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Ovarian Hormones Contribute to High Levels of Binge-Like Drinking by Female Mice

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Abstract

Background—Recently, the incidence of binge drinking by women has increased. Binge drinking is detrimental to women's health, yet the biological mechanisms that promote excessive drinking by women are not well understood. One method of assessing binge-like ethanol (EtOH) consumption in mice is the drinking in the dark (DID) test, in which mice drink sufficient EtOH to achieve intoxication. In this study, we directly compared male, female, and ovariectomized (OVX) mice for DID and tested whether 17β-estradiol (E2) contributes to DID. We also measured whether DID varies throughout the estrous cycle and if repeated intermittent DID impacts the estrous cycle.

Methods—Male, female, and OVX C57BL/6J mice were tested for DID for 2 hours per day on days 1–3 and for 4 hours on day 4 using a single bottle containing 20% EtOH. To measure the effects of E2 on DID, OVX mice were treated with estradiol benzoate (EB) or vehicle daily starting two weeks prior to the drinking test and throughout the DID procedure. In a separate group of experiments, EtOH consumption and estrous cycle phase were measured in freely cycling mice that were drinking EtOH or water 5 days per week for 2 or 6 weeks.

Results—Female mice consumed more EtOH than male and OVX mice. Treatment with EB increased EtOH consumption by OVX mice compared with vehicle-treated controls. However, EtOH intake did not vary across the estrous cycle, nor did long-term DID alter the estrous cycle.

Conclusions—These results demonstrate that ovarian hormones, specifically E2, contribute to increased EtOH consumption by female mice in the DID test. Although ovarian hormones contribute to this behavior, EtOH consumption is not affected by estrous cycle phase in freely cycling mice. This study provides a framework for understanding the factors that contribute to binge drinking in females.

Keywords

alcohol; binge drinking; female; estrogen; sex differences

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INTRODUCTION

Sex differences in the incidence of alcohol use disorder (AUD) have been attributed to both social and biological factors (Becker and Koob, 2016). Though historically men have been more likely than women to drink alcohol and exhibit problematic drinking behavior, recent epidemiological studies of alcohol consumption by different birth year cohorts indicate that this gender gap is narrowing. The percentage of women who binge drink has increased in cohorts born more recently (Keyes et al., 2008, White et al., 2015). These data are particularly concerning given that excessive drinking in women is associated with higher rates of alcoholism-associated diseases such as alcoholic hepatitis, cardiomyopathy and cancers (Wilsnack et al., 2013). Furthermore, women show increased vulnerability to ethanol (EtOH)-induced neurotoxicity and more severe cognitive impairment associated with alcohol abuse (Hashimoto and Wiren, 2008, Wilhelm et al., 2016). It is therefore important to determine the biological factors that contribute to excessive drinking in both sexes in order to find methods of reducing alcohol consumption that will be effective in women and men.

Rodents are commonly used to determine the biological and genetic factors that contribute to excessive drinking. Female rodents consume more EtOH than males in several EtOH consumption tests (Rhodes et al., 2007, Jury et al., 2017, Priddy et al., 2017, Becker and Koob, 2016). One biological factor that may contribute to increased EtOH consumption by females is the presence of circulating ovarian hormones. In the four core genotype mouse model, in which sex chromosome complement and gonadal phenotype have been separated, alcohol drinking is predicted by gonadal phenotype, with gonadal females consuming more alcohol than gonadal males (Barker et al., 2010). Additionally, removal of the ovaries (ovariectomy, OVX) in rats and mice can reduce EtOH consumption (Ford et al., 2002a, Forger and Morin, 1982, Becker et al., 1985), although others have found that this is not the case (Almeida et al., 1998, Vetter-O'Hagen and Spear, 2011). The role of the estrous cycle in altering EtOH consumption is also equivocal. Several studies have found that alcohol consumption is not altered during the estrous cycle in freely cycling rats (Ford et al., 2002b, Priddy et al., 2017, Roberts et al., 1998), although estrous cycle phase does affect operant responding for EtOH in rats whose cycles have been synchronized by treatment with a gonadotropin-releasing hormone receptor agonist (Roberts et al., 1998).

The main circulating form of estrogen in premenopausal females, 17β-estradiol (E2), is likely one hormone that contributes to high levels of EtOH drinking in female rodents. Numerous studies have demonstrated that administration of E2 to rats and mice increases EtOH consumption (Ford et al., 2004, Quirarte et al., 2007, Ford et al., 2002a, Marinelli et al., 2003, Rajasingh et al., 2007, Reid et al., 2002, Reid et al., 2003), although E2 administration has also been shown to decrease EtOH intake (Almeida et al., 1998, Hilakivi-Clarke, 1996, Sandberg et al., 1982, Sandberg and Stewart, 1982). Interestingly, in clinical studies, higher circulating levels of E2 in women are positively correlated with alcohol intake and an association has been found between oral contraceptive use and alcohol consumption (Martin et al., 1999, Muti et al., 1998, Lund and Jacobsen, 1990). Overall, human and animal studies implicate E2 in modulating alcohol drinking.

