Li *et al.*, 2011, Simms *et al.*, 2008). In addition to its simplicity (i.e., no use of sucrose fading; no water/food deprivation), another advantage of this paradigm is that rats—including outbred rats which do not typically consume large quantities of ethanol—exposed to an intermittent 2-bottle-choice access procedure show an escalation in consumption and preference for ethanol (see Carnicella *et al.*, 2014 for review, Simms *et al.*, 2008). On the other hand, females do not show an escalation in ethanol drinking using this intermittent 2-bottle-choice paradigm (Butler *et al.*, 2014). Finally, this drinking paradigm is sensitive to pharmacological manipulation (Li *et al.*, 2011, Sabino *et al.*, 2013, Simms *et al.*, 2008, Steensland *et al.*, 2012); and rats made physically dependent on ethanol increase ethanol consumption using this procedure as well (Li *et al.*, 2011). Therefore, the present study utilized this intermittent 2-bottle choice procedure given that this model reliably produces an escalation in drinking and preference while avoiding the use of forced administration.

The present study determined the effects of 10 days of chronic intermittent ethanol (CIE) exposure, a well-established ethanol dependence model, on subsequent ethanol intake using intermittent exposure to 20% ethanol with the 2-bottle choice paradigm. Male and female Long-Evans rats were used to explore the contributions of sex to dependence-induced anxiety and CIE-related drinking. The time-course of ethanol drinking was monitored during baseline (5 weeks), and then rats were exposed to chronic ethanol via the commonly used intermittent vapor inhalation method (Goldstein and Pal, 1971, Macey *et al.*, 1996, O'Dell *et al.*, 2004, Rogers *et al.*, 1979) which produces withdrawal symptoms consistent with dependence across many species (Finn and Crabbe, 1997, Goldstein and Pal, 1971, Kliethermes *et al.*, 2004, Lack *et al.*, 2007, Macey *et al.*, 1996). This procedure offers exquisite control over blood ethanol concentration (BEC), exposure duration/frequency, and withdrawal duration — all of which have been shown to alter ethanol consumption upon removal from the vapor chamber (Griffin *et al.*, 2009a, Griffin *et al.*, 2009b, Lopez and Becker, 2005, O'Dell *et al.*, 2004). Post-procedure drinking was then examined in both CIE- and air-exposed rats. Lastly, the ability for the cannabinoid CB1 receptor antagonist, SR 141716A (Rimonabant), to reduce ethanol consumption using this intermittent access to 20% ethanol 2-bottle choice paradigm was tested. SR 141716A was chosen because it reduces ethanol drinking in rodents under a variety of experimental conditions (Arnone *et al.*, 1997, Colombo *et al.*, 1998, Dyr *et al.*, 2008, Freedland *et al.*, 2001) and would therefore make a good pharmacological compound to test the sensitivity of this drinking paradigm in ethanol-dependent rats. These investigations will help further our understanding of the neurobiological mechanisms influencing withdrawal-induced drinking and factors leading to increased “risk” for developing an AUD—particularly in under-studied populations, such as females.

2. Material and methods

2.1. Subjects

A total of 56 male and female Long-Evans rats (Harlan Laboratories, Indianapolis, IN) arrived at 8 weeks old. Upon arrival, rats were individually housed and maintained on a reverse 12:12 h light dark cycle (lights on at 9 PM). Standard rat chow and water were available ad libitum throughout the entire experimental procedure. Rats were acclimated to daily handling and weighing for one week prior to the start of the experimental procedure. All animal care procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Wake Forest Animal Care and Use Committee.

2.2. General experimental design

Rats were given intermittent access to a 20% ethanol (prepared in tap water from 95% ethyl alcohol) 2-bottle choice procedure (see below). After 5 weeks, rats were separated into two exposure groups, counter-balanced based on ethanol intake during the baseline period: ethanol vapor (CIE) or air (CON) as previously described (Christian *et al.*, 2012, Lack *et al.*, 2007). Rats in the CIE group were exposed to vapor inhalation for 10 consecutive days, whereas CON rats were similarly handled and housed, except they received room air only (below). After the 10-day CIE exposure, rats were given a 96 h abstinence period prior to having access to the same 2-bottle choice procedure to measure post-dependence drinking. Rats were then injected with the cannabinoid CB1 receptor antagonist, SR 141716A, once weekly for four consecutive weeks (see below).

