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Sex Differences in Ethanol's Anxiolytic Effect and Chronic Ethanol Withdrawal Severity in Mice With a Null Mutation of the 5α-Reductase Type 1 Gene

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Abstract

Manipulation of endogenous levels of the GABAergic neurosteroid allopregnanolone alters sensitivity to some effects of ethanol. Chronic ethanol withdrawal decreases activity and expression of 5α-reductase-1, an important enzyme in allopregnanolone biosynthesis encoded by the 5α-reductase-1 gene (*Srd5a1*). The present studies examined the impact of *Srd5a1* deletion in male and female mice on several acute effects of ethanol and on chronic ethanol withdrawal severity. Genotype and sex did not differentially alter ethanol-induced hypothermia, ataxia, hypnosis, or metabolism, but ethanol withdrawal was significantly lower in female versus male mice. On the elevated plus maze, deletion of the *Srd5a1* gene significantly decreased ethanol's effect on total entries versus wildtype (WT) mice and significantly decreased ethanol's anxiolytic effect in female knockout (KO) versus WT mice. The limited sex differences in the ability of *Srd5a1* genotype to modulate select ethanol effects may reflect an interaction between developmental compensations to deletion of the *Srd5a1* gene with sex hormones and levels of endogenous neurosteroids.

Keywords

neurosteroid; allopregnanolone; GABA; alcohol; hypnosis; metabolism

INTRODUCTION

A variety of evidence indicates that steroid hormone derivatives alter neuronal activity via rapid and potent effects on γ-aminobutyric acid$_A$ receptors (GABA$_A$Rs) and other ligand-

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All institutional and national guidelines for the care and use of laboratory animals were followed.

gated ion channels (Belelli and Lambert, 2005; Finn and Purdy, 2007; Paul and Purdy, 1992; Reddy, 2010). These compounds have been called neuroactive steroids, and their behavioral effects are consistent with the ability to potentiate $GABA_AR$ function (e.g., anxiolytic, anticonvulsant, antidepressant, hypnotic, anesthetic; Finn et al., 2004a; Gasior et al., 1999). The $GABA_AR$-active neuroactive steroids allopregnanolone (ALLO; 3α,5α-tetrahydroprogesterone), 3α,5α-tetrahydrodeoxycorticosterone (THDOC), and 3α,5α-androstanediol are formed from the two step reduction of the parent steroids progesterone, deoxycorticosterone, and testosterone, respectively, by the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD). Because 5α-reduction is an irreversible reaction in mammalian cells (see Celotti et al., 1997), 5α-reductase is considered a rate-limiting enzyme in neuroactive steroid biosynthesis. As a result, 5α-reductase inhibitors have been used to block the metabolism of the parent steroids, decrease levels of ALLO, THDOC or androstanediol, and determine the impact of increased cellular excitability on behavior (reviewed in Finn et al., 2006a).

The pharmacological profile of ALLO is similar to that of ethanol, and both ALLO and ethanol can potentiate $GABA_AR$ function (e.g., Kumar et al., 2009; Spanagel 2009). Taken in conjunction with the finding that ethanol injection and consumption increased endogenous ALLO to pharmacologically active levels in male rodents (Barbaccia et al., 1999; Eva et al., 2008; Finn et al., 2004c; VanDoren et al., 2000; but see Porcu et al., 2010, 2014), the ability of ethanol to increase endogenous ALLO levels may potentiate or prolong ethanol's effects via dual (i.e., ethanol + ALLO) positive modulatory actions at $GABA_ARs$. Likewise, the 5α-reductase inhibitor finasteride decreased the steroidogenic effect of ethanol injection (Morrow et al., 2001) and ethanol consumption (Eva et al., 2008), and it also reduced selective effects of ethanol (Dazzi et al., 2002; Hirani et al., 2005; Sanna et al., 2004; Tokunaga et al., 2003; VanDoren et al., 2000), suggesting that a reduction in ethanol's steroidogenic effect might reduce the action of ethanol at $GABA_ARs$. Consistent with this idea, ethanol was found to have a direct and indirect effect on $GABA_AR$ function, with the indirect effect being due to steroidogenesis in that it was blocked with finasteride (Sanna et al., 2004). Thus, targeting 5α-reductase to decrease endogenous ALLO levels and concomitant GABAergic inhibition should decrease sensitivity to the effects of ethanol that are mediated by $GABA_ARs$.

Chronic ethanol consumption and the induction of physical dependence reduced ethanol's steroidogenic effect (Boyd et al., 2010), and withdrawal from chronic ethanol exposure decreased ALLO levels in rodents (Cagetti et al., 2004; Finn et al., 2004a; Morrow et al., 2001; Snelling et al., 2014; Tanchuck et al., 2009). The withdrawal-induced decrease in endogenous ALLO levels was accompanied by a significant reduction in activity and expression of 5α-reductase and 3α-HSD (Cagetti et al., 2004; Finn et al., 2004a; Tanchuck et al., 2009), and separate studies determined that administration of finasteride increased chronic ethanol withdrawal (Gililland-Kaufman et al., 2008; Tanchuck et al., 2013). These preclinical findings are consistent with results in small cohorts of alcoholic patients in which a decrease in plasma ALLO and THDOC levels corresponded to an increase in the subjective ratings of anxiety and depression during the early withdrawal phase (Romeo et al., 1996). Collectively, the results are suggestive of an inverse relationship between

endogenous $GABA_AR$-active neuroactive steroid levels and behavioral changes in excitability during ethanol withdrawal.

Gene mapping studies found a significant genetic correlation between chronic ethanol withdrawal severity and a region of chromosome 13 (Crabbe, 1998) in which the murine gene for the enzyme 5α-reductase-1 (*Srd5a1*) has been mapped (37 cM; Jenkins et al., 1991). Each provisionally mapped gene controls a small portion of trait variability and is termed a quantitative trait locus (QTL). A subsequent chronic ethanol withdrawal study identified an epistatic interaction between the chromosome 13 QTL and a chromosome 11 QTL that contains several genes encoding proteins for $GABA_AR$ subunits (*Gabra6*, *Gabrb2*, and *Gabrg2*) (Bergeson et al., 2003). These gene mapping studies offer a hint that *Srd5a1* may affect chronic ethanol withdrawal severity, perhaps through a modifying effect on the activity or expression of certain $GABA_AR$ subunit genes on chromosome 11.

Collectively, the evidence described above suggests that 5α-reductase is one rate-limiting enzyme in neuroactive steroid synthesis and that pharmacologically inhibiting 5α-reductase can impact ALLO levels, ethanol's steroidogenic effect, some of ethanol's behavioral effects, and chronic ethanol withdrawal severity. While there are two isoforms of the 5α-reductase enzyme, 5α-reductase-1 is widely expressed in the rodent nervous system, whereas 5α-reductase-2 is primarily expressed in peripheral steroidogenic tissues (Melcangi et al., 1998). Additionally, the expression of *Srd5a1* is similar in males and females and does not appear to be controlled by androgens (see Melcangi et al., 1998), and gene mapping studies suggest that *Srd5a1* may influence chronic ethanol withdrawal severity. For these reasons, the present studies determined whether a deletion mutation of the *Srd5a1* gene in male and female mice would significantly alter select ethanol-related behaviors, ethanol's steroidogenic effect, and chronic ethanol withdrawal severity. We predicted that deletion of *Srd5a1* would reduce ethanol's steroidogenic effect as well as ethanol's anxiolytic effect without altering ethanol's ataxic or hypnotic effect, because finasteride did not alter ethanol-induced ataxia or hypnosis (Khisti et al., 2003, 2004). We also predicted that deletion of *Srd5a1* would increase chronic ethanol withdrawal severity, but that withdrawal would be lower in female versus male mice (e.g., Veatch et al., 2007).

METHODS

ANIMALS

Homologous recombination in mouse embryonic stem cells was used to produce mice with a disruption (null allele) in the *Srd5a1* gene (Mahendroo et al., 1996, 1997, 1999). These B6;129S7-*Srd5a1*^{tm1Mahe} null-mutant mice (i.e., *Srd5a1* knockout, KO) are now maintained as cryopreserved embryos by The Jackson Laboratory (Bar Harbor, Maine), and they were re-derived for our use. Heterozygous breeding pairs were obtained, and upon arrival in Portland, were bred in the Portland Alcohol Research Center animal core. After weaning, same-sex littermates were housed in groups of four in individually filtered and ventilated bedding-lined cages with stainless steel tops with *ad libitum* access to food and water under a 12:12 hr light/dark cycle (lights on at 0600) at 26 ± 1°C. Tail clips were taken in order to genotype littermates prior to testing. Mice were of mixed background strain (C57BL/6J x 129/SvJ), and studies tested adult male and female wildtype (WT) and KO littermates.