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One test for excessive EtOH consumption in mice is drinking in the dark (DID), in which mice are given limited access to EtOH during the dark cycle. In this procedure, mice will routinely drink to intoxication and achieve blood EtOH concentrations (BECs) greater than 100 mg % (Rhodes et al., 2005). Because of the high levels of drinking achieved and pharmacologically relevant BECs obtained, the DID test has become a popular measure of binge-like drinking in mice and is commonly used to test for effects of genetic and pharmacological manipulations on this behavior (Thiele and Navarro, 2014). Female mice drink more EtOH than males in the DID test (Rhodes et al., 2007), similar to other EtOH consumption models, but it is not known whether ovarian hormones or estrous cycle phase alter DID. The goals of this study were to directly compare male and female mice in the DID test, determine if OVX and E2 treatment affect DID, and test whether EtOH drinking changes during different phases of the estrous cycle. We also monitored the estrous cycle during a prolonged DID experiment to determine whether regular bouts of binge-like drinking affect estrous cycle pattern. Our data suggest that ovarian hormones in mice, particularly E2, are responsible for the increased binge-like consumption of EtOH by females compared with males. However, binge-like drinking does not change throughout the estrous cycle in freely cycling mice. The results presented here provide useful information for researchers studying sex differences in AUD and will assist with both experimental design and interpretation of data when sex is included as a biological variable in mouse models of excessive alcohol consumption.

MATERIALS AND METHODS

Animals

C57BL/6J male and female mice (8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME USA) and were single-housed upon arrival. Mice were maintained on a 12-hour reversed light/dark cycle (lights off at 10 am) for the duration of the study. All procedures with animals were conducted according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

Measurement of Estrous Cycle Phase and OVX

Vaginal smears were collected daily and analyzed using a light microscope to identify the phase of the estrous cycle as previously described (Byers et al., 2012). Vaginal smears were collected 4 hours prior to testing drinking (one hour before lights off). Estrous cycle duration was calculated as the number of days between 2 consecutive proestrus phases.

To perform OVX, mice were anesthetized with an intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (8 mg/kg). Lateral incisions were made on the back, parallel to the spine, to expose the abdominal cavity. The ovaries were isolated and dissected away from the uterine horn by cauterization and the uterine horn was placed back into the abdominal cavity. Incisions were closed with sterile sutures and wound clips. Mice received one injection of meloxicam (2 mg/kg, subcutaneously, SC) immediately after surgery, and a second injection the following day. Cessation of the estrous cycle was confirmed by analysis

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of vaginal smears using bright field microscopy for 4–5 days starting 10 days after surgery. Mice were allowed to recover for 2–3 weeks prior to beginning experimental procedures.

E2 Administration

17β-Estradiol-3-benzoate (EB) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in sesame oil vehicle at a concentration of 4 ng/μL and administered to mice by SC injection in a volume of 50 μl to give a dose of 0.2 μg per mouse (~10 μg/kg). Beginning three weeks after OVX, EB was administered once daily, for a period of 2 weeks prior to testing DID, and every day during the DID procedure. On drinking days, EB was given 4 hours before each drinking session.

Drinking in the Dark (DID)

Mice were individually housed for at least 2 weeks prior to the DID procedure. DID was conducted with a single bottle of 20% EtOH, as described previously (Dutton et al., 2017, Rhodes et al., 2007). Briefly, on days 1–3, water bottles were replaced with sipper tubes containing a 20% EtOH solution (v/v in water) for 2 hours, starting 3 hours into the dark phase. Volumes of EtOH consumed were measured at the end of each drinking session. On day 4, mice were allowed to drink EtOH for 4 hours, and volumes consumed were measured after 2 and 4 hours. In Figure 1, 16 mice per group were tested. In Figure 2, 11 OVX and 10 EB-treated mice were tested. In a separate group of gonadally intact mice (10 mice), EtOH drinking was assessed during different phases of the estrous cycle (Figure 3*A*–*E*). In the same experiment, the effect of EtOH drinking on the progression through the estrous cycle was measured (Figure 4). In these experiments, mice were allowed to drink 20% EtOH (or water, as a control) for 2 hours a day, 5 days per week for 6 weeks. Finally, EtOH drinking was also assessed in another group of mice (n=8) for 4 hours per day, 5 days per week for 2 weeks (Figure 3*G*–*F*).