2.3. Ethanol intake procedure

One week after arrival, rats (n=16/sex) were intermittently exposed to a 20% ethanol (Warner Graham Co, Cockeysville, MD) 2-bottle-choice drinking procedure. Fluids were presented in plastic bottles (ACE Co., Allentown, NJ; Model # PC09BH) with no. 8.5 rubber stoppers containing steel ball-bearing sipper tubes (Ancare Corp., Bellmore, NY). Beginning on Monday, a bottle containing 20% (v/v) ethanol in water and a bottle containing water was placed onto the home cage approximately 1 h into the dark cycle. Following a 24 h period, these bottles were replaced with two bottles of water for 24 h. This 2-bottle-choice procedure was repeated on Wednesday and Friday, with rats having unlimited access to two bottles of water on Tuesday, Thursday, and over the weekend. Placement of the ethanol bottle was alternated to prevent any side preferences. Rats were weighed daily and bottles were weighed (to the nearest 0.1 g) before, 30 min, 2 h, and 24 h after being placed onto the home cage.

2.4. Chronic ethanol vapor inhalation exposure

Rats in the CIE condition were housed in their standard home cages, which were placed in larger, custom-built Plexiglas chambers (Triad Plastics, Winston-Salem, NC). Using standard procedures from our laboratory, ethanol-vapor (~35mg/L) was mixed with room air and pumped into the chamber for 12 h/day over 10 consecutive days during the light cycle (beginning at 9 PM) (Christian *et al.*, 2013, Christian *et al.*, 2012, Lack *et al.*, 2007). Animals in the CON group were housed identically—i.e., in their home cages, placed inside

the larger Plexiglas chambers, except they were exposed only to room air. Rats in each condition were weighed daily and tail blood samples were taken periodically (days 3, 7, and 10) throughout the 10-day CIE period (at 9 AM) to monitor blood ethanol concentration (BEC) and allow for adjustment of ethanol vapor as necessary.

2.5. Post-CIE exposure drinking and drug treatment effects

After the 10-day CIE exposure, rats were given a 96 h abstinence period before beginning their post-dependence 2-bottle-choice drinking on the following Monday. Rats were exposed to the same 2-bottle-choice drinking procedure as described above for two weeks. On the Friday of the second week, rats were habituated to an intraperitoneal (i.p.) injection of saline (1 ml/kg). SR 141716A test sessions were conducted on subsequent Fridays using a within-subjects design, with drug doses counterbalanced under a Latin-square design. No drug treatment was administered on Monday and Wednesday to allow for a 1-week washout period between each dose. Rats in each condition (CIE or CON) received all doses of SR 141716A 30 min before bottles were placed onto the home cage. SR 141716A (National Institute of Mental Health [NIMH] Chemical Synthesis and Drug Supply Program) was dissolved in saline (1 ml/kg) with a few drops of Tween 80 (Sigma-Aldrich, St. Louis, MO) and administered i.p. at doses of 0, 1, 3, and 10 mg/kg.

2.6. Elevated Plus Maze

To ensure that 10 days of intermittent ethanol vapor exposure produces dependence-like behaviors in male and female Long-Evans rats, a separate group of age-matched rats ($n=12/\text{sex}$) were exposed to 10 days of CIE vapor or air and tested on the elevated plus maze (EPM) 24 h after the last ethanol vapor exposure. We used standard procedures from our laboratory for EPM testing (Diaz *et al.*, 2011, McCool and Chappell, 2009). Briefly, rats were habituated to the behavioral testing room for at least 20 min prior to being placed on the junction of the EPM. Overhead room lights were turned off; however, each open arm was dimly lit ($\sim 40\text{lux}$). Test sessions ran for 300s, with beam-breaks and time spent in each arm recorded by a computer (Med Associates) connected to the plus maze. Upon completion of each trial, the plus maze was wiped clean with warm water and mild soap and allowed to dry prior to placement of the next rat. To avoid any potential effects on anxiety-like behavior from holding males and females in the same behavioral testing room, males and females were tested on the EPM on different days.

2.7. Blood ethanol determination

Upon completion of the SR 141716A dose-response curve, rats were given an additional week of 2-bottle-choice drinking with no drug treatment. Blood samples were collected from the tail 30 min after bottles were placed onto the home-cage to measure BECs, which were determined using a commercially available assay kit (Carolina Liquid Chemistries, Winston-Salem, NC). One week later, rats were injected with SR 141716A (1 mg/kg) followed 30-min later by an injection of 1 g/kg ethanol (20% v/v, i.p.) or saline. Tail blood samples were taken 30 min post-ethanol injection to examine if SR 141716A affects ethanol metabolism and could contribute to the reductions in ethanol intake following administration of this drug.