Littermates were counterbalanced across all conditions to match hybrid genetic background as closely as possible. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health and were approved by the local Institutional Animal Care and Use Committee.

Genotyping—Genomic DNA was purified from mouse tail tissue following a standard proteinase K and phenol/chlorofom extraction procedure. Genotyping was performed by polymerase chain reaction (PCR) analysis using primers: *Srd5a1*-F: 5′-TAACCGCGTCCTGCTAGCTA-3′ and *Srd5a1*-R: 5′-AGTGGACTTTGGGCAATAGA-3′. PCR was for 35 cycles with an annealing temperature of 55°C. The resulting products were electrophoresized on 1.5% agarose gel, yielding a 360-bp product for the null allele but 400-bp for the WT allele.

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Experiment 1: Behavioral testing—A series of ethanol-related behaviors were examined once per week in adult male and female KO and WT mice that were tested in three experimental passes due to animal availability (Table 1). Mice were tested at weekly intervals to avoid the potential for the development of ethanol tolerance. In the first experimental pass, mice were tested for ethanol's anxiolytic effect (2 g/kg; week 1), ethanol's hypothermic effect (3 g/kg; week 2), ethanol's hypnotic effect (3.6 g/kg; week 3), and ethanol-induced ataxia (2 g/kg; week 4). In the second and third experimental passes, mice were tested for ethanol's anxiolytic effect (2 g/kg; week 1), ethanol's hypnotic effect (2.6 g/kg; week 2), and ethanol's hypothermic effect (3 g/kg; week 3). Doses of ethanol for each behavior were chosen, based on published data (see below).

Prior to initiation of these behavioral studies, mice were moved to a procedure room and weighed a minimum of one hour prior to the initiation of testing. Ethanol was injected intraperitoneally using a 20% v/v solution that was prepared in sterile saline (Baxter Health Care Corporation, Deerfield, IL) from a 200 proof solution (Pharmco Products, Brookfield, CT).

Anxiolytic effect: The elevated plus maze was used as the animal model of anxiety (see Finn et al., 1997 for details). Separate groups of mice were pretreated with saline or 2 g/kg ethanol 10 min prior to being placed on the central platform of the elevated plus maze for a 5 min test. The number of entries into the open and closed arms and the amount of time spent in the open and closed arms were measured. For an arm entry to be measured, all four paws had to be within the arm. Mice normally prefer the closed arms of the plus maze, but anxiolytic drugs increase the proportion of open arm entries and the time spent on the open arms (anxiolysis). Total arm entries were used as an index of activity.

Hypothermic effect: Mice were placed into individual holding chambers for hypothermia testing as described previously (Crabbe et al., 1989). Rectal temperatures were recorded with a Sensortek TH-8 digital thermometer after insertion of a lubricated 0.5 mm probe 2.5 cm into the rectum for 5 seconds. After baseline temperature was taken, all animals were

injected with 3 g/kg ethanol, and then rectal temperatures were taken at 30, 60 and 120 minutes post injection.

Hypnotic effect: The duration of loss of righting reflex (LORR) and blood ethanol concentration (BEC) at the return of the righting reflex (RORR) was used as the index of sensitivity to ethanol's hypnotic effect (see Browman and Crabbe, 2000 for details). Each mouse was injected with a 3.6 g/kg dose of ethanol and placed on its back in a V-shaped Plexiglas trough as soon as it showed signs of ataxia. The mouse was left undisturbed until it was able to right itself (turn over onto all 4 paws) twice within 30 sec, which was defined as the time of recovery or RORR. Then, an orbital blood sample (20 μl) was taken to assess BEC at RORR. Duration of LORR was calculated as the length of time between loss of the righting reflex and recovery.

Ataxic effect: The rotarod test for ataxia was used, as described previously (Finn et al., 1997). The AccuRotor Rota Rod (Accuscan Instruments, Columbus, OH) allowed for testing of 4 mice at a time. Each mouse was placed on the stationary rotarod, which began to accelerate linearly (20 rpm/min) until the mouse fell off. The latency to fall was then used to calculate the speed (rpm) at which the mouse could no longer remain on the rotarod. Mice were given one day of practice on the accelerating rotarod (10 trials each) to establish a stable baseline level of performance. The following day, mice received 3 baseline trials on the rotarod, followed by an injection of 2 g/kg ethanol. Mice were then placed in individual cages prior to testing. At 30 and 90 minutes post injection, each mouse was given 3 additional consecutive tests on the rotarod. Decreased latency (and rpm) corresponded to decreased performance.

Experiment 2: Metabolism—In order to rule out that a potential effect of genotype on ethanol metabolism contributed to the present findings, BEC was measured across time following injection of 2 or 4 g/kg ethanol. Each ethanol dose was tested in a separate cohort of naïve adult male and female WT and KO mice (Table 1). Briefly, mice were restrained by hand, and orbital blood samples (20 μl) were collected from each animal at 30, 60, 120, and 240 min post-injection for BEC determination. Eyes were carefully blotted following the orbital sampling, and eyes were alternated for each time point to minimize trauma. Between sampling, the mouse was returned back to its home cage. Prior work suggests that multiple orbital blood sampling did not significantly alter BECs relative to animals that had received a single blood sampling (Kamens et al., 2006).

BEC determination: Blood samples were processed and assayed for ethanol content by gas chromatography, as described elsewhere (Ford et al., 2005, and companion article). Sample peak area was interpolated against a standard curve derived from seven pairs of ethanol standards (0.25–4.0 mg/ml) to determine sample BEC.

Experiment 3: Ethanol's steroidogenic effect—Experimentally naïve adult male and female KO and WT were tested in two experimental passes (Table 1). Over 3 consecutive days, mice were habituated to handling and injected with saline. On the 4th day, separate groups of mice were injected with either saline or 2 g/kg ethanol. Thirty minutes after the final injection (on day 4), animals were rapidly euthanized, and trunk blood was collected. A

small aliquot (20 μl) of blood was reserved for BEC measurement, whereas the remainder was processed for later evaluation of plasma steroid concentrations. Plasma samples were stored at −80°C until assayed.

Allopregnanolone: ALLO was extracted from plasma (200 μl), as described in detail elsewhere (Finn and Gee, 1994; Tanchuck et al., 2009), and reconstituted in 200 μl assay buffer. Extraction efficiency was determined using 50 μl of redissolved extract by liquid scintillation spectroscopy, while 100 μl was used in the RIA. The RIA utilized [^{3}H] ALLO (10,000 cpm in 100 μl RIA buffer; Perkin Elmer, Akron, OH) and a polyclonal antiserum that was a generous gift from Dr. Kelvin Gee (University of California, Irvine, CA) with minimal cross-reactivity (Finn and Gee, 1994). Counts per minute were normalized and fit to a least-square regression equation produced by log-logit transformation of the standards (0.0195 – 20 ng). Mass of the samples was calculated by interpolation of the standards and correction for extraction efficiency. The minimum detectable limit in the present assay was 25 pg. The intra-assay and inter-assay coefficients of variation averaged 14% and 15%, respectively.

Corticosterone: A commercially available ^{125}I double antibody RIA kit (Immu-Chem Double Antibody Corticosterone for rodents; MP Biomedicals, Solon, OH) was used, as recently described (Ford et al., 2013). CORT levels were single determined from each sample (5 μl) via interpolation from a standard curve derived from six standards (25 – 1000 ng/ml). A plasma volume of 5 μl was analyzed, based on prior experience with mouse samples in this assay. The intra- and inter-assay variability was less than 5%.

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Experiment 4: Ethanol withdrawal severity—Separate groups of naïve adult male and female KO and WT mice were exposed to 72 hr ethanol vapor or air by the Portland Alcohol Research Center Dependence Core, using a standard procedure for inducing physical dependence (Finn and Crabbe, 1999; Tanchuck et al., 2009; Terdal and Crabbe, 1994). Due to animal availability, this study was conducted in three experimental passes (Table 1). Withdrawal severity was assessed by measuring handling-induced convulsions (HICs), as described previously (Gililland and Finn, 2007; Metten and Crabbe, 2005). HIC scores ranged from 0 – 7, with a score of 0 indicating no convulsions, and a score of 7 indicating spontaneous convulsions (no animals received a score of 7 in the present studies). To measure the HIC, each mouse was lifted by the tail. Scores ranging from 1 to 3 required a gentle turn to elicit a very brief tonic or clonic convulsion (1 indicates a facial grimace, 2 indicates a tonic convulsion, and 3 indicates a tonic clonic convulsion), whereas brief convulsions elicited by merely lifting the animal by the tail were scored as 4 to 6 (4 indicates a tonic convulsion, 5 indicates a tonic clonic convulsion, and 6 indicates a severe tonic clonic convulsion).