Measurement of BECs

Blood samples were collected from mice tested in the DID procedure to measure blood EtOH concentrations. Trunk blood was collected immediately after the 4-hour drinking session in a heparinized capillary tube during euthanasia by decapitation. BECs were measured using a NAD-ADH enzymatic essay as previously described (Carnicella et al., 2009).

Statistical Analysis

Data are presented as means ± SEM and were analyzed using Prism 6 (GraphPad Software, La Jolla, CA USA). To analyze the drinking data from the daily 2-hour sessions we used two-way repeated measures (RM) ANOVA followed by *post hoc* Tukey's multiple comparisons test. Drinking data from the 4-hour sessions and BECs were analyzed by Student's *t*-test or one-way ANOVA followed by Tukey's test. Student's *t*-test was used to compare the length of the cycle for EtOH vs. water drinking.

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RESULTS

Females Consume More EtOH in the DID test than Males and OVX Mice

Rhodes *et al* previously demonstrated that female mice of several inbred strains consume more EtOH than males in the DID procedure (Rhodes et al., 2007). To expand on these results and determine if ovarian hormones contribute to increased EtOH consumption by females, we directly compared DID between male, female, and OVX C57BL/6J mice. No significant differences were observed during the 2-hour drinking sessions when comparing these three groups by two-way RM ANOVA (Figure 1*A*, 16 mice per group: group, $F(2, 45) = 1.58$, $p = 0.217$; time: $F(3, 135) = 2.89$, $p = 0.038$; interaction: $F(6, 135) = 1.31$, $p = 0.258$). However, during the 4-hour drinking session on day 4, female mice consumed significantly more EtOH compared with both males and OVX mice (Figure 1*B*, one-way ANOVA: $F(2, 45) = 9.27$, $p = 0.0004$). *Post-hoc* multiple comparisons tests indicated significant differences between gonadally intact females and males ($p = 0.0016$) and between intact and OVX females ($p = 0.0015$), but not between males and OVX mice ($p = 0.999$). We measured BECs in one of the two cohorts tested for DID (8 mice per group). A difference in BECs was also found at the end of the 4-hour drinking session, with OVX mice having significantly lower BECs than males and females (Figure 1*C*; one- way ANOVA: $F(2, 21) = 6.94$, $p = 0.0049$). *Post-hoc* multiple comparisons tests indicated significant differences between OVX and gonadally intact females ($p = 0.0059$), between males and OVX mice ($p = 0.024$), but not between males and intact females ($p = 0.81$). We also measured water consumption a few days prior to performing the DID test and found that water consumption did not differ between males, females, and OVX mice (data not shown). Together, our data indicate that gonadally intact female mice consume more EtOH than males and OVX mice during the 4-hour drinking session, and that there is a pronounced reduction in BECs in OVX mice. These results suggest that ovarian hormones contribute to the higher levels of EtOH consumption observed in females.

E2 Increases Binge-like EtOH Drinking in OVX Mice

To determine if E2 is responsible for the increased EtOH consumption by females, we treated female OVX mice daily with EB (n=10) or sesame oil vehicle (VEH, n=11) and measured DID. The timeline for the experiment is shown in Figure 2*A*. We did not observe a significant difference between groups during the 2-hour drinking sessions (Figure 2*B*, two-way RM ANOVA: treatment, $F(1, 19) = 2.55$, $p = 0.127$; time, $F(3, 57) = 5.01$, $p = 0.0038$; interaction, $F(3, 57) = 0.901$, $p = 0.446$). However, EB-treated mice consumed significantly more EtOH compared with VEH-treated animals during the 4-hour session on day 4 (Figure 2*C*; $t(19) = 3.25$, $p = 0.0042$). Significantly higher BECs were also detected in EB-treated mice at the end of the 4-hour session on day 4 (Figure 2*D*; $t(8) = 4.27$, $p = 0.0027$). Water consumption was measured a few days prior to performing the DID test and did not differ between treatment groups (data not shown). Increased EtOH consumption by EB-treated OVX mice suggests that E2 may be one factor that contributes to the higher levels of EtOH drinking by gonadally intact females compared with males.

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EtOH Intake in the DID Procedure is Not Altered During the Estrous Cycle