2.8. Data analysis

Prior to analysis, baseline intake and EPM data were subjected to an outlier test (± 1.5 standard deviations from the mean), which resulted in the removal of 1 male for baseline intake and 1 animal of each sex for the EPM experiment. Average weekly ethanol and water intake (g/kg and ml/kg, respectively) and preference (ethanol intake in ml / total fluid intake in ml X 100) data were calculated and baseline intake for males and females were analyzed using a mixed factorial ANOVA with sex as a between subjects factor (IBM SPSS, Armonk, NY). Given that expected statistical differences in baseline intake emerged between sexes (see “Results”), subsequent data were analyzed separately for males and females. In post-dependence drinking analyses, CIE and CON groups served as between-subjects factors. Significant effects and interactions were explored further using Tukey’s post hoc test. The EPM data in males and females were analyzed via unpaired t-tests as percent open arm time [open arm time/(open arm time + closed arm time)] and percent open arm entries [open arm entries/(open arm entries + closed arm entries)]. Data are expressed as mean ($\pm$ SEM) and significance was defined as $p < 0.05$.

3. Results

3.1. Effect of chronic ethanol on anxiety-like behavior is similar in males and females

To confirm that 10 days of CIE exposure is sufficient to produce dependence-like behavior, male and female rats were tested on the elevated plus maze 24 h after the last ethanol vapor exposure. In males, there were significant effects of condition in the analysis of open arm time (%) [$t(8)=2.39$, $p < 0.05$] and a trend for percent open arm entries [$t(8)=2.16$, $p=0.06$], with CIE withdrawal (WD) animals spending significantly less time in and trending toward entering the open arms less than their air-exposed male counterparts (Figure 1, left panels). Similarly, females in the withdrawal condition spent significantly less time in the open arms of the plus maze relative to air-exposed controls [$t(8)=2.51$, $p < 0.05$], with no effect of entries into the open arms (Figure 1, right panels). There were no differences between conditions in junction time/entries in either males or females.

3.2. Baseline ethanol intake, preference, and water intake

Analysis of baseline intake at the 30 min and 2 h time points revealed that females consumed more ethanol than males across the entire baseline drinking period [main effect of sex: g/kg/30 min: $F(1,29)=15.78$, $p < 0.0001$ and g/kg/2 h: $F(1,29)=33.55$, $p < 0.001$]. At the 24 h time point, a main effect of sex [$F(1,29)=6.66$, $p < 0.05$] and a sex X week interaction [$F(4,116)=3.78$, $p < 0.01$] revealed that females consumed more ethanol than males during the first three weeks. Additionally, males showed an escalation in their drinking with significant differences in intake observed at the fourth and fifth weeks compared to the first week. Ethanol intake in females was not significantly different across weeks (Figure 2, top panels). At the 30 min time point, males and females showed a significant increase in preference for ethanol during the fourth and fifth weeks [main effect of week $F(4,116)=2.50$, $p < 0.05$], with similar findings observed at the 2 h time point [week X sex interaction $F(4,116)=2.53$, $p < 0.05$]. Preference data at the 24 h time point mimicked the ethanol intake data, with males demonstrating an escalation in preference for ethanol over water whereas no difference was observed in females across the five weeks [sex X week interaction:

$F(4,116)=5.24, p<0.0001$] (Figure 2, middle panels). Generally, females also consumed more water than males at the three time points tested and water intake decreased in both sexes across weeks. At 30 min, a significant sex X week interaction [$F(4,29)=3.40, p<0.01$] revealed that females consumed more water than males during almost all weeks (i.e., 1, 2, 3, and 5), with this sex difference diminishing at the 2 h time point [sex X week interaction: $F(4,29)=5.80, p<0.001$]. The 24 h water intake paralleled the ethanol and preference data in males, with water intake significantly decreasing by the third week [$F(4,29)=4.95, p<0.01$] (Figure 2, bottom panels).

3.3. CIE Exposure Enhances Drinking in Males but not Females

Analysis of the drinking data following 10 days of CIE vapor exposure revealed a significant week X condition interaction in males for each time point [30 min: $F(1,13)=5.44, p<0.05$, 2 h: $F(1,13)=7.47, p<0.05$, 24 h: $F(1,13)=4.76, p<0.05$] (Figure 3, top panels). Post hoc tests demonstrated that while air-exposed control males consumed ethanol at similar levels before (“pre”) and after (“post”) air exposure, CIE rats exposed to 10 days of intermittent ethanol vapor consumed more ethanol after this exposure compared to their baseline intake (Figure 3, top panels). In females, CIE exposure had no effect on ethanol intake at any of the time points examined. Instead, a main effect of week [$F(1,14)=8.27, p<0.05$] emerged at the 24 h time point, showing that regardless of CIE exposure history, females consumed more ethanol after this short, 10-day ‘abstinence’ period from 20% ethanol drinking sessions than they did at baseline (Figure 3, bottom panels).

