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Gonadal hormones and sex chromosome complement differentially contribute to ethanol intake, preference, and relapse-like behavior in Four Core Genotypes mice

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Abstract

Alcohol use and high-risk alcohol drinking behaviors among women are rapidly rising. In rodent models, females typically consume more ethanol (EtOH) than males. Here, we used the Four Core Genotypes (FCG) mouse model to investigate the influence of gonadal hormones and sex chromosome complement to EtOH drinking behaviors. FCG mice were given access to escalating concentrations of EtOH in a two-bottle, 24-h continuous access drinking paradigm to assess consumption and preference. Relapse-like behavior was measured by assessing escalated intake following repeated cycles of deprivation and re-exposure. Twenty-four hour EtOH consumption was greater in mice with ovaries (*Sry*-), relative to those with testes, and in mice with the XX chromosome complement, relative to those with XY sex chromosomes. EtOH preference was higher in XX vs. XY mice but not influenced by gonad type. Escalated intake following repeated cycles of deprivation and re-exposure emerged only in XX mice (vs. XY). These results demonstrate that aspects of EtOH drinking behavior may be independently regulated by sex hormones and chromosomes and inform our understanding of the neurobiological mechanisms which contribute to EtOH dependence in male and female mice. Future investigation of the contribution of sex chromosomes to EtOH drinking behaviors is warranted.

Keywords: alcohol deprivation effect, Four Core Genotypes mouse, sex chromosomes, sex differences, sex hormones

Introduction

Risky drinking behaviors and the development of Alcohol Use Disorder (AUD) is a prevalent health issue in the United States and worldwide [1]. Recent research demonstrates that alcohol use and high-risk alcohol drinking behaviors among women are rapidly rising [2]. Further, women may progress from initial alcohol experience to alcohol dependence more quickly than men [3]. Paralleling the effects observed in humans, female rodents are known to be more vulnerable to a range of addictive behaviors [4]. For example, female rodents consume more ethanol (EtOH) than males [5–8] and are more likely to consume EtOH despite the risk of punishment [9–11]. The neurobiological mechanisms contributing to these behavioral differences are still relatively unknown.

One potential mediator of sex differences in alcohol drinking behaviors is gonadal hormones. Elevated levels of estrogens have been associated with higher levels of alcohol consumption in adolescent [12] and adult women [13]. Recent work also suggests that rising progesterone levels may protect against alcohol intake in some women and that gonadal hormone levels may be associated with drinking alcohol to cope with negative emotional states [14]. In rodents, elevated levels of EtOH consumption and EtOH reward in females is disrupted by gonadectomy [15–17]. These effects appear to be mediated by estradiol, which promotes drinking [18–23] and EtOH reward [24].

Gonad type is influenced by the presence or absence of the *Sry* gene, which is located on the Y chromosome and is responsible for the development of testes in male mice and the secretion of testosterone [25,26]. As such, gonad differentiation is the result of only one of a number of chromosomal differences between males and females. Indeed, the human Y chromosome encodes 27 proteins and the X chromosome encodes approximately 1500 proteins that could potentially contribute to sex differences in alcohol drinking behaviors and a large number of X-linked genes are important for brain development and function [27–29]. Differentiating the influences of gonadal hormones and sex chromosomes on behavior is, however, difficult and there is very little data on sex chromosome contributions to alcohol drinking.

Fortunately, the Four Core Genotypes (FCG) mouse model, in which the *Sry* gene is absent from the Y chromosome and inserted onto an autosome in the same mice, allows the influences of sex

chromosomes and gonadal hormones on behavior to be assessed independently [25,26]. Without the *Sry* transgene, mice have female gonads (ovaries) regardless of sex chromosome complement, whereas *Sry*+ mice have male gonads (testes) (**Fig. 1**). This results in four groups: XX/*Sry*-, XY/*Sry*-, XX/*Sry*+, and XY/*Sry*+. Using this mouse model, sex chromosomes have been found to influence aggression, parenting, nociception, social interaction, and habit formation [25,30]. In regard to EtOH drinking, female gonads (*Sry*-) have been associated with increased consumption (paralleling the effects of gonadal hormones described above) while sex chromosomes influenced the development of habitual vs. goal-directed responding for EtOH [31].

To explore the contributions of gonadal hormones and sex chromosomes to alcohol consumption, preference, and relapse-like behavior, we examined 24-h EtOH intake and the alcohol deprivation effect in FCG mice. Our results suggest an important role for sex chromosomes in mediating female vulnerability to EtOH drinking behaviors.

Methods

Subjects

39 FCG mice (PND 60+) were generated from breeding pairs consisting of *Sry*+ male and C57BL/6J (wild type) female mice at the Laboratory of Animal Resources at Miami University [26]. FCG breeding pairs used to generate experimental mice were obtained from UCLA. This breeding scheme results in four groups XX/*Sry*- (n = 10), XY/*Sry*- (n = 10), XX/*Sry*+ (n = 9), and XY/*Sry*+ (n = 10) (**Fig. 1**). Mice were tested in adulthood (XX/*Sry*-: 27.05 ± 1.85; XY/*Sry*-: 25.15 ± 2.46; XX/*Sry*+: 18.57 ± 1.63; XY/*Sry*+: 20.12 ± 1.84 weeks at the start of testing [all data mean ± SEM]).