Since fluctuations in E2 occur throughout the estrous cycle, with the highest levels observed during late diestrus and proestrus (Nilsson et al., 2015), we next sought to determine if EtOH consumption is altered during the estrous cycle. We tested 10 freely cycling female mice in the DID procedure for 2 hours per day, 5 days per week for 6 weeks in order to obtain sufficient power to detect differences at each of the four stages of the estrous cycle. Representative images of vaginal cellular composition are shown in Figure 3*A–D*. EtOH consumption did not change during the different phases of the cycle (Figure 3*E*, one-way ANOVA: $F(3, 214) = 0.286$, $p = 0.836$). Because we observed a significant increase in ethanol drinking by OVX mice treated with EB after 4 hours, but not 2 hours of DID (Figure 2), we performed a second, independent DID experiment in naturally cycling mice (n = 8) given access to EtOH for 4 hours daily for 5 days per week for a period of two weeks. Similar to what we observed in the 2 hour drinking sessions, ethanol intake during 4 hours was not altered by estrous cycle phase (Figure 3*F*, one-way ANOVA: $F(3, 76) = 1.58$, $p = 0.201$), nor did ethanol consumption differ by estrous cycle phase in the last 2 hours of the drinking session (Figure 3*G*, one-way ANOVA: F (3, 76) = 2.03, $p = 0.117$). Together these data indicate that the hormonal fluctuations occurring during the estrous cycle in naturally cycling mice are not sufficient to affect EtOH intake in the DID procedure.

EtOH Drinking During DID Does Not Affect the Length and Pattern of the Estrous Cycle

Chronic EtOH consumption disrupts the estrous cycle in rats (Emanuele et al., 2001). To determine if long-term binge-like EtOH consumption affects the estrous cycle in mice, we first compared the duration of the estrous cycle in mice drinking 20% EtOH (n=10) or water as a control (n=10) for 2 hours a day, 5 days a week for 6 weeks. Estrous cycle length did not differ between the EtOH- and water-drinking groups (Figure 4*A*, $t(51) = 0.72$, $p = 0.47$). The average cycle length in the water-drinking group was 4.39 ± 0.13 days, while the length of the cycle in the EtOH-drinking group was 4.52 ± 0.13 days. We next examined the percentage of time spent in estrus and diestrus between the EtOH- and water-drinking groups and found that the percentage of days in diestrus and estrus did not differ between groups (Figure 4*B*). The water-drinking group spent 47.9% of days in estrus and 22.9% of days in diestrus, while the EtOH-drinking group spent 48.9% of days in estrus and 23.2% of days in diestrus. These percentages are similar to those reported in another study of young adult C57BL/6 mice (Caligioni, 2009). Together, these data suggest that EtOH drinking in the DID procedure does not alter the pattern of the estrous cycle, even after long-term drinking.

DISCUSSION

We demonstrate here that adult C57BL/6J mice exhibit sex differences in EtOH consumption in the DID test, with females drinking significantly more alcohol than males during a 4-hour session. Rhodes et al also observed an increase in EtOH consumption by C57BL/6J females compared with males in the DID test, although the results were not statistically significant (Rhodes et al., 2007). Female mice drink more EtOH than males in other types of limited-access EtOH consumption procedures (Strong et al., 2010, Cozzoli et al., 2014, Zhou et al., 2017, Middaugh and Kelley, 1999), and numerous studies have shown

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that female mice drink more EtOH than male mice in a two-bottle choice test (Jury et al., 2017, Middaugh et al., 1999, Meliska et al., 1995). This phenomenon is not limited to mice, as female rats also drink more than males in different models of alcohol drinking (Lancaster et al., 1996, Priddy et al., 2017, Lancaster and Spiegel, 1992, Li and Lumeng, 1984, Moore and Lynch, 2015).

Interestingly, we found significant differences in EtOH consumption between females and males only after the 4-hour drinking session on the fourth day of drinking, whereas EtOH consumption after the 2-hour drinking sessions on the first three days was not significantly different. These data suggest that sex differences in EtOH drinking manifest after longer access to EtOH. Others have found that sex differences in EtOH consumption depend on the length of the drinking session, with females drinking more than males during longer periods of access to EtOH (Middaugh et al., 1999, Strong et al., 2010). It is possible that the pattern of drinking in males and females differs such that the male mice consume more EtOH during the early part of the session and drink only enough to maintain intoxicating BECs for the remainder of the session, whereas female mice may continue to consume large amounts of EtOH throughout the entire drinking session. For example, studies of the microstructure of DID behavior in C57BL/6J mice showed that both male and female mice “front load” EtOH during the first 15–30 minutes of the drinking session, but males tend to decrease contacts with the ethanol bottle more than females during the next 30 minutes (Wilcox et al., 2014, Rhodes et al., 2007).

A possible explanation for sex differences in EtOH consumption might be altered alcohol pharmacokinetics between male and female rodents. Several studies have shown that alcohol metabolism is faster in female rats and mice (Robinson et al., 2002, Peterson et al., 1991, Kishimoto et al., 2002, Middaugh et al., 1992) and that gastric EtOH absorption may also be more rapid in females (Desroches et al., 1995). Female rodents may consume more EtOH than males in order to maintain intoxicating BECs since they are metabolizing EtOH at a faster rate. This is in line with our data showing that intact female mice drink more EtOH than male mice, but the BECs measured at the end of the drinking session did not differ between the two groups. Interestingly, however, Crippens et al found that while female rats eliminated EtOH from the blood more rapidly than males, the levels of EtOH in the brain did not differ by sex (Crippens et al., 1999). The lack of difference in brain EtOH levels argues that the increased EtOH consumption by female rodents may not solely be due to differences in EtOH pharmacokinetics but possibly some other biological factor(s).