3.4. SR 141716A (Rimonabant) Differentially Modulates Ethanol and Water Intake in Males and Females

Analysis of 20% ethanol consumption in males revealed a main effect of dose at each time point examined [30 min: $F(3,59)=4.51, p<0.01$, 2 h: $F(3,59)=6.96, p<0.001$, and 24 h: $F(3,59)=3.63, p<0.05$]. Post hoc tests demonstrated reductions in ethanol intake after administration of 3 and 10 mg/kg at 30 min and 2 h; however, during the 24 h time point, only the 10 mg/kg dose was still effective at reducing ethanol intake. Water intake in males was similarly reduced at these doses (Figure 4, left panels). In females, however, SR 141716A significantly reduced ethanol intake at lower doses. A main effect of dose at 30 min and 2 h [$F(3,63)=12.08, p<0.0001$ and $F(3,63)=9.97, p<0.0001$, respectively] showed that females consumed significantly less ethanol after all doses tested (1, 3, and 10 mg/kg). Ethanol intake in females was still significantly suppressed after 3 and 10 mg/kg at 24 h. Similar to males, water intake was likewise reduced after injections of SR 141716A (Figure 4, right panels).

Given that the ethanol drinking data suggest sex differences in pharmacological sensitivity to SR 141716A, a percent of control (100%) statistical analysis was used to facilitate direct sex comparisons in ethanol intake (Figure 5). Bonferroni’s tests were then performed to determine the locus of effects relative to control in males and females while correcting for multiple comparisons. At the 30 min time point, a main effect of dose [$F(3,72)=16.94, p<0.0001$] and a sex X dose interaction [$F(3,72)=3.71, p<0.05$] emerged. Although the 1 mg/kg dose was ineffective at reducing ethanol intake in males, it did

suppress consumption in females, and the 3 and 10 mg/kg doses similarly reduced intake in both sexes (Figure 5, left panel). At the 2 h time point, a main effect of dose emerged [$F(3,72)=28.10$, $p<0.0001$]. Significant reductions in ethanol intake were observed after 3 and 10 mg/kg in males. All doses tested reduced ethanol intake in females at the 2 h time point (Figure 5, middle panel). The analysis of ethanol intake at 24 h revealed a main effect of dose [$F(3,72)=14.05$, $p<0.0001$], with males showing reductions at 3 and 10 mg/kg whereas intake in females was still significantly suppressed at all doses tested. There was also a trend for females to consume less ethanol than males 24h after administration of SR 141716A (trend for main effect of sex: $p=0.07$) (Figure 65, right).

3.5. Acute SR 141716A Does Not Alter Blood Ethanol Concentrations

Bloods were taken periodically throughout the 10 day CIE exposure. In males, BECs on days 3, 7, and 10 averaged 188.26 ± 3.99 , 219.03 ± 7.02 and 203.5 ± 15.08 . In females, BECs averaged 200.71 ± 20.12 , 260.21 ± 29.36 and 260.10 ± 29.34 . BECs on the morning of the last day of CIE vapor exposure (i.e., day 10) were not significantly different between males and females ($p=0.11$). On the Monday after the last SR 141716A injection, rats were given a week of intermittent access (M, W, F) to 20% ethanol and blood samples were collected that Friday immediately following the drinking session. BECs were significantly correlated with ethanol consumption after 30 min of ethanol access in both males ($r=0.84$, $p<0.01$) and females ($r=0.77$, $p<0.05$) (Figure 6).

At the completion of the drinking study, male and female rats were given an injection of SR 141716A (1 mg/kg) 30 min prior to an ethanol challenge (1 g/kg, i.p.) to determine if SR 141716A altered ethanol metabolism. The results showed no significant differences in BECs after an ethanol challenge injection between vehicle (114.55 ± 11.26) and SR 141716A (102.13 ± 6.58)-injected animals in either sex (ns main effect of dose: $p=0.18$). Interestingly, males had significantly lower BECs than females (73.11 ± 10.99 and 131.51 ± 3.29 , respectively), regardless of SR 141716A injection challenge [main effect of sex: $F(1,20)=59.33$, $p<0.0001$].