Prior to experimentation, mice were group housed by gonad type (*Sry*- or *Sry*+). One week before the first experimental session mice were individually housed in standard shoe box udel polysufone rectangular mouse cages (18.4 cm x 29.2 cm x 12.7 cm) outfitted with 2-bottle cage tops. Mice were given standard care and had access to LabDiet 5001 standard chow and reverse-osmosis (RO) filtered water *ad libitum*. Mice were kept on a 12:12 hour light:dark cycle (lights on at 7 AM). All mice were cared for in

accordance with the guidelines set by the National Institute of Health and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Miami University.

24-h home cage EtOH drinking and deprivation

Mice were given access to ethanol (EtOH) in their home cage ~3.5 h into the light cycle (i.e., 11:30 AM). Mice had access to two bottles for 24 h. One bottle contained reverse-osmosis (RO) drinking water and the other bottle contained EtOH in RO water (v/v). EtOH concentrations increased over drinking sessions from 5%, 10%, 15%, to 20% (5 drinking sessions/ concentration) (**Fig. 2A**). After the last 20% EtOH session, mice underwent a 6-day EtOH deprivation period. After the deprivation period, mice were reintroduced to 20% EtOH for 24 h. This cycle of deprivation and re-exposure was then repeated for a total of 5 deprivation sessions (**Fig. 3A**). All bottles were weighed every 24 h. Every 48 h mice were weighed and fresh solutions were made. Bottles were alternated daily to equate side biases. Two "dummy" cages were outfitted with bottles to account for spillage and evaporation.

24-h home cage water and sucrose drinking

At least two weeks following the last re-exposure session, mice were presented with RO water for one 24-h session. The following day mice were presented with a bottle of 2.5% sucrose or RO water for a total of five 24-h drinking sessions (**Fig. 4A**).

Blood EtOH concentration (BEC) analysis

At least two weeks following sucrose consumption (**Fig. 4A**), a subset of mice (n = 28, XX/*Sry*- = 7, XY/*Sry*- = 7, XX/*Sry*+ = 7, XY/*Sry*+ = 7) were given access to 15% EtOH at the onset of the dark cycle (i.e., 7:00 PM) for a total of 27 h. Bottles were weighed 24 h into the session and at the end of the session. At the end of the session, blood samples were obtained by using the modified tail-clip technique [32]. Mice were placed on a cage top and were allowed to explore while the researcher used a sterile razor blade to remove 1 mm from the tip of the tail. A heparinized capillary tube was used to collect 70 µL of blood. Blood was dispensed into a centrifuge tube and placed on ice. Immediately following collection, the blood was spun in an accuSpin Micro 17 microcentrifuge (Fisher Scientific) at 3000 rpm for 8 min. The serum

was then pipetted out into a centrifuge tube and stored at -80 °C until testing. BECs were assessed using an AM1 alcohol analyzer (Analox Technologies).

Data Analysis

Consumption was calculated as *(Initial Bottle Weight – Post Bottle Weight) – Average of Dummy Bottles*. EtOH consumption was expressed as grams of EtOH consumed per kilogram of body weight. Preference was calculated as ((*Volume of EtOH*)/ (*Volume of EtOH + Water Consumption*))*100. Consumption and preference for each concentration was computed by averaging across the five drinking sessions. Total water or EtOH consumption was calculated by summing consumption across all drinking sessions for each individual mouse then averaging for each group. Water and sucrose data were expressed as milliliters of water consumed per kilogram of body weight.

For consumption and preference data, a Mixed Effects analysis of variance (ANOVA) (in the case of missing values) or a Three-Way ANOVA was used with *Sry* (gonad type) and sex chromosome complement as between-subjects factors and EtOH concentration as the within-subjects factor. For total consumption of water or EtOH, a Two-Way ANOVA was used with *Sry* (gonad type) and sex chromosome complement as between-subjects factors. In cases where the assumption of sphericity was violated ($\varepsilon < 0.75$), the Greenhouse-Geisser correction was applied. For *post hoc* tests, a Holm-Sidak multiple comparisons test was used to compare differences between groups. All data are shown as mean ± SEM and the alpha level was set at $p < 0.05$. All analyses were conducted in GraphPad Prism v. 8.3 (La Jolla, CA). All images were created using GraphPad Prism, BioRender, and GNU Image Manipulation Program (GIMP) 2.10.

Results

Sex hormones and chromosomes influence EtOH consumption.

Analysis of EtOH consumption across concentrations revealed influences of both *Sry* (gonad type) and sex chromosomes. A Three-Way ANOVA identified main effects of the *Sry* gene ($F_{(1, 35)} = 4.704$, $p = 0.037$), chromosome complement ($F_{(1, 35)} = 9.839$, $p = 0.004$), and EtOH concentration ($F_{(2.439, 85.353)} = 55.046$, $p < 0.0001$). There were interactions with EtOH concentration for the *Sry* gene ($F_{(3, 105)} = 3.156$, p

= 0.028) and chromosome complement ($F_{(3, 105)} = 2.838$, $p = 0.042$). The Three-Way interaction of *Sry* gene X sex chromosomes X EtOH concentration did not reach significance. Average consumption of EtOH and water at each concentration for all four genotypes is presented in **Tables 1 and 2**.