Sex differences in alcohol drinking in rats emerge around puberty, indicating that gonadal hormones may modulate drinking (Lancaster et al., 1996). In addition, EtOH consumption studies using the four core genotype mouse model, which separates gonadal phenotype and chromosome complement, have demonstrated that gonadal phenotype is responsible for increased EtOH consumption by female mice (Barker et al., 2010). Several studies in rats and mice have demonstrated that OVX, which depletes circulating ovarian hormones, reduces alcohol intake to levels similar to that of males (Ford et al., 2002a, Forger and Morin, 1982, Becker et al., 1985). In accordance with these observations, we found that OVX C57BL6/J mice drank less EtOH in the DID test than gonadally intact female mice and that the amount of EtOH intake by OVX mice was comparable to male mice. These results

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suggest that ovarian hormones contribute to the increased EtOH consumption by female C57BL/6J mice. The ability of OVX to reduce EtOH consumption is not universal, however, because others have not found OVX to alter EtOH drinking (Vetter-O'Hagen and Spear, 2011, Almeida et al., 1998, Hilakivi-Clarke, 1996). Reasons for this discrepancy may have to do with several factors, such as the timing of OVX (adolescence vs. adulthood), length of access to EtOH (limited vs. continuous access), correction for baseline levels of EtOH consumption prior to OVX, or strain of rat or mouse used.

In addition to finding that OVX reduced EtOH consumption in the DID test, we observed a dramatic difference in BECs between OVX mice and gonadally intact males and females. OVX mice drank 24% less EtOH compared with gonadally intact females, but exhibited a 64% decrease in BECs. Interestingly, BECs in OVX mice were also lower than in males despite these two groups consuming similar amounts of EtOH. Although BECs correlate with EtOH consumption, they are variable and do not always reflect cumulative intake during a drinking session (Wilcox et al., 2014). In fact, BECs correlate better with EtOH intake after a 2-hour drinking session than a 4-hour session. BECs also show a stronger association with the number of drinking bouts early in the session vs. later in the session (Rhodes et al., 2005, Wilcox et al., 2014). These data suggest that there might be differences in the bout structure of EtOH drinking during the 4-hour session between males, females, and OVX mice. However, differences in EtOH metabolism may also have contributed to the large difference between BECs in OVX females and gonadally intact male and female mice.

One ovarian hormone that contributes to increased EtOH consumption by female rodents is E2. Here, we found that treatment of OVX mice with EB increased binge-like EtOH drinking during the 4-hour session and also resulted in higher BECs. These data suggest that E2 may be the primary ovarian hormone that drives higher levels of binge-like EtOH drinking by female C57BL/6J mice. Our findings agree with several studies in mice and rats demonstrating that E2 treatment increases EtOH intake (Ford et al., 2004, Quirarte et al., 2007, Ford et al., 2002a, Marinelli et al., 2003, Rajasingh et al., 2007, Reid et al., 2002, Reid et al., 2003). However, there are several instances in which E2 treatment decreased EtOH consumption (Almeida et al., 1998, Hilakivi-Clarke, 1996, Sandberg et al., 1982, Sandberg and Stewart, 1982). Reasons for these contradictory findings may have to do with such factors as the dose of E2 used, route of E2 administration, and duration of E2 treatment prior to initiating EtOH drinking. In this study we treated mice with daily EB injections for two weeks prior to testing drinking. In studies where E2 elicited a suppression of EtOH consumption, the suppression was often transient and observed at high doses of E2. The dose of EB that we used in this study (0.2 μg per mouse, ~10 μg/kg) is likely much lower than the dose used in a previous study in CD-1 mice, in which mice were supplemented with subcutaneous 0.25 mg E2 slow-release pellets (Hilakivi-Clarke, 1996). Ingberg et al found that serum E2 levels after implantation with pellets are initially supraphysiological and decline to levels in the high physiological range after a few weeks (Ingberg et al., 2012). We conducted an experiment measuring E2 levels in the serum of OVX mice treated with EB compared with gonadally intact mice in proestrus using a commercially available immunoassay kit. Treatment with 0.2 μg EB resulted in levels of E2 in the serum 4 hours after injection that roughly corresponded to serum levels measured during proestrus, when E2 levels peak (Vandegrift et al., 2017). The other possibility is that the opposite effects of

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E2 in the Hilakivi-Clarke study and our study on EtOH consumption is due to mouse strain differences (CD-1 vs. C57BL/6J) or procedural differences in measuring EtOH consumption (continuous two-bottle choice of 5% EtOH over 7 days vs. DID with 20% EtOH).