On the first day of the experiment, all animals were scored for a baseline HIC. The ethanol-exposed animals were weighed and injected with ethanol (1.5 g/kg) and pyrazole hydrochloride (pyrazole, 68.1 mg/kg; Sigma-Aldrich Company, St. Louis, MO) prior to exposure to ethanol vapor (5.5 mg ethanol per liter of air) inside inhalation chambers for 72

hrs. Pyrazole is an alcohol dehydrogenase inhibitor that is used to stabilize BECs during the ethanol vapor exposure. Air-exposed animals were treated similarly, but received only pyrazole injections and were exposed to air for 72 hrs. At 24 and 48 hrs, all mice were briefly removed from the chambers, weighed, injected with pyrazole and placed back into the chamber. Tail blood samples (20 μl) were collected from a subset of the ethanol-exposed animals each day and from all ethanol-exposed animals upon retrieval from the inhalation chambers at 72 hrs to determine BEC (target BEC was 1.5 mg/ml). Immediately prior to the blood sample taken at 72 hrs, a HIC score was obtained, then the animals were placed back into their home cages and taken to a procedure room for HIC scoring, which was performed hourly for the first 12 hrs and at hr 24 and 25 as described above.

DATA ANALYSIS

Data are expressed as mean ± SEM. For the chronic ethanol withdrawal data, area under the curve (AUC 25) was calculated for each animal (hrs 0 – 25), using the trapezoidal method, as previously described (Metten and Crabbe, 2005). The general statistical strategy was to utilize analysis of variance (ANOVA) to assess genotype (KO, WT) and sex effects on all the dependent variables, with drug/treatment as an additional factor in the analysis of the plus maze and chronic ethanol withdrawal AUC data, and time as a repeated measure for the analysis of the rotarod, hypothermia, metabolism, and chronic ethanol withdrawal HIC data. Data across experimental passes were combined, as there was no effect of experimental pass or interaction with genotype or sex. Significant interactions were pursued with Simple Main Effects analysis and post-hoc tests. Because we were predicting that there would be sex and genotype differences, trends for a significant interaction also were followed up with post-hoc tests. Significance was set at $p \leq 0.05$.

RESULTS

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Experiment 1: Behavioral testing—At the beginning of these studies, mean ± SEM body weights were: 21.19 ± 0.83 g (female KO), 21.83 ± 0.77 g (female WT), 26.85 ± 1.10 g (male KO), and 29.02 ± 1.31 g (male WT). Body weights were significantly lower in female versus male mice [$F(1,60) = 37.269$, $p < 0.001$], but there was no effect of genotype and no interaction with genotype.

Anxiolytic effect: For the analysis of the elevated plus maze data, the percent (%) open arm entries was used as the index of ethanol's anxiolytic effect (Figure 1A), although similar results were found for % open arm time. The 3-way ANOVA indicated that ethanol increased % open arm entries [$F(1,56) = 7.048$, $p = 0.01$], but there was no effect of genotype or sex. However, the trend for an interaction between sex and dose [$F(1,56) = 3.273$, $p = 0.076$] suggested that there were sex differences in ethanol's anxiolytic effect. Subsequent analyses indicated the ethanol exerted an anxiolytic effect only in female mice [$F(1,29) = 13.397$, $p = 0.001$], with post-hoc tests showing that ethanol significantly increased % open arm entries in WT ($p = 0.003$), but not in KO female mice.

Total arm entries on the elevated plus maze was used as an index of activity (Figure 1B). While genotype or sex did not alter total arm entries, there was a trend for ethanol to increase total arm entries [$F(1,56) = 2.959$, $p = 0.09$] as well as a significant interaction between genotype and dose [$F(1,56) = 4.033$, $p < 0.05$]. Subsequent analyses indicated that ethanol increased total arm entries only in WT mice [$F(1,33) = 12.937$, $p = 0.001$], with post-hoc tests showing that the increase in total arm entries was significant in male WT mice ($p = 0.002$) and was at the level of a trend in female WT mice ($p = 0.086$).

Hypothermic effect: Body temperature measurements after ethanol injection were used as an index of ethanol-induced hypothermia (Figure 2B). Baseline body temperature was significantly higher in female versus male mice [$F(1,47) = 6.398$, $p = 0.015$], but there was no effect of genotype and no interaction between genotype and sex. Repeated measures ANOVA indicated that body temperature was significantly altered across time [$F(3,141) = 87.438$, $p < 0.001$], with a trend for temperatures to be higher in females versus males [$F(1,47) = 3.884$, $p = 0.055$], which likely was due to the higher baseline temperatures in the female mice. There were no interactions with genotype and no 3-way interaction, indicating that ethanol-induced hypothermia was similar in male and female, KO and WT mice. This conclusion is supported by re-analysis of the data as a change from baseline, which only revealed a main effect of time [$F(2,94) = 17.592$, $p < 0.001$] (i.e., no effect of sex or genotype and no interactions; data not shown).

Hypnotic effect: LORR duration and BEC at RORR were used as the index of ethanol's hypnotic effect (Figure 3). While there was a trend for LORR duration to be lower in female versus male mice [$F(1,59) = 3.434$, $p = 0.069$], there was no overall effect of genotype and no interaction between genotype and sex. There also were no significant differences in BEC at RORR (i.e., no effect of sex or genotype and no interaction).

Ataxic effect: Rotarod performance following ethanol injection was used as the index of ataxia. The analysis of latency to fall and speed (rpm) at which the mouse could no longer remain on the rotarod produced identical results, so rpm analysis will be described, and these data are depicted in Figure 2A. Baseline performance on day 2 (i.e., prior to ethanol injection) was not influenced by genotype or sex, and there was no interaction between genotype and sex. Similarly, repeated measures ANOVA revealed that performance was significantly altered across time [$F(2,42) = 13.066$, $p < 0.001$], but there was no interaction with genotype or sex and no 3-way interaction. When data were re-analyzed, collapsed across sex, there again was only a main effect of time [$F(2,46) = 14.822$, $p < 0.001$].

Experiment 2: Metabolism—For the 2 g/kg study, mean ± SEM body weights were: 22.99 ± 1.38 g (female KO), 20.70 ± 0.42 g (female WT), 26.37 ± 1.84 g (male KO), and 27.35 ± 2.14 g (male WT). For the 4 g/kg study, mean ± SEM body weights were: 22.43 ± 0.68 g (female KO), 22.90 ± 1.66 g (female WT), 30.20 ± 0.89 g (male KO), and 33.65 ± 3.35 g (male WT). Overall, body weights were significantly lower in female versus male mice [2 g/kg: $F(1,28) = 10.248$, $p = 0.003$; 4 g/kg: $F(1,18) = 45.832$, $p < 0.001$], but there was no effect of genotype and no significant interaction with genotype.

BEC data across the four time points following injection of 2 and 4 g/kg ethanol are depicted in Figure 4. For the 2 g/kg data (Figure 4A), BECs changed significantly across time $F(3,84) = 510.678$, $p < 0.001$], and there was a trend for an interaction between time and genotype [$F(3,84) = 2.438$, $p = 0.070$]. Subsequent analyses suggested that the interaction was due to the tendency for BECs to be higher in KO versus WT mice [$F(1,30) = 2.902$, $p = 0.099$]. For the 4 g/kg data (Figure 4B), BECs again changed significantly across time [$F(3,54) = 194.397$, $p < 0.001$], and there was a significant interaction between time and sex [$F(3,54) = 3.473$, $p < 0.05$]. While the interaction suggests that BECs were changing across time differently in the male and female mice, subsequent analyses conducted on each sex revealed only a main effect of time [$F(3,15) = 102.806$, $p < 0.001$ for males; $F(3,39) = 174.007$, $p < 0.001$ for females] and no interaction with genotype in either sex.

Experiment 3: Ethanol's steroidogenic effect—Prior to ethanol or saline injection on day 4, mean ± SEM body weights were: 23.01 ± 0.56 g (female KO), 23.43 ± 1.00 g (female WT), 31.73 ± 1.71 g (male KO), and 29.00 ± 1.08 g (male WT). Body weights were significantly lower in female versus male mice [$F(1,80) = 41.409$, $p < 0.001$], but there was no effect of genotype and no interaction with genotype.

Injection of 2 g/kg ethanol produced an expected rise in BECs. Mean ± SEM BECs were 1.70 ± 0.11 mg/ml (male WT, n=10), 1.66 ± 0.23 mg/ml (male KO, n=10), 1.33 ± 0.13 mg/ml (female WT, n=10), and 1.34 ± 0.11 mg/ml (female KO, n=14). BECs did not vary as a factor of genotype, but they were significantly lower in female versus male mice [$F(1,40) = 5.593$, $p < 0.05$].