Additionally, because there were no interactions between *Sry* and chromosomes, data were collapsed across these factors for visualization and follow-up analyses. When comparing all *Sry*- to *Sry*+ mice, a Two-Way ANOVA revealed significant main effects of *Sry* gene ($F_{(1, 37)} = 4.189$, $p = 0.048$) and EtOH concentration ($F_{(2.487, 92.00)} = 53.100$, $p < 0.0001$) and a significant interaction of these two factors ($F_{(3, 111)} = 3.057$, $p = 0.031$). *Post hoc* Holm-Sidak tests showed that *Sry*- mice consumed more EtOH than *Sry*+ mice at the 20% concentration only ($p = 0.003$) (**Fig. 2B**).

When comparing XX mice to XY mice, a Two-Way ANOVA revealed significant main effects of sex chromosomes ($F_{(1, 37)} = 8.960$, $p = 0.005$) and EtOH concentration ($F_{(2.410, 89.16)} = 53.280$, $p < 0.0001$) and a significant interaction of these two factors ($F_{(3, 111)} = 2.695$, $p = 0.050$). *Post hoc* Holm-Sidak tests showed that XX mice consumed more EtOH than XY mice at the 15% concentration only ($p = 0.004$) (**Fig. 2C**).

Sex hormones and chromosomes influence preference for EtOH vs. water.

Analysis of EtOH preference demonstrated an influence of sex chromosomes. A Three-Way ANOVA revealed a main effect of chromosome complement ($F_{(1, 35)} = 8.130$, $p = 0.007$) and EtOH concentration ($F_{(2.248, 78.673)} = 47.188$, $p < 0.0001$). There was also a significant *Sry* gene X EtOH concentration interaction ($F_{(3, 105)} = 3.169$, $p = 0.027$). Because there were no interactions between *Sry* gene and sex chromosomes, data were collapsed across these factors for visualization and follow up analyses.

When comparing all *Sry*- to *Sry*+ mice, a Two-Way ANOVA revealed a main effect of EtOH concentration ($F_{(2.309, 85.44)} = 47.330$, $p < 0.0001$) and an *Sry* gene X EtOH concentration interaction ($F_{(3, 111)} = 3.120$, $p = 0.029$). In *post hoc* analyses, the comparison between *Sry*- and *Sry*+ mice at the 10% concentration approached the threshold for significance, with *Sry*+ mice exhibiting higher preference than *Sry*- mice ($p = 0.089$) (**Fig. 2D**). For comparisons of XX and XY mice, a Two-Way ANOVA revealed a main effect of EtOH concentration ($F_{(2.39, 88.43)} = 45.270$, $p < 0.0001$) and a main effect of sex chromosomes

($F_{(1, 37)} = 6.774$, $p = 0.013$). The interaction was not significant ($F_{(3, 111)} = 1.808$, $p = 0.150$) but a *post hoc* Holm-Sidak test suggested that preference was higher in XX vs. XY mice specifically for the 15% concentration ($p = 0.008$) (**Fig. 2E**).

Higher EtOH consumption in *Sry*- mice without corresponding increases in EtOH preference suggested that gonad type also influences water consumption. A Two-Way ANOVA of water consumption during the 24-h EtOH drinking paradigm in *Sry*- and *Sry*+ mice demonstrated that *Sry*- mice consumed more water than *Sry*+ mice, as evidenced by a main effect of *Sry* gene ($F_{(1, 25)} = 6.721$, $p = 0.016$). There was also a significant main effect of EtOH concentration ($F_{(2.210, 55.254)} = 24.510$, $p < 0.0001$). The interaction of *Sry* gene and EtOH concentration approached the threshold for significance ($F_{(3, 75)} = 2.432$, $p = 0.072$).

Sex chromosomes influence the alcohol deprivation effect.

Mice were re-exposed to 20% EtOH following 6-day cycles of deprivation for 5 total re-exposures. Previous research suggests that this regimen promotes increases in intake known as the alcohol deprivation effect [33]. Analysis of EtOH consumption at baseline (= the five sessions immediately preceding the beginning of the first deprivation period) and on the five deprivation sessions demonstrated that sex chromosomes influenced the magnitude of the alcohol deprivation effect. A Three-Way ANOVA revealed main effects of the *Sry* gene ($F_{(1, 35)} = 12.075$, $p = 0.001$), sex chromosome complement ($F_{(1, 35)} = 13.202$, $p = 0.0009$), and session ($F_{(3.119, 109.174)} = 7.947$, $p < 0.0001$). Follow up Dunnett's tests (control condition = baseline) showed that mice in the XX/*Sry*- group consumed more EtOH on re-exposure sessions 4 ($p = 0.039$) and 5 ($p = 0.047$) and mice in the XX/*Sry*+ group consumed more EtOH on re-exposure session 5 ($p = 0.039$) compared to baseline (**Fig. 3B**). Mice in the XY/*Sry*+ group consumed less EtOH compared to baseline on re-exposure session 4 ($p=0.032$) compared to baseline.

Mice with the XX chromosome complement continued to consume more EtOH than mice with the XY chromosome complement during the deprivation phase of the experiment. Comparison of all XX to XY mice with a Two-Way ANOVA identified main effects of sex chromosome complement ($F_{(1, 37)} = 10.651$, $p = 0.002$) and re-exposure session ($F_{(3.226, 119.377)} = 8.029$, $p < 0.0001$). *Post hoc* Holm-Sidak tests

showed XX mice (vs. XY) consumed greater amounts of EtOH at re-exposure sessions 2 ($p = 0.025$) and 4 ($p = 0.005$).