4. Discussion

The current experiments explored the effects of CIE vapor exposure on subsequent ethanol intake and anxiety-like behavior in male and female rats. The results show that, while 10 days of CIE exposure was sufficient to induce a withdrawal phenotype in both sexes as evidenced by an increase in anxiety-like behaviors on the elevated plus maze, only males demonstrated an increase in ethanol intake after ethanol exposure in this dependence model. Another goal was to pharmacologically test the 2-bottle choice drinking paradigm with a compound reliably shown to reduce ethanol consumption under a variety of drinking conditions in male rodents (i.e., SR 141716A). Our results indicate that, as expected, male rats showed a reduction in ethanol consumption after administration of SR 141716A at 3 and 10 mg/kg. In females, however, ethanol intake was significantly reduced at these same time points following all doses tested (e.g., 1, 3, and 10 mg/kg). We also demonstrated that this reduction in ethanol consumption—while not an ethanol-specific effect—was not due to SR

141716A's ability to alter the pharmacokinetics of ethanol, as BECs did not differ between vehicle or SR 141716A animals after an ethanol challenge injection.

The baseline drinking data obtained in the current experiment complement those previously reported in male Long-Evans rats exposed to several drinking sessions with access to 20% ethanol in a 2-bottle choice paradigm (Carnicella *et al.*, 2008, Meyer *et al.*, 2013, Simms *et al.*, 2008). For instance, males significantly escalated their ethanol intake from week 1 (3.0 g/kg/24 h) to week 5 (4.7 g/kg/24 h), which was mimicked by their increased ethanol preference across time. Females, however, did not show an escalation in their drinking, instead, they showed a slight (but non-significant) reduction from week 1 (5.7 g/kg/24 h) to week 5 (5.1 g/kg/24 h). Given that there are limited studies utilizing an intermittent access to ethanol paradigm in females (Butler *et al.*, 2014, Hwa *et al.*, 2011, Rosenwasser *et al.*, 2014), it is difficult to compare our data to others. However, in a continuous access model, adult males consumed significantly more ethanol by the last (fourth) week of testing whereas an escalation was not observed in adult females—a similar effect was observed for ethanol preference (Bell *et al.*, 2006). Likewise, in a study similar to ours, there was no evidence for escalated ethanol intake in adult female Long-Evans rats exposed to intermittent 20% ethanol (Butler *et al.*, 2014). It is possible that females cannot escalate their intake across sessions like males reliably do because females are already drinking at maximal levels. For instance, several groups have documented 24 h ethanol intake in adult, female Long-Evans rats and show that their average intake is approximately 5 g/kg (Butler *et al.*, 2014, Ford *et al.*, 2002a). However, female rats selectively bred for high alcohol consumption (e.g., alcohol preferring [P] and high alcohol drinking [HAD]) can (and do) consume up to 9 g/kg/24 h; although, similar to our findings and what others have reported (Butler *et al.*, 2014) using Long-Evans rats, an escalation across drinking sessions did not occur in females of either high consuming line with intermittent access to 10% ethanol (Rosenwasser *et al.*, 2014). Yet, there was a sex-specific increase in ethanol preference across time, with males demonstrating an increase in 24 h ethanol preference compared to females (Rosenwasser *et al.*, 2014)—an effect that was similar to the current experiment (i.e., males showed an increase in ethanol preference across time at the 24 h time point whereas females did not). It is likely that this increase in ethanol intake and preference across drinking sessions in males was facilitated by a concomitant decline in their water intake, which was less dramatic in females (Figure 2). While it has been established that the 20% intermittent access paradigm engenders an escalation in ethanol consumption in males (Simms *et al.*, 2008), even in studies directly comparing intermittent to continuous access paradigms (Mill *et al.*, 2013), others comparing these groups have shown escalations in intake across time in high alcohol consuming rat lines under continuous access conditions as well (Rosenwasser *et al.*, 2014) and escalations in preference under both drinking paradigms. Importantly, our data further support the observation that males escalate ethanol intake and preference in the intermittent access paradigm. However, we cannot definitively state that this escalation is specific to the intermittent ethanol paradigm given that we did not include a continuous access comparison group.

In the current experiment, we employed an ethanol dependence model consisting of a relatively short 10 day CIE vapor exposure. It is noteworthy that longer exposures have been

used in similar dependence models (Finn *et al.*, 2007, O'Dell *et al.*, 2004, Rimondini *et al.*, 2002, Valdez *et al.*, 2002). Despite this shorter duration, our data are consistent with the CIE exposure producing a dependence-like state given the BECs during CIE exposure and elevated anxiety-like behavior evident during withdrawal (Gilpin *et al.*, 2008). The BECs during our 10 day CIE exposure (175–250 mg/dl) were very similar to other reports using longer exposures (150–250mg/dl) (Rimondini *et al.*, 2002, Valdez *et al.*, 2002). Furthermore, BECs in this range typically result in physical signs of withdrawal (e.g., tail stiffness, abnormal gait) which persist for up to 72 h post-withdrawal (Macey *et al.*, 1996). Although somatic signs of withdrawal do dissipate within a few days of ethanol termination (Gilpin *et al.*, 2008), evidence for elevated anxiety-like behavior is observed both during acute withdrawal (Lack *et al.*, 2007) and after protracted periods of several weeks (Rasmussen *et al.*, 2001, Valdez *et al.*, 2002). Increased anxiety-like behavior upon termination of chronic ethanol exposure is a commonly used marker for dependence in rodent models given the strong relationship between alcohol relapse and anxiety in humans (Driessen *et al.*, 2001, Schellekens *et al.*, 2015). Therefore, while our CIE duration was shorter, our BECs and behavioral results strongly suggest it was sufficient to produce a dependence-like state.

