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Null Mutation of 5α-Reductase Type I Gene Alters Ethanol Consumption Patterns in a Sex-Dependent Manner

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Abstract

The neuroactive steroid allopregnanolone (ALLO) is a positive modulator of $GABA_A$ receptors, and manipulation of neuroactive steroid levels via injection of ALLO or the 5α-reductase inhibitor finasteride alters ethanol self-administration patterns in male, but not female, mice. The *Srd5a1* gene encodes the enzyme 5α-reductase-1, which is required for the synthesis of ALLO. The current studies investigated the influence of *Srd5a1* deletion on voluntary ethanol consumption in male and female wildtype (WT) and knockout (KO) mice. Under a continuous access condition, 6 and 10 % ethanol intake was significantly greater in KO versus WT females, but significantly lower in KO versus WT males. In 2-h limited access sessions, *Srd5a1* deletion retarded acquisition of 10 % ethanol intake in female mice, but facilitated it in males, versus respective WT mice. The present findings demonstrate that the *Srd5a1* gene modulates ethanol consumption in a sex-dependent manner that is also contingent upon ethanol access condition and concentration.

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Human and Animal Rights and Informed Consent: All institutional and national guidelines for the care and use of laboratory animals were followed.

Keywords

Neurosteroid; Alcohol; Lickometer; Saccharin; Drinking patterns

Introduction

The neurosteroid allopregnanolone (ALLO; 3α-hydroxy-5α-pregnan-20-one) is a potent positive modulator of γ-aminobutyric acid$_A$ receptors (GABA$_A$Rs) that is formed from the 2-step reduction of progesterone (e.g., Belelli and Lambert 2005). This profile may underlie its ability to modulate the abuse-related effects of ethanol, particularly as an anticonvulsant, anxiolytic, locomotor stimulant, and hypnotic. The major route of progesterone metabolism in the rodent brain is via 5α-reduction; the 5α-reduction of progesterone to form 5α-dihydroprogesterone (5α-DHP) is an irreversible reaction in mammalian cells (see Celotti et al. 1997). Based on conversion of progesterone to 5α-DHP, the following pattern of 5α-reductase activity was observed in mouse brain: midbrain tegmentum > hypothalamus > hippocampus > cerebral cortex (Roselli and Snipes 1984). A similar brain regional distribution of 5α-reductase activity was found in rat brain (e.g., Li et al. 1997). Of the two isoforms of the 5α-reductase enzyme, type 1 (5α-reductase-1, *Srd5a1*) is widely expressed in the rodent nervous system; the expression is similar in males and females and does not appear to be controlled by androgens (see Melcangi et al. 1998). In contrast, 5α-reductase-2 is primarily expressed in peripheral steroidogenic tissues.

There are some physiological and behavioral ramifications of *Srd5a1* gene disruption in mice. Two initial studies with the null mutant model determined that female *Srd5a1* knockout (KO) mice exhibited a parturition defect of maternal origin that was attributable to the inability to reduce androgens into steroid metabolites with a 5α stereo-configuration, and that ultimately resulted in excess estrogen production to levels that undermine pregnancy (Mahendroo et al. 1996, 1997). Subsequent work in ovariectomized females revealed no significant changes between wildtype (WT) and KO mice in basal levels of seizure susceptibility, locomotion, time spent in open arm of the elevated plus maze, latency to paw lick, and latency and duration of immobility in the forced swim test (Frye et al. 2002a, 2004). Similarly, gonadectomized male KO and WT mice did not differ in basal levels of aggression or seizure susceptibility (Frye et al. 2001, 2002b). However, it was noted that female and male KO mice lacked significant behavioral responses to exogenous progesterone or testosterone, respectively, within these tasks that were apparent in WT mice. This later observation suggests that the 5α-reductase-1 enzyme plays a role in mitigating some of behavioral effects of progesterone and testosterone.