Because ovarian hormones appear to contribute to higher levels of EtOH consumption in female mice, we measured whether EtOH intake would vary over different phases of the estrous cycle, since hormonal profiles are different at each phase (Nilsson et al., 2015). We measured EtOH drinking in the DID test over a period of 6 weeks. We found that levels of EtOH intake did not vary with different phases of the estrous cycle in female C57BL/6J mice, suggesting that cyclic hormonal fluctuations are not sufficient to affect binge-like EtOH intake in freely cycling mice. This finding is in agreement with studies in rats (Priddy et al., 2017, Ford et al., 2002b, Roberts et al., 1998), although one study did find that EtOH intake and preference changed over the estrous cycle (Forger and Morin, 1982). Interestingly, however, Roberts et al discovered estrous cycle differences in operant EtOH self-administration in rats whose cycles had been synchronized with gonadotropin-releasing hormone (Roberts et al., 1998), suggesting that under certain conditions estrous cycle effects can be unmasked. The lowest EtOH reinforcers were earned during proestrus and estrus. Consistent with most of the animal experiments, the majority of clinical experiments conducted in women have not discovered alterations in EtOH drinking at different phases of the menstrual cycle (Holdstock and de Wit, 2000, Terner and de Wit, 2006, Pomerleau et al., 1994), although one study found increased alcohol consumption during the luteal phase as measured by self-report diaries (Harvey and Beckman, 1985). In summary, estrous cycle phase may play a subtle, but not major role, in EtOH consumption by rodents.

Studies in rats, monkeys, and humans have found that chronic EtOH drinking alters estrous/ menstrual cycle phase (Emanuele et al., 2001, Emanuele et al., 2002). We wondered whether the pattern of the estrous cycle pattern would be altered in mice during long-term binge-like EtOH consumption using the DID protocol. We found that the pattern of the estrous cycle pattern is not disrupted by 6 weeks of DID, indicating that this type of “chronic” drinking is not extreme enough to elicit changes in the estrous cycle.

Together, the results presented here indicate that ovarian hormones contribute to binge-like drinking in the DID test in C57BL/6J mice, with E2 promoting EtOH drinking. Our results support findings in rats and mice that have been presented in a large body of literature indicating that ovarian hormones, in particular E2, promote high levels of EtOH consumption in various EtOH drinking models. Although limited, the few clinical studies that have been done suggest that high levels of E2 are correlated with increased drinking by women (Martin et al., 1999, Muti et al., 1998, Lund and Jacobsen, 1990). Binge drinking by women is becoming more prevalent, demonstrating the importance of determining the biological underpinnings of EtOH consumption by women. Future studies will determine which of the estrogen receptors are involved in promoting EtOH consumption and the neurocircuitry in which these receptors act.

Mice are commonly used to understand the biological and genetic contributors to AUD and the DID test is an easily employed model of binge-like drinking by mice. Our data indicates that although ovarian hormones contribute to binge-like drinking by female mice, the level

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of EtOH intake in the DID test does not significantly vary across the estrous cycle. This knowledge greatly simplifies experimental design when comparing sexes in the DID test, since estrous cycle need not necessarily be tracked. Inclusion of females in drinking studies will greatly facilitate understanding of the molecular mechanisms that drive excessive drinking in both sexes.

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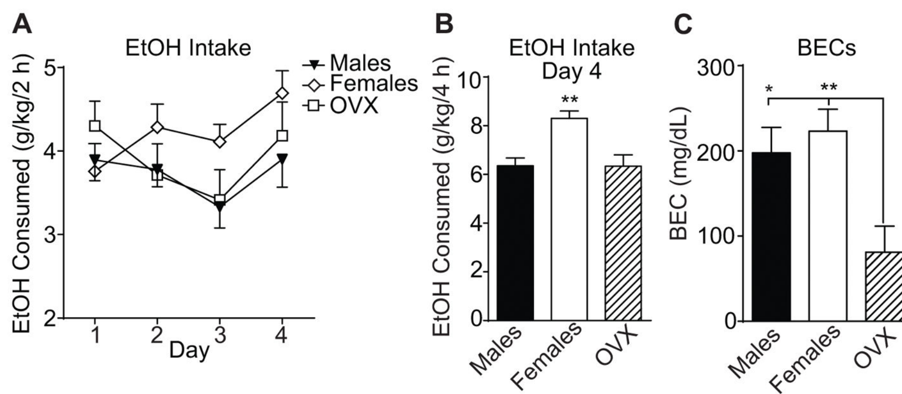

Fig. 1.