Plasma ALLO levels revealed only a main effect of sex [$F(1,71) = 6.321$, $p < 0.05$], with males exhibiting significantly greater levels than females (Figure 5B), whereas plasma CORT levels were significantly increased by ethanol injection [$F(1,66) = 49.259$, $p < 0.001$], and they were higher in WT versus KO mice [$F(1,66) = 7.633$, $p < 0.01$] and in female versus male mice [$F(1,66) = 6.783$, $p = 0.01$] (Figure 5A). There was a significant interaction between sex and treatment [$F(1,66) = 4.991$, $p < 0.05$] and a trend for a 3-way interaction [$F(1,66) = 3.145$, $p = 0.08$], indicating that there were sex and genotype differences in the effect of ethanol injection on plasma CORT levels. In female mice, plasma CORT concentrations were significantly increased by ethanol injection [$F(1,34) = 28.424$, $p < 0.001$] and were higher in WT versus KO mice [$F(1,34) = 4.798$, $p < 0.05$]. The lack of significant interaction between treatment and genotype confirmed that ethanol injection produced a similar increase in plasma CORT levels in KO and WT female mice. In contrast, ethanol injection differentially altered plasma CORT levels in the male KO versus WT mice. While CORT concentrations were significantly increased by ethanol injection [$F(1,32) = 26.021$, $p < 0.001$), they tended to be higher in WT versus KO mice [$F(1,32) = 3.38$, $p = 0.075$], and there was a strong trend for a 2-way interaction between treatment and genotype [$F(1,32) = 3.947$, $p = 0.056$]. Post-hoc analyses confirmed that ethanol injection significantly increased plasma CORT levels only in the male KO mice [$F(1,16) = 29.00$, $p < 0.001$], because the increase in male WT mice only reached the level of a strong statistical trend [$F(1,16) = 4.277$, $p = 0.055$].

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Experiment 4: Ethanol withdrawal severity—Prior to the initiation of exposure to 72 hr ethanol vapor or air, mean ± SEM body weights were: 22.48 ± 0.63 g (female KO air), 22.86 ± 0.84 g (female KO ethanol), 21.95 ± 0.63 g (female WT air), 23.73 ± 0.66 g (female WT ethanol), 30.04 ± 0.75 g (male KO air), 28.58 ± 0.80 g (male KO ethanol), 28.17 ± 0.75 g (male WT air), and 28.83 ± 0.74 g (male WT ethanol). Body weights were significantly lower in female versus male mice [$F(1,99) = 136.123$, $p < 0.001$], but there was no effect of genotype or treatment and no significant interactions with sex.

Following exposure to 72 hr ethanol vapor, BECs were not significantly influenced by sex or genotype (nor was there an interaction of these factors). In general values approximated the 1.5 mg/ml target BEC. The mean ± SEM BEC for each genotype and sex was: 1.39 ± 0.16 (female KO, n=13), 1.67 ± 0.15 (female WT, n=14), 1.39 ± 0.15 (male KO, n= 15), and 1.48 ± 0.16 (male WT, n=15).

Hourly HIC scores were significantly higher in the ethanol- versus air-exposed mice [$F(1,97) = 114.16$, $p < 0.001$] (Figure 6A & B), indicating that the animals exposed to 72 hrs of ethanol vapor were undergoing withdrawal upon removal from the inhalation chamber. Exposure to air + pyrazole produced a slight enhancement of HIC within the early phase of withdrawal (i.e., 1st 12 hrs), which subsequently decreased to baseline levels. This finding is consistent with the report by Homanics et al. (1998) in which control 129/SvJ mice exhibited marked HIC that peaked approximately 2–3 hr post-removal from the inhalation chamber and returned to baseline at 8 hr. Thus, all of our experiments employed the use of pyrazole in the air controls to offset any potential confound due to a small proconvulsant effect of pyrazole in some genotypes.

Neither genotype nor sex had a significant impact on HIC scores, but there was a significant interaction between treatment and sex [$F(1,97) = 8.977$, $p = 0.003$]. HIC scores changed significantly across time [$F(14,1358) = 44.928$, $p < 0.001$], and the pattern across time was dependent upon treatment [$F(14,1358) = 16.848$, $p < 0.001$ for the time x treatment interaction]. As is apparent in Figure 6A and 6B, hourly HIC scores remained elevated for a longer period of time after withdrawal in the ethanol-exposed mice and decreased across time in the air-exposed mice. Also, the pattern of change in HIC scores across time differed in the male and female mice exposed to ethanol vapor or air. This conclusion is supported by the significant interaction of sex and time [$F(14,1358) = 2.133$, $p = 0.008$] and of sex, treatment, and time [$F(14,1358) = 1.911$, $p < 0.05$]. The interactions with sex were pursued with Simple Main Effects analyses. In the ethanol exposed mice, hourly HICs were significantly higher in male versus female mice [$F(1,53) = 12.357$, $p = 0.001$], and they changed significantly across time [$F(14,472) = 24.884$, $p < 0.001$]. The significant interaction between sex and time [$F(14,742) = 1.924$, $p < 0.05$] was due to the fact that HICs remained elevated across time in the males, but decreased across time in the female mice. In contrast, there was no sex difference in HIC scores in the air-exposed mice. However, HICs did change significantly across time [$F(14,672) = 33.470$, $p < 0.001$], and the significant interaction between sex and time [$F(14,672) = 2.039$, $p < 0.05$] indicated that the pattern of change in HIC scores differed across time in the air-exposed male and female mice.

Analysis of AUC 25, an index of total ethanol withdrawal severity, yielded similar results to that for the hourly HIC scores (Figure 6C). AUC 25 was significantly higher in the ethanol- versus air-exposed mice [$F(1,99) = 105.078$, $p < 0.001$], and there was a significant interaction between sex and treatment [$F(1,99) = 7.349$, $p < 0.01$]. Subsequent analyses revealed that AUC 25 was significantly lower in the ethanol-exposed female versus male mice [$F(1,53) = 11.22$, $p = 0.001$], but AUC 25 did not differ in the air-exposed male and female mice.

DISCUSSION

The present studies examined a series of acute ethanol-related behaviors, ethanol metabolism, ethanol's steroidogenic effect, and chronic ethanol withdrawal severity in *Srd5a1* KO and WT mice to determine the correspondence between deletion of *Srd5a1* and pharmacological inhibition of 5α-reductase with finasteride, which inhibits both isoforms of the enzyme in the rodent (Finn et al., 2006a). Consistent with results with finasteride, *Srd5a1* deletion significantly reduced ethanol's anxiolytic effect and did not alter ethanol-induced hypnosis or ataxia. However, chronic ethanol withdrawal severity did not differ in KO versus WT mice, nor did ethanol exert a steroidogenic effect in either genotype. Thus, the presence of 5α-reductase-2 in the KO mice may have contributed to some of these differences, when compared with results following finasteride.

Basal levels of activity, anxiety, rotarod performance, body temperature, or HICs were not influenced by *Srd5a1* genotype. These results are consistent with earlier work in which male KO and WT mice did not differ in basal levels of aggression or seizure susceptibility to pentylenetetrazol (Frye et al., 2001, 2002b) and female KO and WT mice did not differ in basal levels of activity, anxiety, nociceptive behavior, depressive symptoms, or seizure susceptibility to pentylenetetrazol (Frye et al., 2002a, 2004). Work by Frye and colleagues also determined that basal hippocampal ALLO levels did not differ in KO versus WT male and female mice that were gonadectomized (Osborne and Frye, 2009) and that basal ALLO levels in plasma, whole brain or various brain regions did not differ in KO versus WT female mice that were ovariectomized or in diestrus (Frye et al., 2004; Koonce et al., 2012; Koonce and Frye, 2013, 2014). We also did not observe a sex or genotype difference in plasma ALLO levels following saline injection, suggesting that the female mice were in diestrus at the time of euthanasia. Thus, the similar baseline behavioral measures in KO and WT mice were not influenced by basal ALLO levels.

Our original hypothesis for a genotype difference in chronic ethanol withdrawal and/or ethanol's anxiolytic effect in the *Srd5a1* mutant mice was based on the supposition that endogenous levels of the GABAergic neurosteroid ALLO might differ in the KO versus WT mice following acute or chronic ethanol exposure. With regard to ethanol's steroidogenic effect, male and female KO and WT mice were resistant to the ability of an acute 2 g/kg ethanol injection to increase plasma ALLO levels. We had previously found that a 2 g/kg ethanol injection increased brain ALLO levels by 1.7-fold in male C57BL/6J mice (Finn et al., 2004c), but more recent work indicated that ethanol injection did not increase ALLO levels in plasma, hippocampus or cortex of male C57BL/6J and DBA/2J mice (Porcu et al., 2010, 2014). Regardless, the present findings indicate that a 2 g/kg ethanol injection did not

significantly increase plasma ALLO levels in mice that were on a C57BL/6J x 129/SvJ background, irrespective of *Srd5a1* genotype or sex, and the levels in female mice suggest that the females were in diestrus. Data indicate that chronic ethanol vapor exposure, did not alter plasma ALLO levels in dependent male C57BL/6J mice or during withdrawal (Finn et al., 2000, 2004a), but it did disrupt normal estrous cycling in female mice so that the animals remained in diestrus during withdrawal (Veatch et al., 2007). Since data are not available in 129/SvJ mice, we do not know whether chronic ethanol exposure and withdrawal would decrease endogenous ALLO levels in the C57BL/6J x 129/SvJ hybrid (WT), and how *Srd5a1* genotype and sex would impact the effect of chronic ethanol exposure and withdrawal on ALLO levels.