Sry- mice also continued to consume more EtOH than *Sry*+ mice during the deprivation phase of the experiment. Comparison of all *Sry*- to *Sry*+ mice with a Two-Way ANOVA identified main effects of *Sry* gene ($F_{(1, 37)} = 9.823$, $p = 0.003$) and re-exposure session ($F_{(3.251, 120.303)} = 7.770$, $p < 0.0001$). *Post hoc* Holm-Sidak tests revealed *Sry*- mice (vs. *Sry*+) consumed greater amounts of EtOH at re-exposure sessions 1 ($p = 0.049$), 2 ($p = 0.049$), 3 ($p = 0.049$), and 4 ($p = 0.008$).

Sex hormones influence water but not sucrose consumption.

To determine whether the effects of the *Sry* gene on water consumption could be replicated without concurrent access to EtOH, water consumption in the home cage during a 24-h period was measured in all mice. A Two-Way ANOVA revealed that *Sry*- mice drank more water vs. *Sry*+ mice, as evidenced by a main effect of *Sry* ($F_{(1, 35)} = 13.339$, $p < 0.001$) (**Fig. 4B**).

A final control experiment was conducted to assess whether the observed differences in EtOH consumption and preference were specific to EtOH reward. Mice drank a 2.5% sucrose solution in a two-bottle choice paradigm for five 24-h sessions. Two-Way ANOVAs identified no group differences in average consumption (**Fig. 4C**) or preference for sucrose.

Blood EtOH concentrations did not differ between genotypes

Two weeks following sucrose consumption, mice were re-exposed to 15% EtOH for 27 h and blood samples were collected at the end of the session. A Two-Way ANOVA revealed no significant differences in consumption between groups at the 24 h or 3 h timepoints. BECs were significantly correlated with 3-h consumption ($R^2 = 0.343$, $p = 0.001$) (**Fig. 4D**). A Two-Way ANOVA also identified no significant differences in BECs between groups (XX/*Sry*-: 38.31 13.91, XX/*Sry*+: 37.18 20.28, XY/*Sry*-: 18.93 9.54, XY/*Sry*+: 24.27 19.24 [all data mean mg/dl SEM]).

Discussion

Using the FCG mouse model in a 24-h continuous access drinking paradigm, we explored how sex hormones and sex chromosomes contribute to female vulnerability to EtOH consumption, preference, and

relapse-like behavior and uncovered novel influences of sex chromosomes. Twenty-four hour EtOH consumption was greater in mice with female gonads (*Sry*-) and in mice with the XX chromosome complement. EtOH preference was higher in XX vs. XY mice. Escalated intake following repeated cycles of deprivation and re-exposure emerged only in XX mice (vs. XY). Follow-up control studies demonstrated that *Sry*- mice consumed more water than their *Sry*+ counterparts did and that drinking resulted in similar blood EtOH levels among the genotypes. These results concur with prior findings concerning the role of ovarian hormones in female vulnerability to EtOH drinking while revealing an underappreciated role for sex chromosomes in these behaviors.

Because the presence or absence of *Sry* determines the development of ovaries (*Sry*-) vs. testes (*Sry*+), behavioral differences between the *Sry* genotypes are primarily driven by levels of gonadal hormones. It is well established that ovarian hormones such as estradiol promote consumption and seeking of alcohol in rats and mice [15,18–21,23]. Our observation of higher intake in *Sry*- mice similarly suggests that female gonads promote EtOH consumption. This finding also replicates an earlier study in FCG mice that reported higher 30-min EtOH consumption in *Sry*- mice [31]. In the current study, the effect of *Sry* on consumption was most pronounced at the highest concentration of EtOH (20%), which supports a previous study from our lab that found higher consumption in female vs. male C57BL/6J mice only at concentrations above 10% [11]. Thus, the current results provide further evidence that, at least in mice, female vulnerability to EtOH consumption is concentration dependent.

Interestingly, the effect of *Sry* (gonad type) on EtOH consumption did not extend to EtOH preference. Some other studies have seen increased preference in female rodents [15] while others have not [8,10]. In the current study, the lack of an effect on preference was driven by elevations in water intake in *Sry*- mice during EtOH drinking sessions, relative to *Sry*+ mice. Higher water intake in *Sry*- mice was also observed in a separate, EtOH-free session. We have observed a similar pattern of greater consumption but not preference in female vs. male C57BL/6J mice using a drinking in the dark paradigm [8] and similar findings have been seen in rats [34,35]. Further, estrogens have been shown to influence water consumption in rodents [36,37]. The influence of gonad type on water intake is an important consideration for studies of EtOH

drinking behaviors, since variations in water intake could influence blood EtOH levels. It also raises the possibility that mice with female gonads may simply drink more fluids. The observation of equal sucrose consumption across genotypes could argue against this conclusion, although we cannot rule out that consumption levels in that experiment reached a ceiling and obscured any effects.

In addition to gonadal influences on EtOH consumption, we report a novel role for sex chromosomes in influencing both consumption and preference. In one other study of 30-min consumption of 10% EtOH in FCG mice, sex chromosome complement did not influence consumption [31]. In the current study, differences between XX and XY mice were greatest at the 15% concentration. Thus, the effects of chromosomes may be dependent on the drinking paradigm used or the concentration of EtOH presented. Importantly, sex chromosomes did not influence water or sucrose intake, suggesting that the observed effects are specific to EtOH. When considered alongside our findings regarding *Sry* influences on consumption but not preference, these results suggest that sex chromosome differences between males and females play a critical, and perhaps primary, role in driving sex differences in some EtOH drinking behaviors.