Preclinical dependence models have consistently shown that animals will increase their alcohol intake after chronic ethanol exposure (O'Dell *et al.*, 2004, Rimondini *et al.*, 2002, Valdez *et al.*, 2002). Our results support these previous reports in males and also demonstrate that a shorter CIE exposure regimen of 10 days is sufficient to increase ethanol consumption in males, but not females (Figure 3). Females given the same exposure showed increases in anxiety-like behavior on the elevated plus maze. It is thus unlikely that females failed to show a dependence-induced increase in ethanol consumption because they were somehow resistant to the chronic ethanol exposure. Unfortunately, research exploring the effects of ethanol dependence on subsequent drinking in females is generally lacking. One study found a five-fold increase in ethanol consumption after 4 weeks of ethanol vapor exposure in female Wistar rats whereas males showed an almost 2.5-fold increase in ethanol intake (Aufreire *et al.*, 1997). Therefore, it is possible that our shorter CIE exposure simply revealed a differential sensitivity to 'dependence' duration with respect to subsequent ethanol consumption. This is particularly interesting given that both sexes expressed significant withdrawal-associated anxiety following our shorter CIE. Another possibility for the lack of effect of ethanol dependence on increased drinking in females may be due to estrous cycle. Estrous cycle was not collected in the current experiment in order to maintain similar stress/environmental exposures between males and females and reduce the likelihood of stress effects on ethanol intake in one sex. Regardless, others have shown that chronic ethanol exposure (2–8 weeks) does indeed disrupt estrous cycle in females (Emanuele *et al.*, 2001, Sanchis *et al.*, 1985). Importantly for our findings, estrous cycle can modulate the microstructure of ethanol drinking behavior (e.g., bout size and frequency) but does not appear to dramatically affect total ethanol intake (Ford *et al.*, 2002b). Therefore, while estrous cycle was not examined, it is unlikely that total ethanol intake in females in our study would have been affected in a cycle-dependent manner, even if our shorter 10 day CIE exposure was sufficient to disrupt estrous cycle.

The intermittent 2-bottle choice to 20% ethanol paradigm has been pharmacologically validated with drugs that target the opioid, glutamate, and GABA systems, and reductions in ethanol intake have been observed following administration of these system-specific compounds (Li *et al.*, 2011, Simms *et al.*, 2008). We therefore decided to expand upon this literature by pharmacologically validating this drinking paradigm using the cannabinoid CB1 receptor antagonist, SR 141716A, and including males and females. Although SR 141716A does not appear to be a promising pharmacotherapy in humans (George *et al.*, 2010, Soyka *et al.*, 2008), it does consistently reduce ethanol consumption in rodents (Arnone *et al.*, 1997, Colombo *et al.*, 1998, Colombo *et al.*, 2007, Dyr *et al.*, 2008), which supports a role for endocannabinoids in mediating alcohol consumption possibly by altering its reinforcing properties (Cohen *et al.*, 2002, Hungund *et al.*, 2003).

As expected, we found that SR 141716A administration 30 min prior to bottle placement resulted in significantly reduced levels of ethanol intake in males and females; however, we also observed that water intake was likewise reduced after administration of 3 and 10 mg/kg. Others have also reported non-ethanol specific effects of SR 141716A on water, sucrose, and food intake (Arnone *et al.*, 1997, Colombo *et al.*, 1998, Freedland *et al.*, 2001); although, this is not always observed (Robinson and McCool, 2015). While these data suggest that SR 141716A may have general suppressant effects on fluid consumption, the results from the current study do show a stronger effect on ethanol intake than water, particularly in females. For instance, at the 2 h time point (where SR 141716A reduced water and ethanol consumption in both sexes), intake (% control) by males following 1, 3, and 10 mg/kg SR 141716A was 88.29%, 87.66%, and 63.86% for ethanol whereas it was 86.20%, 96.86%, and 77.60% for water. Similarly, SR 141716A dose-dependently reduced ethanol to a greater extent than water in females after 1, 3, and 10 mg/kg: ethanol: 70.89%, 69.86%, and 65.42%, water: 93.36%, 75.55%, and 114.71%. It is also possible that SR 141716A-mediated intake reductions might be due to general locomotor suppressing effects. Although not tested in the current study, our laboratory has reported that 3 mg/kg SR 141716A does not alter locomotor-related behaviors, latency to first lick or latency to first lever response in an operant self-administration paradigm (Robinson and McCool, 2015). Higher doses like 10 mg/kg SR 141716A have been shown to reduce locomotor activity (horizontal beam breaks) (Craft *et al.*, 2012). Thus, while it is possible that decreased locomotor activity may have contributed to the reductions in fluid consumption at the highest dose, it is unlikely that it caused a reduction in consumption at the lower doses tested.