In contrast to the reduced behavioral effects of progesterone or testosterone in the KO versus WT mice (Frye et al., 2001, 2002a, 2002b, 2004), *Srd5a1* genotype did not influence sensitivity to ethanol-induced hypothermia, ataxia, or hypnosis. Ethanol metabolism also was not altered by genotype or sex, and the results following 4 g/kg were similar to our previous data in intact male and female C57BL/6J mice (Gililland and Finn, 2007). Overall, these results are not surprising, when one considers that ethanol injection did not significantly increase plasma ALLO levels in the KO or WT mice in the present study and that pharmacological manipulation of endogenous ALLO levels did not alter ethanol-induced ataxia or hypnosis in earlier work (Khisti et al., 2003, 2004). Collectively, the similar acute sensitivity to ethanol-induced hypothermia, ataxia, and hypnosis in KO and WT mice was independent of an ethanol-induced change in endogenous ALLO levels.

An interesting finding was that ethanol exerted a significant anxiolytic effect only in female WT but not female KO mice; ethanol was not anxiolytic in male KO or WT mice. We predicted that ethanol would exert an anxiolytic effect in WT but not in KO mice, based on previous work in male rats in which the anxiolytic effect of ethanol was potentiated by treatments that increased endogenous ALLO (e.g., ALLO or progesterone pretreatment) and blocked by treatments that decreased endogenous ALLO (e.g., finasteride or adrenalectomy; Hirani et al., 2005). So, the lack of an anxiolytic effect in WT males was not anticipated. The WT mice in the present studies were on a mixed C57BL/6J x 129/SvJ background, and recent work indicated that genetic background influenced several ethanol-related phenotypes (but anxiolytic effect not assessed) in male WT mice (Blednov et al., 2010); differences were observed in mice that were backcrossed onto 129S1/SvimJ versus C57BL/6J backgrounds. Thus, it is possible that the genetics of the background strains contributed to the lack of anxiolytic effect in the male mice.

With regard to the elevated plus maze results in female mice, recent work found that stage of the estrous cycle influenced behavior on the elevated plus maze in female *Srd5a1* KO and WT mice that were congenic on a C57BL/6 background and exhibiting normal 4–5 day long estrous cycles (Koonce et al., 2012). While open arm time tended to be higher in WT versus KO females, it was increased in KO and WT mice during proestrus versus diestrus (although the increase was lower in the KO mice, and open arm time did not differ between KO and WT mice during diestrus). Additionally, plasma progesterone did not differ in WT and KO mice during proestrus or diestrus, but plasma estradiol levels were significantly higher in KO versus WT mice, and cortical and hippocampal ALLO levels were significantly lower in

KO versus WT mice during proestrus (but not diestrus). Although we did not monitor estrous cycle phase in the present study, we presume that the KO and WT mice on a mixed C57BL/6J x 129/SvJ background also exhibited normal 4–5 day estrous cycles as in Koonce et al. (2012). So, the similar % open arm entries in the saline-injected KO and WT mice suggest that the mice were in diestrus. When compared to the results in male mice, it is possible that the genetics of the background strains interacted with stage of estrous cycle and higher estradiol levels in the KO versus WT mice to impact the genotype differences in sensitivity to ethanol's anxiolytic effect in female mice.

Following the 72 hr of chronic ethanol vapor exposure, BEC upon the initiation of withdrawal did not differ among groups. These data indicate that the significant sex difference in chronic ethanol withdrawal severity was not due to variations in ethanol exposure level. Notably, the lower withdrawal in female versus male mice is consistent with earlier work (e.g., Veatch et al., 2007). The persistent elevation in HIC scores during withdrawal in the male mice also is consistent with work showing that seizure susceptibility during withdrawal remained elevated in male rodents for a longer duration (Alele and Devaud, 2007; Devaud and Chadda, 2001). Collectively, the studies are indicative of a faster recovery from ethanol withdrawal in female rodents.

Since the WT mice are a hybrid of C57BL/6J and 129/SvJ background strains, we were unsure whether ethanol withdrawal in this genotype would be mild (similar to C57BL/6J) or moderate to severe (similar to 129/SvJ) and whether the air-controls would exhibit mild HICs as has been reported for 129/SvJ genotypes (Homanics et al., 1998; Metten and Crabbe, 2005). The pattern of hourly HIC scores in the ethanol- and air-exposed mice in the present study (Figure 6A & 6B) was comparable to that in the 129/SvJ male mice (Homanics et al., 1998; Metten and Crabbe, 2005), consistent with the contribution of genes from 129/SvJ strains to the HIC phenotype in the KO and WT mice. For the air-exposed KO and WT mice, the slight elevation in hourly HICs returned to baseline levels by 8 hrs post-removal from the inhalation chambers as was shown for 129/SvJ mice (Homanics et al., 1998), and the AUC 25 was identical to that reported for 129S1/Sv1mJ mice (Metten and Crabbe, 2005). Consistent with the chronic ethanol withdrawal phenotype following 72 hr ethanol vapor exposure for 129 strain genotypes (e.g., Homanics et al., 1998; Metten and Crabbe, 2005), hourly HICs remained elevated across the 25 hr period in the KO and WT mice. It is possible that the high withdrawal in the male WT mice made it difficult to observe a significant increase in withdrawal severity in the KO mice due to a "ceiling" effect. However, it is unlikely that a "ceiling" effect can explain the lack of a genotype effect in the female mice that exhibited relatively moderate chronic ethanol withdrawal severity. Another potential explanation for the lack of genotype difference in chronic withdrawal is that the presence of 5α-reductase-2 in the KO mice compensated for any impact of *Srd5a1* deletion on ethanol withdrawal severity. We have found that microinjection of finasteride into discrete brain regions, which would only inhibit *Srd5a1*, was required to significantly increase chronic ethanol withdrawal severity (Gililland-Kaufman et al., 2008; Tanchuck et al., 2013), whereas systemic finasteride did not significantly alter chronic ethanol withdrawal severity in male and female C57BL/6J mice (Finn et al., 2004b). Future studies could pharmacologically inhibit the type 2 enzyme in the

Srd5a1 mutants or test the double mutants that have been developed (mice lacking *Srd5a1* and *Srd5a2*; Mahendroo et al., 2001) and determine the impact on chronic ethanol withdrawal severity.

Another possibility is that $GABA_AR$ expression or sensitivity could have changed during development or adulthood to compensate for the deletion of *Srd5a1* in the mutant mice. For instance, we have found that sensitivity to ALLO's anticonvulsant effect and to effects of ALLO on $GABA_AR$ function was significantly decreased during ethanol withdrawal in genotypes with high chronic ethanol withdrawal (Finn et al., 2000, 2006b). The expression of many $GABA_AR$ subunits is altered following chronic ethanol exposure and withdrawal (see Kumar et al., 2009), but data are not available on the expression of $GABA_AR$ subunits in control KO or WT mice or during withdrawal. Thus, the lack of genotype difference in ethanol withdrawal severity suggests that $GABA_AR$ subunit expression was not differentially altered following chronic ethanol exposure in the KO versus WT mice. The results also suggest that it is unlikely that a developmental compensation for the deletion of the *Srd5a1* gene exerted a modifying effect on the activity or expression of certain $GABA_AR$ subunit genes to influence chronic ethanol withdrawal.

The *Srd5a1* deletion mutation was generated using 129/SvJ stem cells and was preserved in C57BL/6J x 129/SvJ hybrid mice (Mahendroo et al., 1996). We presume that the initial characterization of the levels of the two isoforms of the 5α-reductase enzyme has not changed, because we obtained heterozygote breeding pairs from re-derived cryopreserved embryos from The Jackson Laboratory. The original analysis of *Srd5a1* mRNA in WT mice suggested that there was no sexual dimorphism present in the levels of type 1 mRNA from 12 tissues of male and female mice, including brain (Mahendroo et al., 1996). In contrast, type 2 mRNA expression was sexually dimorphic in WT mice, with at least 30-fold lower expression in female versus male adrenal tissue. No stable type 1 mRNAs were detected in KO mice, and the 5α-reductase type 2 gene did not appear to be induced to compensate for the loss of the type 1 gene in the KO animals. However, we cannot rule out the possibility that the limited genotype differences we have seen are associated with linked genes originating from the 129/SvJ strain, since the mutation is in linkage disequilibrium with genes from that strain (see Gerlai, 1996; Wolfer et al., 2002).