Another major finding of this work is that sex chromosomes and hormones are responsible for greater relapse-like behavior in female mice. The alcohol deprivation effect has been demonstrated previously in male rats and mice [33,38,39], and is characterized by escalation of EtOH consumption following repeated cycles of deprivation and re-exposure. The alcohol deprivation effect has also been observed in female rats and WSC-1 mice following a single two-week deprivation [40,41]. In the latter study, escalations in drinking following deprivation were similar in male and female mice [41]. As such, our results are the first demonstration of greater female susceptibility in this model of relapse-like behavior.

As in the 24-h continuous portion of the experiment, EtOH consumption during re-exposure sessions was higher for mice with XX (vs. XY) chromosomes and *Sry*- (vs. *Sry*+) mice. Escalations in drinking following deprivation (assessed by comparing consumption on re-exposure sessions to baseline consumption of 20% EtOH) were only observed in mice with XX chromosomes. The observation of the alcohol deprivation effect on re-exposure session 4 in XX/*Sry*- but not XX/*Sry*+ mice further suggests that

the presence of female gonads facilitates the effects of deprivation on drinking behavior. One important consideration is that the alcohol deprivation effect was completely absent in XY/*Sry*+ mice, which are most similar to the male mice first used to establish this paradigm in the C57BL/6J line [33]. Interestingly, delayed emergence of escalated drinking (during re-exposure session 6-7) was observed in a study by Melendez and colleagues (2006) when variants of the paradigm were used, including when a higher concentration of EtOH was available. It is possible that a similar delay occurred here in the XY/*Sry*+ mice because we used 20% EtOH, as opposed to 15%. Thus, while we cannot be certain, we think it likely that escalated drinking emerges more quickly (i.e., following fewer deprivation cycles) in vulnerable animals.

An important limitation of this study is that the estrous cycle was not assessed in *Sry*- mice. Estrous phase is typically unrelated to EtOH consumption levels in freely cycling rodents [9,18,23,42,43]. Further, because we averaged intake and preference for each concentration of EtOH over 5 sessions, there are unlikely to be influences of estrous on the 24-h, continuous drinking results. We cannot, however, exclude the possibility that estrous cycle phase influenced results from the re-exposure sessions. Complicating the issue is the fact that extended EtOH exposure can alter the estrous cycle [44–46].

A sex difference driven by sex chromosome complement may be due to genes on the Y chromosome, an extra dose of genes on the X chromosome in females, or paternal imprinting of X-linked genes [47,48]. The mechanisms through which sex chromosomes influence EtOH drinking behaviors remain to be determined. Prior studies in the FCG mice have uncovered a role for sex chromosomes in the development of habit formation [31,49], locomotor activation to cocaine [50], and in the density of tyrosine hydroxylase-expressing neurons in the midbrain [47,51]. It is thus possible that genes on the X and/or Y chromosomes produce sex differences in EtOH consumption, preference, relapse-like behavior, and/or habit formation via influences on the development of brain reward systems.

In sum, the current results confirm an involvement of gonadal hormones and highlight a currently underappreciated contribution of sex chromosomes in EtOH drinking behaviors. In addition to continued exploration of the mechanisms by which sex hormones facilitate EtOH drinking, it is critical that future studies begin to explore sex-specific genetic influences on these behaviors.

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Author Contributions:

AKR, EAS, LNR, NGC, ASJ, and NJO designed the experiments. EAS, LNR, NGC, ASJ, and NJO conducted the experiments. HH and APA provided the FCG breeding pairs. AKR, EAS, LNR, NGC, ASJ, and NJO analyzed data. AKR, EAS, and APA interpreted the findings. EAS drafted the manuscript. All authors edited, contributed to, and approved the manuscript.

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Figure Legends

Figure 1. Breeding scheme of the Four Core Genotypes (FCG) mice. Breeding XX (wild-type) females with a XY/*Sry*+ mice results in four groups of offspring. XX/*Sry*- and XY/*Sry*- mice have ovaries and secrete female hormones regardless of chromosome complement. XX/*Sry*+ and XY/*Sry*+ mice have testes and secrete male hormones regardless of chromosome complement. Key: Purple chromosomes: mouse X chromosomes; gray chromosomes: mouse Y chromosomes; for *Sry*- mice the female mouse reproductive system is shown and for *Sry*+ mice the male mouse reproductive system is shown. The reproductive systems consist of gonadal (ovaries or testes) and non-gonadal tissues.

Figure 2. Differential effects of gonadal hormones and sex chromosomes on EtOH intake and preference. A) Mice drank EtOH, 5%, 10%, 15%, and 20% concentrations, for 24 h across 5 drinking sessions per concentration. **B)** *Sry*- and **C)** XX mice consumed greater amounts of EtOH. **D-E)** XX chromosomes were associated with heightened preference for EtOH vs. water. * $p < 0.05$, ** $p < 0.01$ (Holm-Sidak or 3-Way ANOVA).

Figure 3. XX chromosomes promote the alcohol deprivation effect. A) Following the last 20% EtOH session, mice underwent 6 sessions of deprivation and were then re-exposed to 20% EtOH for one 24-h session. This cycle of deprivation and re-exposure was repeated five times. **B)** XX/*Sry*- and XX/*Sry*+ mice escalated intake compared to drinking at baseline (= 5 sessions preceding deprivation; BL). # $p < 0.05$ vs. BL (Dunnett's), ** $p < 0.01$ (3-Way ANOVA).