Females consume more ethanol (EtOH) in the drinking in the dark test than males and ovariectomized (OVX) mice. (**A**) EtOH consumed (g/kg) during 2-hour drinking sessions on days 1–4. (**B**) EtOH intake (g/kg) during the 4-hour session on day 4. $^{**}p < 0.01$ between males and females and between females and OVX, n = 16 per group (**C**) Blood EtOH concentrations (BECs, mg/dL) at the end of the 4-hour drinking session on day 4, n = 8 per group. $^{*}p < 0.05$ between males and OVX and $^{**}p < 0.01$ between females and OVX. Data are presented as mean ± SEM.

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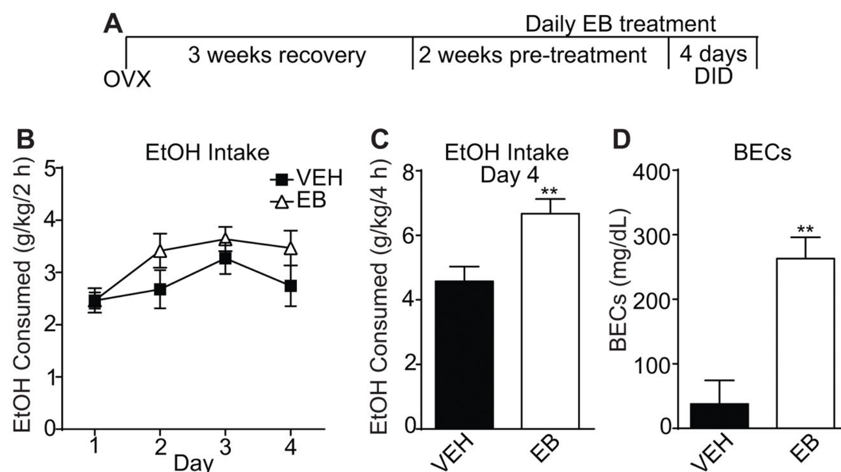

Fig. 2.
17β-estradiol benzoate (EB) increases binge-like EtOH drinking in ovariectomized (OVX) mice. (**A**) Timeline of the experiment. EB injections were given daily for two weeks prior to testing drinking and before each drinking session (**B**) EtOH intake (g/kg) during each 2-hour drinking session from days 1–4 in OVX mice treated with vehicle (VEH) or EB. (**C**) EtOH consumed during the 4-hour session on day 4, n = 10–11 per group. (**D**) Blood EtOH concentrations (BECs, mg/dL) after the 4-hour session on day 4, n = 4–6 per group. **$p < 0.01$. Data are presented as mean ± SEM.

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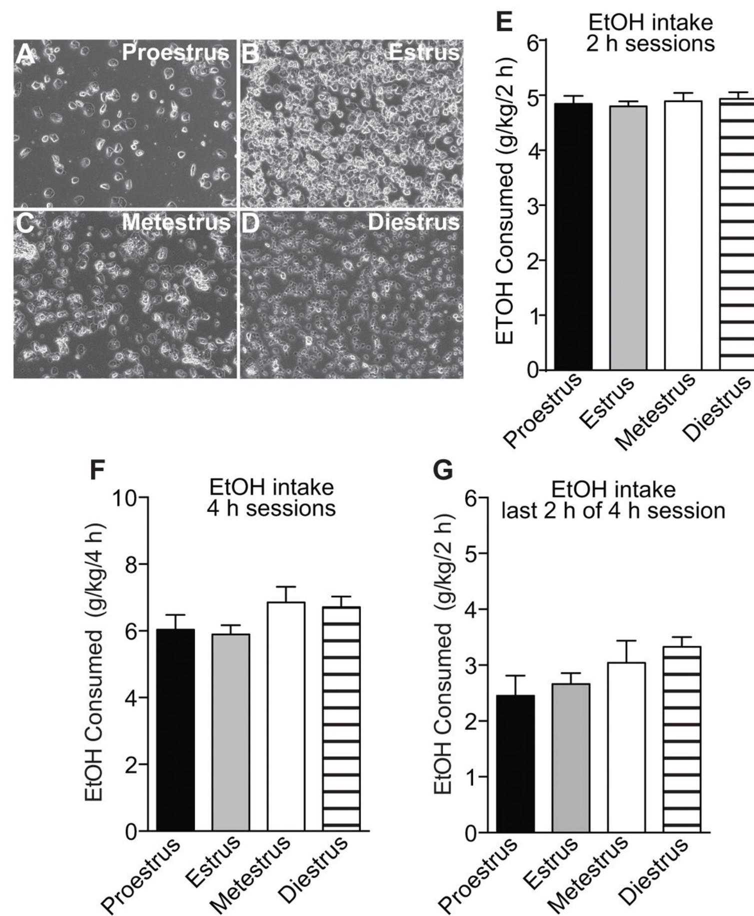

Fig. 3.