In conclusion, deletion of the *Srd5a1* gene significantly decreased ethanol's anxiolytic effect in female mice, and chronic ethanol withdrawal severity was lower in female versus male mice. Taken in conjunction with data indicating that deletion of the *Srd5a1* gene altered various measures of ethanol consumption in a sex dependent manner (Ford et al., companion paper), sex differences in the ability of *Srd5a1* genotype to modulate ethanol's anxiolytic effect and ethanol drinking behavior may reflect an interaction between developmental compensations to deletion of the *Srd5a1* gene with sex hormones and levels of endogenous $GABA_AR$-active neurosteroids.

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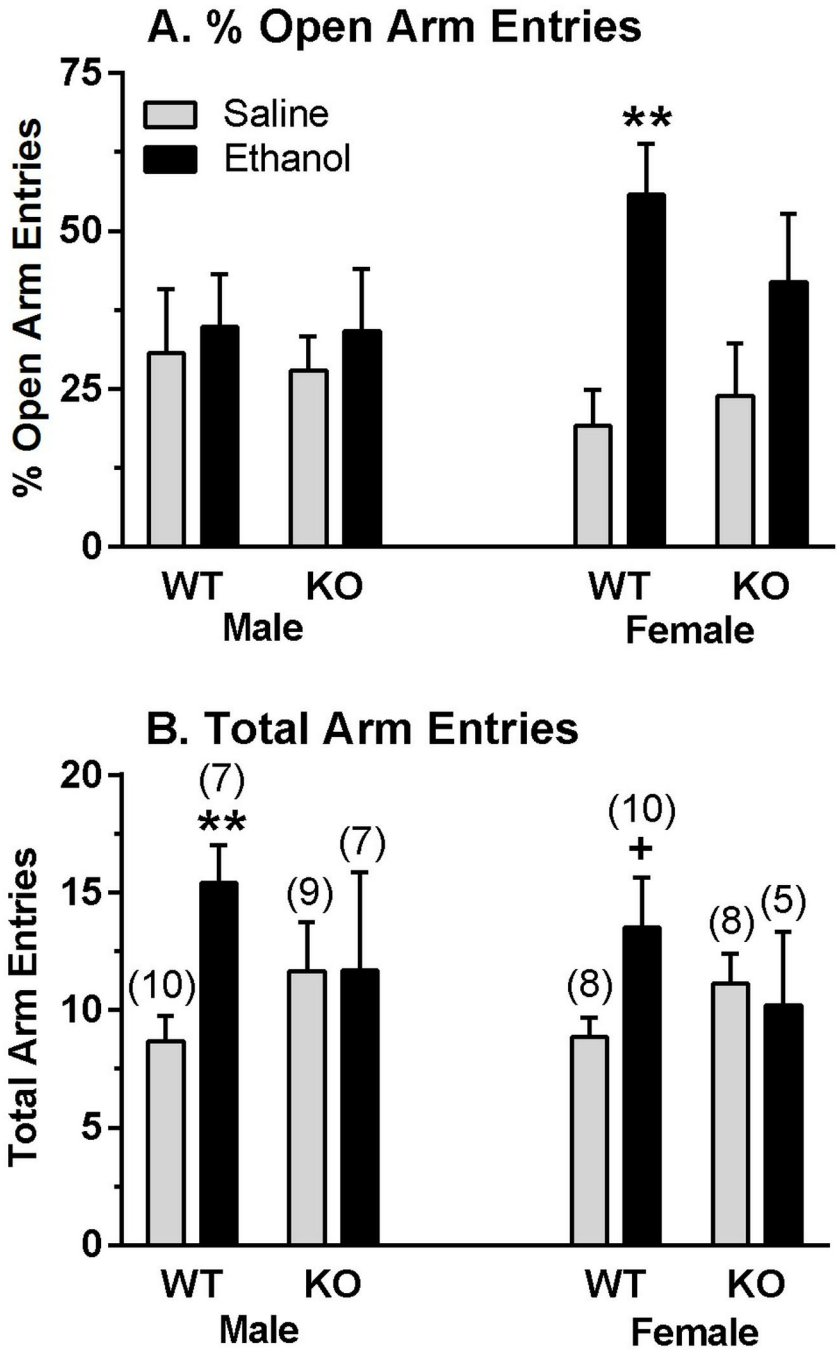

FIGURE 1.

The effect of acute ethanol (2 g/kg) on elevated plus maze behavior in male and female *Srd5a1* knockout (KO) and wildtype (WT) mice. Deletion of the *Srd5a1* gene significantly decreased ethanol's anxiolytic effect, measured by percent (%) open arm entries in female KO versus WT mice **(A)**, and decreased ethanol's effect on total entries, an index of activity, in KO versus WT mice **(B)**. Values are mean ± SEM for the number of animals in parentheses. $^{+}p<0.10$, $^{**}p<0.01$ versus respective saline group

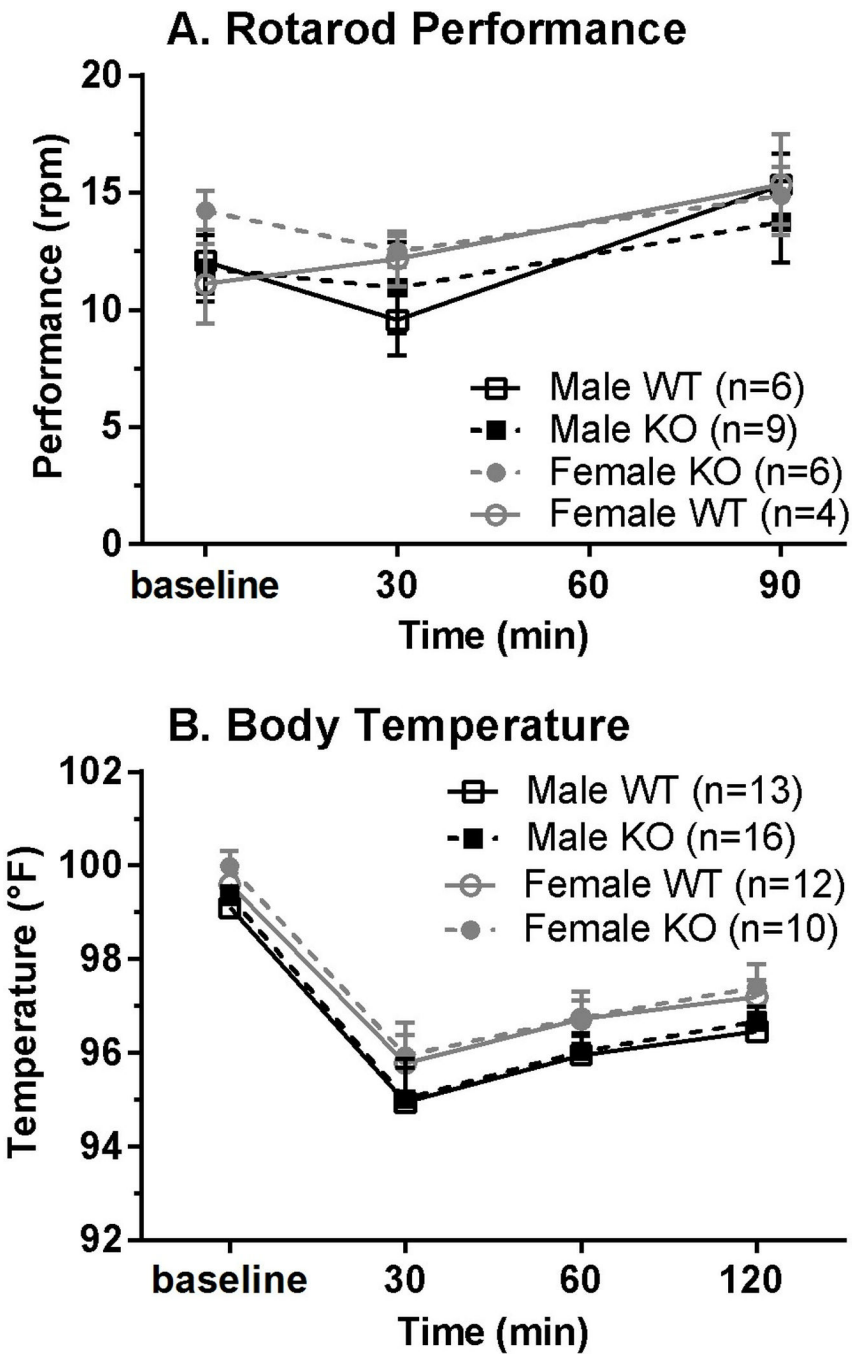

FIGURE 2.