Figure 4. Female gonads (*Sry*-) promote water but not sucrose intake. A) Mice were given access to water for a 24-h session followed by five sessions with 2.5% sucrose. Two weeks after, mice were re-exposed to 15% EtOH for one 27-h session and blood samples were collected immediately after. **B)** *Sry*- mice consumed more water than *Sry*+ mice. ** $p < 0.01$ (2-Way ANOVA). **C)** No differences were

observed in sucrose consumption. **D**) 3-h consumption of 15% EtOH was significantly correlated with BEC measurements ($p < 0.001$).

Table Legends

Table 1. Ethanol consumption by concentration in Four Core Genotypes mice. Female gonad type and XX chromosome complement are associated with greater total EtOH intake. *Sry*- mice consumed more 5% and 20% EtOH. XX mice consumed more 10% and 15% EtOH. *Sry*- and XX mice consumed more total EtOH. [a] main effect of *Sry* gene, $p < 0.05$ (2-Way ANOVA). [b] main effect of chromosomes, $p = 0.05$ (2-Way ANOVA). *$p < 0.05$ vs. XY/*Sry*+ mice (Holm-Sidak). Data are averages ± standard error of the mean (SEM).

Table 2. Water consumption in Four Core Genotypes mice. Gonad type predicted water consumption. *Sry*- mice consumed more water when 5% or 10% EtOH was present. *Sry*- and XY mice consumed more water when 15% EtOH was present. *Sry*- mice consumed more total water. [a] main effect of *Sry* gene, $p < 0.05$ (2-Way ANOVA). [b] main effect of chromosomes, $p = 0.05$ (2-Way ANOVA) *$p < 0.05$ vs. XX/*Sry*+ mice (Holm-Sidak). Data are averages ± standard error of the mean (SEM).

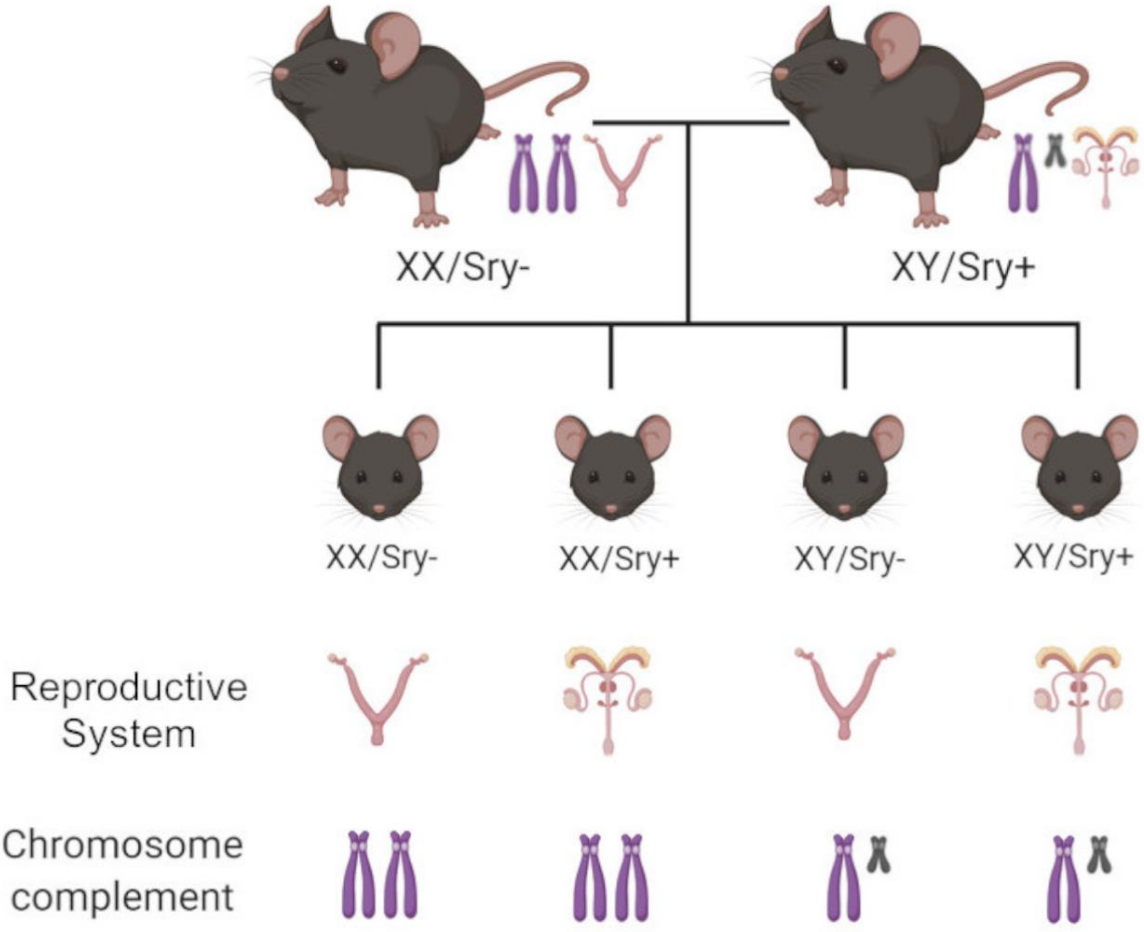
XX/Sry-
XY/Sry+
XX/Sry-
XX/Sry+
XY/Sry-
XY/Sry+
Reproductive System
Chromosome complement

Figure 1.