Finally, the results from our ethanol metabolism experiment suggest that the reductions in ethanol intake were not due to SR 141716A's effects on pharmacokinetics because we did not observe differences in BECs between vehicle- and SR 141716A-injected animals (see "Results" section 3.5). While we only examined one time point, and in animals with a history of ethanol exposure, others have reported (in ethanol-naïve Wistar rats) that BECs did not differ as a function of SR 141716A administration across several time points (30–240 min post-ethanol administration) (Colombo *et al.*, 1998).

Importantly, we found significant sex differences in sensitivity to SR 141716A on ethanol intake. After 1 mg/kg, females consumed significantly less ethanol than males at the 30 min

time point. There was also a trend ($p=0.07$) for females to consume less ethanol than males at the 24 h time point. In adult animals, sex hormones—particularly estradiol—exert a strong influence over the endocannabinoid system (Lopez, 2010). Although CB1 receptor density reaches similar levels between adult males and females, sex differences are evident during the preweanling and early adolescent period (Craft *et al.*, 2013). Not surprisingly, sex differences in body fat composition may also modulate the effects of the hydrophobic drug depending on the percentage of body fat between males and females (Fattore and Fratta, 2010). Therefore, it is possible for female rats to be more sensitive to pharmacological manipulation of the CB1 receptor because, unlike primates, they have less body fat (%) than male rats (Cortright *et al.*, 1997)., SR 141716A potency is greater in females than males when administered systemically (Craft *et al.*, 2012), suggesting higher binding affinity for cannabinoid drugs with CB1 receptors in females. To our knowledge, this is the first preclinical study to examine the effects of SR 141716A on ethanol intake in both females and males simultaneously. Furthermore, while females in the current study demonstrated longer-lasting effects (i.e., into the 24 h time point) of SR 141716A on ethanol intake compared to males, research exploring sex differences in pharmacokinetics (at least at lower doses) might be beneficial for the continued use of this compound in males and females. For example, one study using FAAH (fatty acid amide hydrolase) knockout mice—which rapidly degrades CB endogenous ligands, anandamide N-arachidonylethanolamide and 2-arachidonylglycerol—showed that female knockouts consumed significantly more 12% and 20% ethanol than did wild-type controls, whereas no effect was found in males (Basavarajappa *et al.*, 2006). Unlike males, female knockout mice in this study were also less impaired by the acute intoxicating effects of ethanol which may have contributed to the elevated ethanol consumption seen in these mutant mice. These data together suggest that males and females do differ in their sensitivity to manipulation of the cannabinoid system with regards to ethanol consumption and ethanol-mediated behaviors. Future experiments exploring the effects of CB1 receptor antagonism should include female rodents to determine if differences in sensitivity do exist in terms of mediating ethanol-related behaviors and consumption.

5. Conclusions

Overall, these results support and extend previous reports of CIE-dependent drinking in males. Males in the current study showed an escalation in ethanol intake (24 h) across drinking sessions. We also confirmed that male rodents exposed to a CIE ethanol vapor inhalation dependence model express an anxious phenotype when tested 24 h into withdrawal. Interestingly, while others have consistently reported that several weeks and months of CIE exposure increases ethanol drinking in males, we saw significant increases in ethanol consumption after just 10 days of CIE exposure. We have shown that 10 days of CIE exposure is sufficient to alter glutamatergic transmission compared to air-exposed control animals (Christian *et al.*, 2013, Christian *et al.*, 2012) and to produce anxiety-like behavior (current experiment, Lack *et al.*, 2007). This suggests that the neurobiological alterations that ultimately contribute to the transition from ethanol use to dependence, like increased alcohol consumption, develop over a relatively short exposure timeframe in males. Conversely, while females in the current experiment showed behavioral signs consistent

with ethanol dependence as evidenced by withdrawal-related behaviors on the elevated plus maze, ethanol consumption was not affected. It is thus possible that females may require a longer duration of CIE exposure to enhance ethanol drinking but not withdrawal anxiety. Future studies should examine these sex-specific effects in more detail. Finally, we replicated data in males showing that CB1 receptor antagonism with SR 141716A reduces ethanol (and water) intake; however, we showed these effects using the 2-bottle choice to 20% ethanol paradigm. Interestingly, females appeared to be sensitive to lower doses of the CB1 receptor antagonist using under these test conditions. It would be beneficial for future studies to identify the contributing factors influencing differences in ethanol intake and possibly other ethanol-related behaviors in males and females after antagonism of the CB1 receptor.