Manipulation of endogenous ALLO levels also has been shown to influence some measures of ethanol sensitivity, such as ethanol's anxiolytic effect (Hirani et al. 2005), and this involved an interaction at GABA$_A$Rs (e.g., Kumar et al. 2009; Morrow et al. 2001). Recently, we examined the influence of *Srd5a1* deletion in intact male and female mice on several acute effects of ethanol and found that genotype and sex did not differentially alter ethanol-induced hypothermia, ataxia, hypnosis, or metabolism (see companion article; Tanchuck-Nipper et al. 2014). However, deletion of the *Srd5a1* gene significantly decreased

ethanol's effect on total entries in the elevated plus maze, which is an index of activity, versus WT mice and significantly decreased ethanol's anxiolytic effect, measured by percent open arm entries and time, in female KO versus WT mice. These elevated plus maze data are consistent with the work of Hirani and colleagues (2005), in which the 5α-reductase inhibitor finasteride significantly reduced ethanol's anxiolytic effect.

Despite the limited effect of *Srd5a1* deletion on the measures of ethanol sensitivity described above, a sizeable base of literature indicates that 5α -reduced steroids influence ethanol self-administration and related behaviors. First, the 5α -reduced pregnane neurosteroid ALLO enhanced limited access ethanol self-administration when administered at low dose, but suppressed drinking at higher doses in both male mice under a two-bottle home cage drinking condition (Ford et al. 2005b) and male rats under an operant condition (Janak et al. 1998; Janak and Gill 2003). Recent work with ganaxolone, a synthetic derivative of ALLO that exhibits metabolic resistance, reported a similar bimodal influence of this neurosteroid on operant ethanol self-administration in male alcohol preferring (P) rats (Besheer et al. 2010). Curiously, female C57BL/6J mice were unresponsive to these modulatory effects of ALLO on limited access drinking in a procedure identical to that reported for C57BL/6J males (Ford et al. 2008a; also see Finn et al. 2010). Second, treatment with finasteride dose-dependently attenuated the acquisition (Ford et al. 2008b) and the maintenance (Ford et al. 2005a) of limited access ethanol intake in male mice, but a much higher dose was required to decrease ethanol drinking in female mice. It was notable that the finasteride-induced decrease in ethanol intake was due to a sex-specific mechanism of drinking pattern alteration (Ford et al. 2005a, 2008a; also see Finn et al. 2010). Third, ALLO and ganaxolone facilitated reinstatement of ethanol seeking (Finn et al. 2008; Nie and Janak 2003; Ramaker et al. 2014) and ALLO as well as other $GABA_AR$-positive modulatory steroids, including pregnanolone and allotetrahydrodeoxycorticosterone, substituted for the discriminative stimulus effects of ethanol (Grant et al. 1996, 1997; Bowen et al. 1999; Hodge et al. 2001). Collectively, these findings outline an important role for 5α -reduced steroids in ethanol self-administration, seeking, and discrimination. Thus, an exploration of the functional implications of the 5α -reductase enzyme via use of *Srd5a1* null mutant mice should further inform our current understanding of 5α -reduced steroid involvement in these ethanol-related behavioral processes.

A comprehensive review of genetic manipulations and their influence on ethanol drinking and related behaviors noted that of the neurotransmitter systems, neuromodulators, and signaling molecules examined, GABA was one of several systems identified as playing a major role in ethanol's behavioral effects (Crabbe et al. 2006). Ethanol consumption, as measured by various iterations of a two-bottle choice procedure, was reduced in mutant mice with deletions in $GABA_AR$ subunits α_1, α_2, α_5, or δ, but not in mutants devoid of the $GABA_AR$ β_2 subunit or the GABA transporter, when compared to WT littermates. Interestingly, when both male and female KO mice were examined for their drinking phenotype, sex differences were often observed: only female α_2 subunit KO mice exhibited a reduced intake versus WT, only male α_5 subunit KO mice consumed less than WT, and male δ subunit mutants demonstrated a more pronounced reduction in intake than female mutants versus respective WT mice. Further, more recent work with KO mice for glutamic

acid decarboxylase type 2 (*Gad2*) reported that loss of this crucial GABA biosynthetic enzyme enhanced ethanol self-administration (Blednov et al. 2010). Curiously, the influence of *Gad2* deletion on ethanol intake hinged upon the self-administration procedure employed; augmented ethanol intakes in *Gad2* KO versus WT mice were found only when the mice were permitted continuous access, but not during a limited access condition. Crabbe and colleagues (2006) also noted that the common implementation of continuous access to ethanol during 2-bottle preference testing was in large part historically based, and hinted on the paucity of KO mice studies that examined ethanol drinking phenotypes in more than one self-administration procedure. In the current work, the effects of *Srd5a1* deletion was examined in male and female mice under continuous and limited access conditions so that issues of sex-dependency and drinking contingency could be incorporated into the interpretation of this gene's influence on ethanol consumption phenotype.