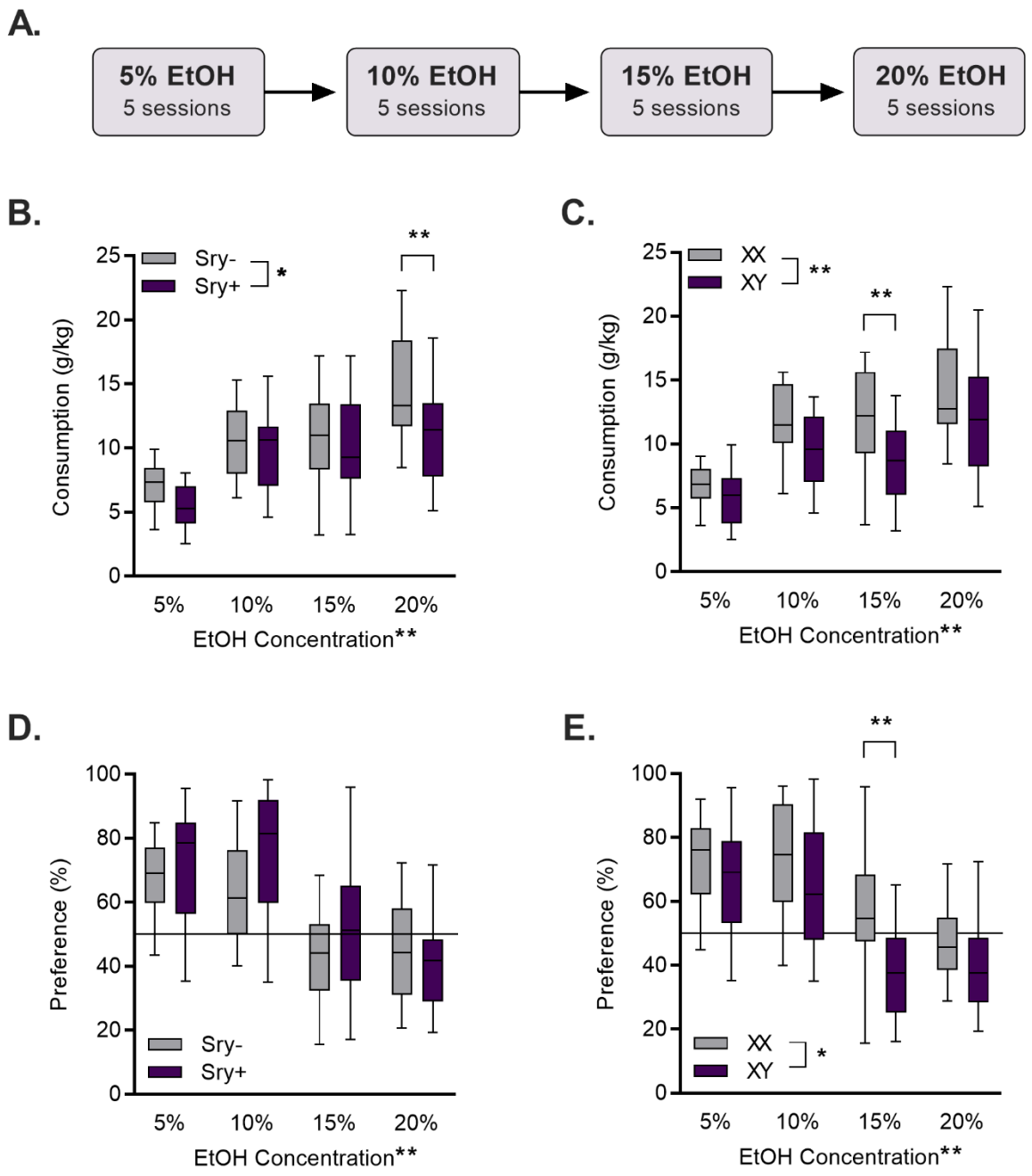
A.
5% EtOH
5 sessions
10% EtOH
5 sessions
15% EtOH
5 sessions
20% EtOH
5 sessions
B.
Sry-
Sry+
*
**
Consumption (g/kg)
EtOH Concentration**
C.
XX
XY
**
**
Consumption (g/kg)
EtOH Concentration**
D.
Preference (%)
Sry-
Sry+
EtOH Concentration**
E.
**
Preference (%)
XX
XY
*
EtOH Concentration**

Figure 2.

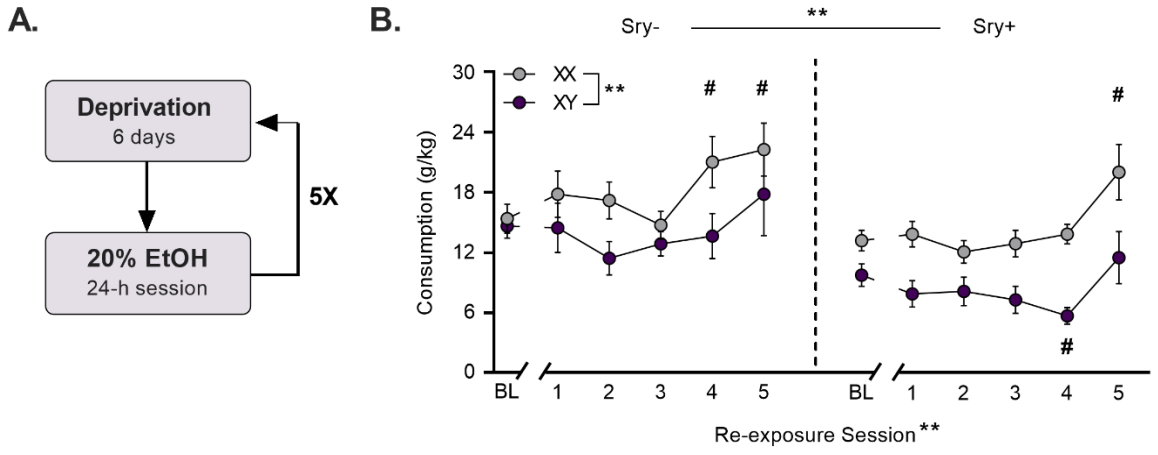
A.
Deprivation
6 days
20% EtOH
24-h session
5X
B.
Sry-
**
Sry+
XX
XY
**
#
#
#
#
Consumption (g/kg)
30
24
18
12
6
0
BL
1
2
3
4
5
BL
1
2
3
4
5
Re-exposure Session**