Sensitivity to ethanol's ataxic (2 g/kg) effect **(A)** and hypothermic (3 g/kg) effect **(B)** in male and female *Srd5a1* knockout (KO) and wildtype (WT) mice. **(A)** The ethanol-induced decrease in rotarod performance at 30 min did not differ significantly in KO versus WT mice. **(B)** While body temperature was higher in female versus male mice, the ethanol-induced decrease in body temperature did not differ in KO versus WT mice. Depicted are the mean ± SEM for the number of animals in parentheses.

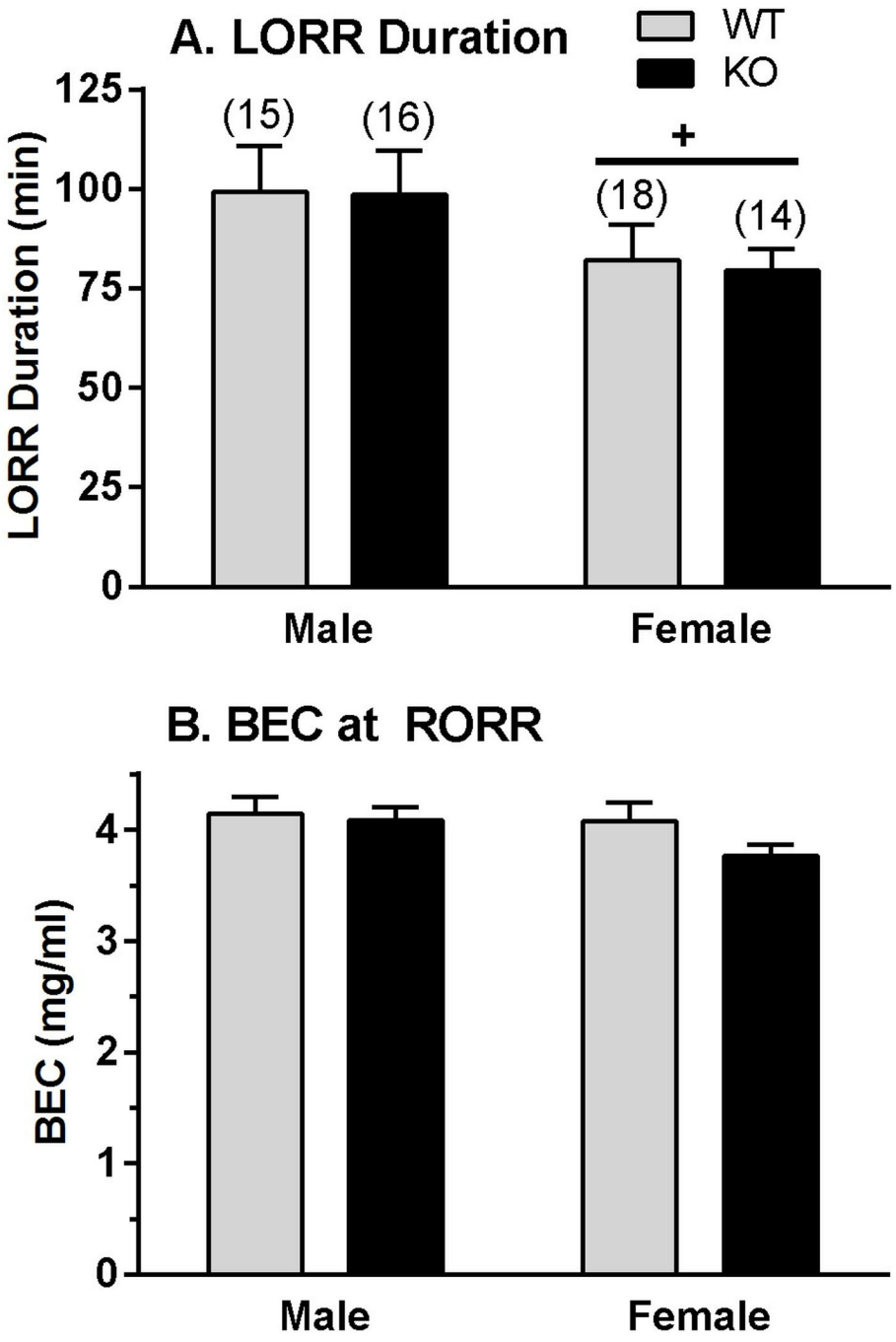

FIGURE 3.

Ethanol's hypnotic (3.6 g/kg) effect did not differ in male and female *Srd5a1* knockout (KO) and wildtype (WT) mice, measured by loss of righting reflex (LORR) duration **(A)** and blood ethanol concentration at return of righting reflex; BEC at RORR **(B)**. Shown are mean ± SEM for the number of animals in parentheses. $^{+}p<0.10$ versus males

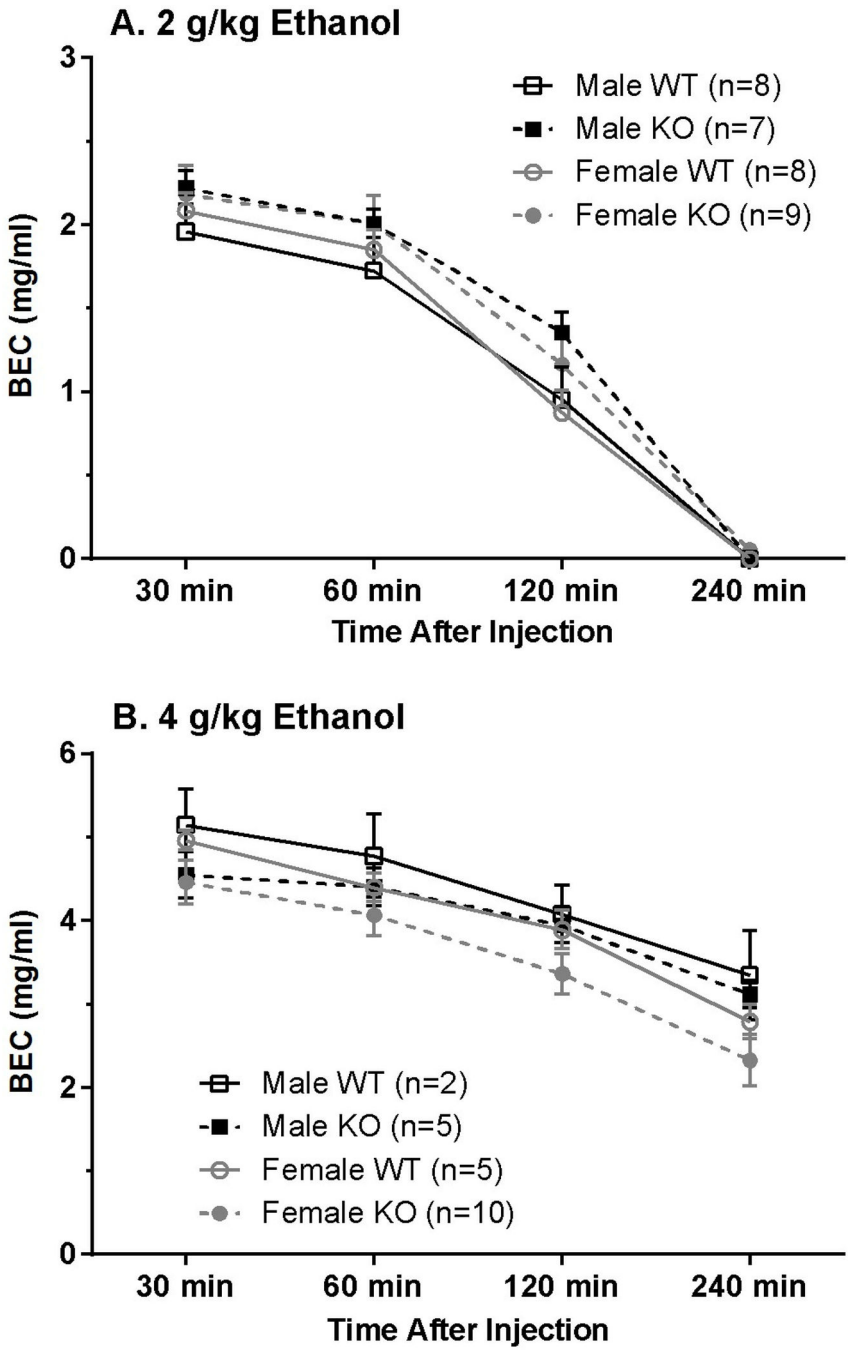

FIGURE 4.

Srd5a1 genotype and sex did not significantly alter ethanol metabolism following an acute 2 g/kg injection **(A)** or an acute 4 g/kg injection **(B)**. Values represent the mean ± SEM for the number of animals in parentheses.