Based on recent findings of continuous access preference drinking differences in *Gad2* KO mice (Blednov et al. 2010) and limited access, operant self-administration differences in mouse lines selected for differences in ethanol withdrawal severity (Ford et al. 2011), our *a priori* hypothesis was that the influence of *Srd5a1* genotype on ethanol consumption would be largely contingent on the concentration of the ethanol solution presented. Further, earlier findings following pretreatment with ALLO (Ford et al. 2005b, 2007) and finasteride (Ford et al. 2005a, 2008a, b) to mice indicated that the onset of ethanol drinking in limited access ethanol consumption would be particularly sensitive to *Srd5a1* deletion, and so latency to first bout and licking that occurred in the initial 20-min of access were given particular attention.

Methods

Animals

Mice with a disruption (null allele) in the steroid 5α-reductase-1 (*Srd5a1*) gene were generated via a process involving homologous recombination of embryonic stem cells, as previously described (Mahendroo et al. 1996, 1997). In brief, an area including the promoter region and 36 amino acids of the 3′ end of exon 1 for the *Srd5a1* gene was supplanted by a neomycin resistance gene cassette. The resultant null-mutant mice (B6;129S7-*Srd5a1*^{tm1Mahe}) were maintained as cryopreserved embryos by The Jackson Laboratory (Bar Harbor, Maine), and were re-derived on a mixed C57BL/6 × 129/SvJ background for our use. Heterozygous breeding pairs were obtained and subsequently bred in the Portland Alcohol Research Center animal core. After weaning, same-sex littermates were initially group-housed in standard shoebox cages with ad libitum access to food and water under a 12:12 h light/dark cycle (lights on at 0600, except when noted otherwise) at 22 ± 1 °C. Tail clips were taken in order to genotype mice prior to testing. In all studies, male and female KO and WT mice were tested. The local Institutional Animal Care and Use Committee reviewed and approved all procedures in compliance with the guidelines set forth in the *Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research* and the U.S. National Institutes of Health.

Continuous access drinking in the home cage

Male and female mice were individually housed and given unlimited access to two fluid-filled 25-ml graduated drinking tubes. In a similar manner to that previously described (Yoneyama et al. 2008), one drinking tube always contained tap water and a second 'drug' tube contained the following solutions in the sequence given: 3 % v/v ethanol (3E; days 1–4), 6E (days 5–8), 10E (days 9–12), 20E (days 13–16), 0.2 % w/v saccharin (S; days 17–20), 3E + S (days 21–24), 6E + S (days 25–28), 10E + S (days 29–32) and 20E + S (days 33–36). Thus, the same mice were used for the assessment of all solutions, and each solution was presented for a 4-day period prior to the introduction of the next solution in the sequence. The positioning of the 'drug' tube was alternated between the left and right sides on the cage top every other day. Food was distributed adjacent to both drinking tubes to avoid a food-associated side preference for the tubes. Volume displacement was measured daily from each drinking tube at 1000 h. Placement of water and 'drug' drinking tubes on two vacant cages allowed for the measurement of leakage and evaporation throughout each session. Although every attempt was made to conduct this procedure with an approximately equal mix of male and female mice and representation of genotypes, the paucity of available male KO mice from the breeding colony at study onset dictated the lower number of mice run in this group. At procedure onset the age of mice ranged from 38 to 68 days old.

Limited access drinking in lickometer chambers

Custom chambers were constructed as previously described (Ford et al. 2005a, b, 2008a, b). Briefly, the chamber consisted of a four-walled Plexiglas insert with a hinged top, elevated stainless steel wire floor (VWR, Tualatin, OR), and two access ports along the back panel for the insertion of drinking sippers. The raised floor and perforated ceiling panel allowed for sufficient air flow. Preference drinking tubes were constructed from stainless steel sippers with double ball-bearings (Ancare, Bellmore, NY) to minimize leakage and 10-ml polystyrene serological pipettes (VWR, Tualatin, OR). This custom drinking tube allowed for volume measurements to the nearest 0.05 ml. The wire floor of the chamber and each sipper constituted an open electrical circuit that was hard-wired to a lickometer device (MED Associates, Inc., St. Albans, VT); this circuit permitted the generation of individual cumulative records for the contacts (licks) recorded from each sipper. Lickometers were interfaced to an IBM compatible computer running MED-PC IV software (MED Associates, Inc.).