Figure 3.

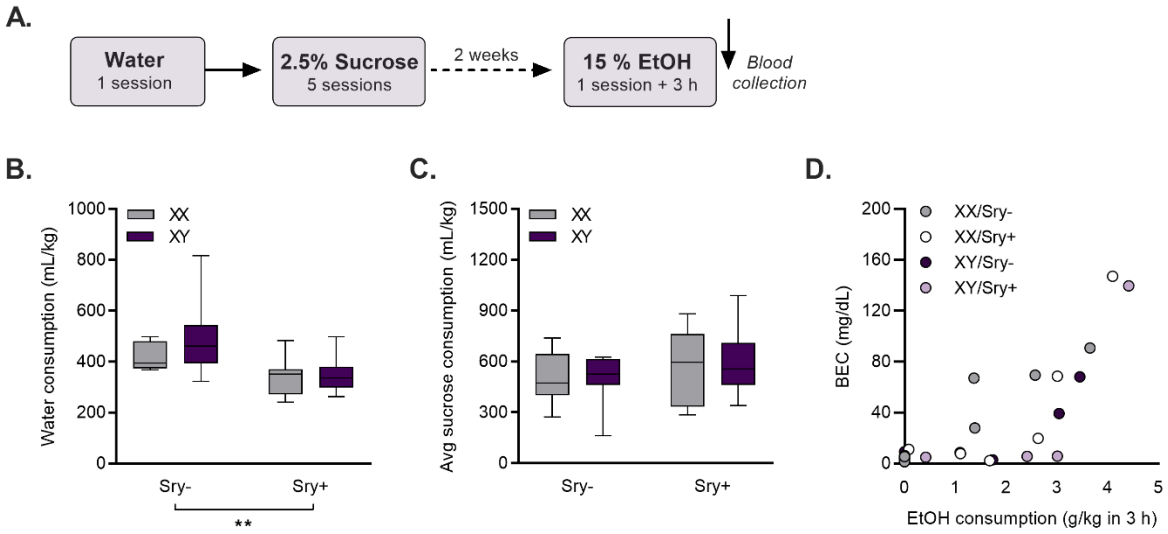
A.
Water
1 session
2.5% Sucrose
5 sessions
2 weeks
15 % EtOH
1 session + 3 h
Blood
collection
B.
Water consumption (mL/kg)
XX
XY
Sry-
Sry+
**
C.
Avg sucrose consumption (mL/kg)
XX
XY
Sry-
Sry+
D.
BEC (mg/dL)
XX/Sry-
XX/Sry+
XY/Sry-
XY/Sry+
EtOH consumption (g/kg in 3 h)

Figure 4.

Table 1. Average EtOH Consumption

Genotype	5% EtOH (g/ kg)[a]	10% EtOH (g/ kg)[b]	15% EtOH (g/ kg)[b]	20% EtOH (g/ kg)[a]	Total EtOH (g/ kg)[ab]
XX/Sry-	6.98 ± 0.53	11.43 ± 0.96	12.37 ± 1.41*	15.39 ± 1.45*	230.29 ± 17.52*
XY/Sry-	6.93 ± 0.67	10.28 ± 0.79	8.90 ± 0.90	14.64 ± 1.20*	201.58 ± 13.11
XX/Sry+	6.23 ± 0.49	11.58 ± 0.76	12.45 ±1.00*	13.23 ± 1.05	217.44 ± 12.37*
XY/Sry+	4.81 ± 0.56	8.53 ± 0.95	8.04 ± 1.02	9.78 ± 1.13	154.66 ± 12.53

Table 2. Average Water Consumption

Group	5% EtOH (mL/ kg)[a]	10% EtOH (mL/ kg)[a]	15% EtOH (mL/ kg)[ab]	20% EtOH (mL/ kg)	Total Water (mL/ kg)[a]
XX/Sry-	88.33 ± 13.93*	88.65 ± 15.66*	120.02 ± 11.21*	129.78 ± 14.40	2109.04 ± 220.50*
XY/Sry-	77.95 ± 8.45	95.82 ± 17.06*	153.91 ± 19.45*	139.10 ± 25.69	2333.89 ± 310.04*
XX/Sry+	41.02 ± 6.07	32.79 ± 10.70	61.65 ± 9.93	90.84 ± 8.06	1131.48 ± 102.75
XY/Sry+	67.09 ± 14.46	54.95 ± 12.07	107.94 ± 9.92	118.12 ±4.49	1740.47 ± 140.87