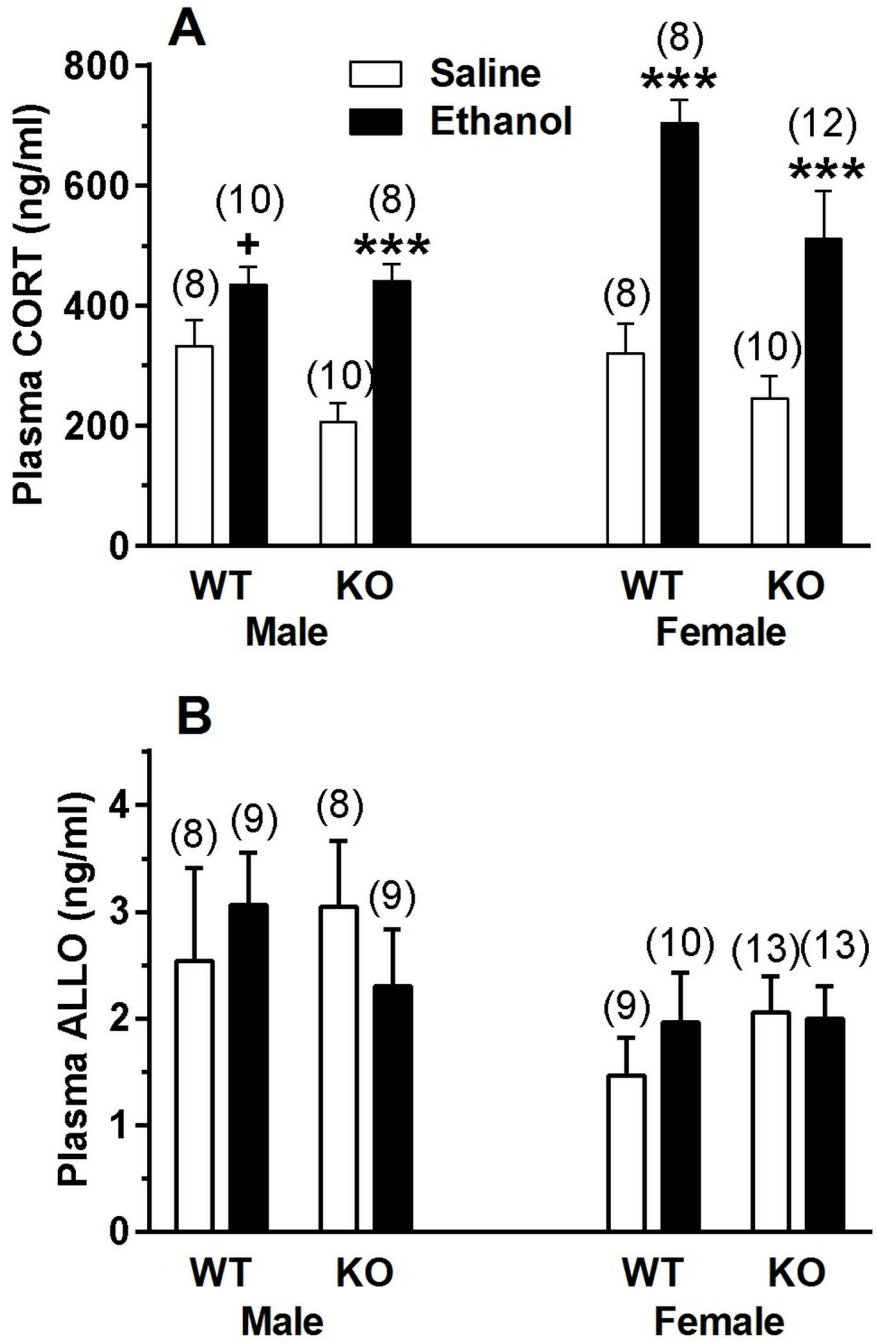

FIGURE 5.

Effect of systemic ethanol injection (2 g/kg) on plasma corticosterone (CORT) levels **(A)**, and plasma allopregnanolone (ALLO) levels **(B)** in male and female *Srd5a1* knockout (KO) and wildtype (WT) mice. Separate groups of mice were injected with ethanol or saline and euthanized 30 min later. Values are the mean ± SEM, and group sizes are provided in parentheses. $^{+}p<0.10$, ***$p<0.001$ versus respective saline group

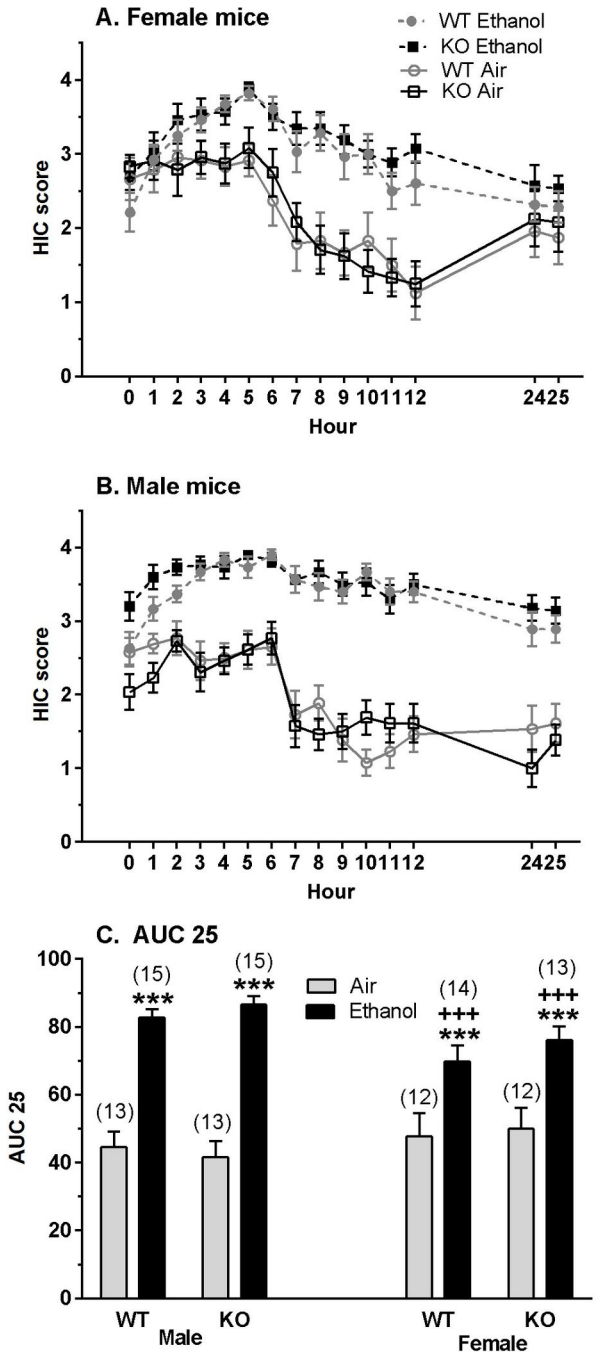

FIGURE 6.

Chronic ethanol withdrawal severity, measured by hourly handling-induced convulsion (HIC) scores **(A & B)** and area under the curve (AUC 25) **(C)**, in male and female *Srd5a1* knockout (KO) and wildtype (WT) mice. Baseline HIC scores (not shown) did not differ in male and female KO and WT mice. While 72 hrs of ethanol vapor exposure significantly increased HIC scores and AUC 25, these measures of chronic withdrawal severity were

significantly lower in female versus male mice. Values represent the mean ± SEM for the number of animals in parentheses.

Table 1

Experimental Details

Experiment	Cohort	Experimental Measurements + (Ethanol Dose)
1	A	Elevated plus maze (2 g/kg), body temperature (3 g/kg), LORR duration (3.6 g/kg), rotarod (2 g/kg)
	B	Elevated plus maze (2 g/kg), LORR duration (3.6 g/kg), body temperature (3 g/kg)
	C	Elevated plus maze (2 g/kg), LORR duration (3.6 g/kg), body temperature (3 g/kg)
2	A	Ethanol metabolism (2 g/kg)
	B	Ethanol metabolism (4 g/kg)
3	A	Ethanol's steroidogenic effect (2 g/kg)
	B	Ethanol's steroidogenic effect (2 g/kg)
4	A	Chronic ethanol withdrawal (72 hr ethanol vapor)
	B	Chronic ethanol withdrawal (72 hr ethanol vapor)
	C	Chronic ethanol withdrawal (72 hr ethanol vapor)

Due to animal availability, separate cohorts of experimentally naïve male and female knockout and wildtype mice were tested in each experiment. In Experiment 1, the ethanol-related behaviors were examined once per week in the order shown for each cohort. Note that rotarod performance was measured only in cohort A, resulting in a lower group size for that dependent measure. Data were combined for Experiments 1, 3 & 4, as each cohort of mice received the same ethanol doses. Data were not combined in Experiment 2, because each cohort of mice was injected with a different dose of ethanol. LORR = loss of righting reflex