The observed sex differences in pharmacological blockade of the CB1 receptor on ethanol consumption coupled with differences in escalation and dependence-induced drinking potentially illustrate the complexity for developing treatments in males and females at various stages of the alcohol dependence/consumption cycle (e.g., the transition between “regular” drinking to development of an AUD). For instance, while men tend to drink more alcohol and are almost twice as likely to develop an AUD compared to women (SAMHSA, 2013), the data on the prevalence of transition from social drinking to alcohol dependence between men and women are mixed. For instance, there is evidence demonstrating that while women may initiate drinking at a later age, their transition to alcohol dependence is shorter compared to men. However, others have shown no significant difference in transition time to dependence between women and men (Alvanzo *et al.*, 2011, Randall *et al.*, 1999, Schuckit *et al.*, 1998, Zilberman *et al.*, 2003). The discrepant findings on sex differences in the transition to AUDs along with the clear sex differences that exist in both drinking patterns and AUD prevalence demonstrate a strong need for pre- and clinical research and screening/treatment options that focus specifically on both men and women. Our current study in a rodent model adds to the existing preclinical literature demonstrating that adult females consume more ethanol than males under a variety of experimental drinking paradigms (Lancaster *et al.*, 1996, Lancaster and Spiegel, 1992, Vetter *et al.*, 2007). By additionally examining post-dependence drinking, we found that, unlike males, female drinking was unaffected by 10 days of CIE vapor exposure despite enhanced withdrawal-anxiety expressed in both sexes. While the alcohol field has yielded crucial findings on the relationship among ethanol dependence, withdrawal, and drinking in males, our preclinical findings support the complexity of these relationships when both sexes are examined.

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Figure 1.

Withdrawal from CIE exposure increases anxiety-like behaviors regardless of sex. Effect of the ethanol dependence model on anxiety-like behavior was assessed on the elevated plus maze in male (left panels) and female (right panels) rats. Bar graphs represent time spent (%) and number of entries (%) into the open arms of the elevated plus maze. Data are presented as mean $\pm$ SEM. Asterisk (*) indicates a significant difference between CON (n=5) and WD (n=5) conditions, $p < 0.05$, *a* indicates approaching significance ($p = 0.06$).

Figure 2.

Males escalate ethanol consumption, but females do not, in an intermittent access drinking model. Average weekly ethanol intake (g/kg, top row) preference (% , middle row) and water intake (ml/kg, bottom row) are shown for the 30 min (left column), 2 h (middle column), and 24 h (right column) time points in male (n=15) and female (n=16) rats. Line graphs represent male and female intake patterns across the 5 baseline weeks. @ indicates a significant sex difference. † indicates a significant difference from week one in males only, ^ in females only, or ‡ for a significant difference from week one in males and females, $p < 0.05$.

Figure 3.

Chronic ethanol exposure in an intermittent vapor inhalation dependence procedure enhances ethanol drinking in male rats but not in females. Bar graphs represent mean ethanol intake (g/kg) before (pre) and after (post) 10 days of CIE or air-exposure (CON) in male (top panels; $n=7-8$ /condition) and female (bottom panels; $n=8$ /condition) rats. * indicates a significant difference at $p<0.05$.

Figure 4.

The CB1 receptor antagonist SR 141716A differentially regulates ethanol (g/kg) and water (ml/kg) intake in male (left) (n=15) and female (right) (n=16) rats. # indicates a significant difference from vehicle, $p < 0.05$.

Figure 5.

SR 141716A decreases ethanol intake, expressed as percent of control, at lower doses in female rats compared to male rats. Bar graphs represent mean ethanol intake at the 30min (left), 2h (middle), and 24h (right) time points in males (n=15) and females (n=16). @ indicates a significant sex difference, # indicates a significant difference from vehicle, p<0.05.

Figure 6.

Blood ethanol concentrations (BEC) after a 30 min drinking session were closely correlated with ethanol intake in both male (n=8) and female (n=8) rats. Pearson r correlations in males ($r=0.84, p<0.01$) and females ($r=0.77, p<0.05$).