Ethanol (EtOH) intake in the drinking in the dark procedure is not altered during the estrous cycle. Representative bright field microscopic images (10x magnification) of vaginal smears are shown from mice in proestrus (**A**), estrus (**B**), metestrus (**C**) and diestrus (**D**). (**E**) Average EtOH intake (g/kg) during 2-hour daily sessions at each stage of the cycle. Cycle stage information was collected from 10 mice, 5 days a week for 6 weeks. Proestrus, n = 44; Estrus, n =104; Metestrus, n =14; Diestrus, n = 57. (**F**) Average EtOH intake (g/kg) during 4-hour daily sessions at each stage of the cycle by 8 mice, 5 days a week for 2 weeks. Proestrus, n = 13; Estrus, n = 40; Metestrus, n = 7; Diestrus, n = 18. (**G**) Average EtOH intake (g/kg) in the last two hours of the 4-hour drinking session shown in (**F**). All data are shown as means ± SEM.

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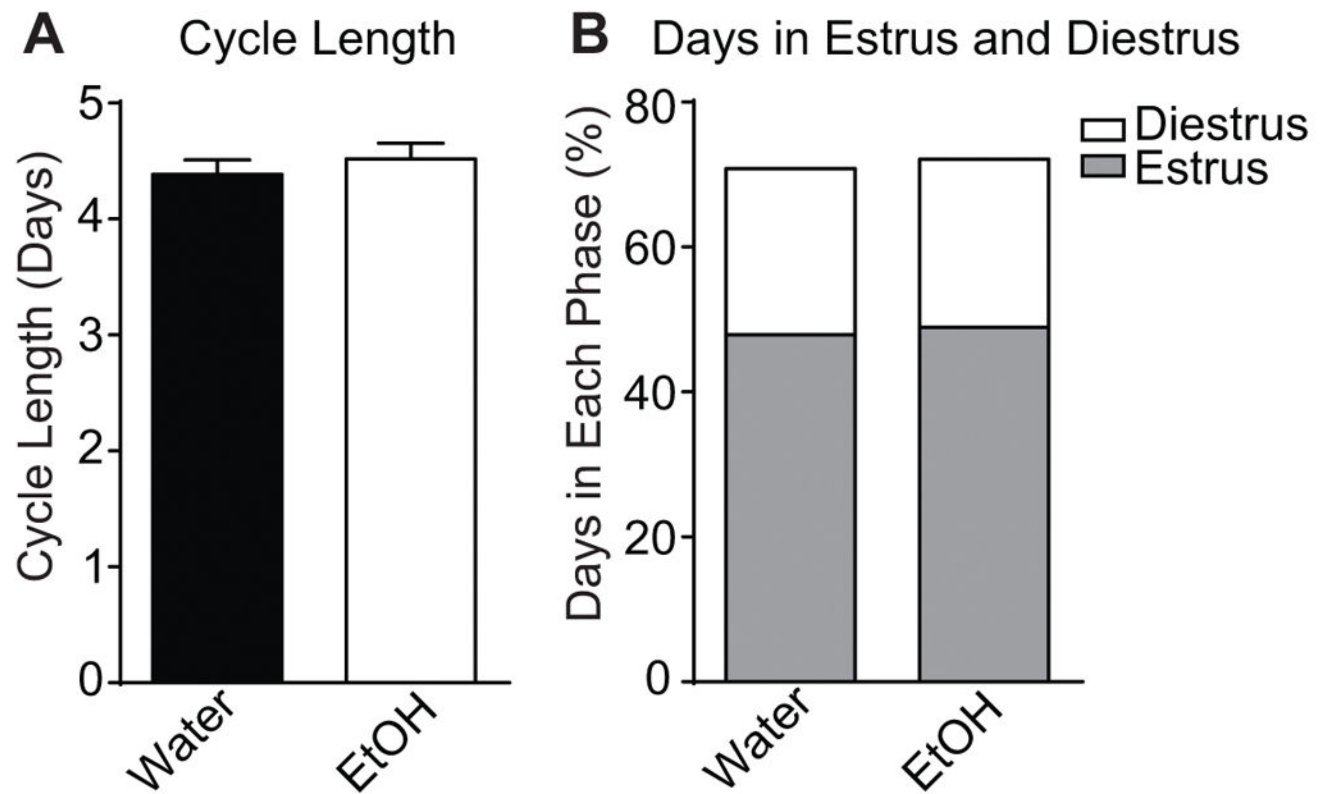

Fig. 4.

Ethanol (EtOH) drinking during drinking in the dark does not affect the length and pattern of the estrous cycle. (**A**) Length of the estrous cycle and (**B**) number of days spent in estrus and diestrus in mice drinking 20% ETOH or water in the DID procedure for 2 hours daily, 5 days per week for 6 weeks. Data are expressed as mean ± SEM from n =10 mice per group.