Mice were singly-housed within lickometer chambers and acclimated to a reverse 12:12 h light/dark schedule (lights off at 0900) over a 7-day period. During chamber acclimation, each mouse was provided continuous access to two 50-ml water bottles and ad libitum rodent chow. A baseline measure of water consumption was measured during the last 3 days of acclimation. Following acclimation, daily 2-h limited access sessions were conducted starting at 1100 h. During these sessions one sipper contained tap water and a second sipper contained 10E. Mice were weighed immediately prior to each session to facilitate calculation of g/kg ethanol consumed. Five consecutive sessions were run to assess acquisition of 10E consumption. At the conclusion of session 5, blood samples (20 μl) were collected from the orbital sinus into glass microcapillary pipets for subsequent measurement of blood ethanol concentrations (BECs). The 10E sipper position was counter-balanced

between chambers to account for potential side preferences, but each sipper remained fixed on the left or right side throughout the study to permit mice to establish a drinking routine. Due to the limited number of available lickometer chambers, it was necessary to run mice through this limited access acquisition procedure over three experimental passes. Each of the three cohorts contained an approximately equal mix of genotype (KO, WT) and sex (male, female). At procedure onset the age ranges of the mice varied by cohort as follows: cohort 1 (59–77 days), cohort 2 (78–102 days) and cohort 3 (40–70 days).

Srd5a1 genotyping

Genomic DNA was purified from mouse tail biopsies following digestion in a proteinase K-containing lysis buffer and subsequent phenol/chloroform extraction, as previously described in detail (Tesson et al. 2002). Genotyping was performed by polymerase chain reaction (PCR) using primers *Srd5a1*-F (5′-TAACCGCGTCCTGCTAGCTA-3′) and *Srd5a1*-R (5′-AGTGGACTTTGGGCAATAGA-3′). Thirty-five cycles of PCR were run with an annealing temperature of 55 °C. Electrophoresis of products on a 1.5 % agarose gel yielded a 360-bp amplicon for the KO allele and a 400-bp amplicon for the WT allele.

Blood ethanol concentrations (BECs)

Samples were processed as described elsewhere (Ford et al. 2005a). Each sample was first transferred to a chilled microcentrifuge tube containing 50 μl of 5 % w/v $ZnSO_4$ and then further constituted with 50 μl of 0.3 N $Ba(OH)_2$and 300 μl of deionized water. Samples were agitated by brief vortex and then centrifuged for 5 min at 12,000 rpm. The resultant supernatants were isolated and assayed for BEC by gas chromatography. Seven sets of known standards (0.25–4.00 mg/ml ethanol concentrations) were assessed in parallel to generate a standard curve from which unknown concentrations of samples were interpolated.

Data analysis

For the continuous access preference drinking experiment, the dependent variables were ethanol or saccharin intake (g/kg) and preference ratio (volume of ethanol, saccharin, or ethanol + saccharin solution divided by total fluid intake). Intakes (g/kg) were calculated from the ethanol or saccharin volume consumed and the body weight recorded from each mouse. The 4-day averages of intakes and preference ratios for each solution were calculated and analyzed. ANOVA was used to assess the effects of the following factors on the dependent variables: *Srd5a1* genotype, sex, and ethanol concentration (repeated measure). Significant ‘genotype × sex’ interactions were pursued with post hoc tests. Separate ANOVAs were performed for the unsweetened ethanol, saccharin only, and sweetened ethanol phases of the experiment.

For the limited access lickometer experiment, the dependent variables were ethanol intake (g/kg), ethanol preference ratio, 10E and water licks, and BEC (mg/ml). Additional ethanol bout parameters included latency to 1st bout (min), bout frequency, bout size (licks), lick rate (licks/min), and inter-bout interval (min). Based on previous work (Ford et al. 2005a, b; 2008a, b), an ethanol bout was experimentally defined as a minimum of 20 licks with no more than a 60-s pause between successive licks. Lick rates are reported as the average rate of all bouts expressed, and does not include session time and isolated licking that fell outside

of defined bouts. Ethanol intakes (g/kg) and preference ratio were calculated as described above for the continuous access preference drinking experiment. Since the acquisition of ethanol intake across consecutive limited access sessions was of particular interest, an ANOVA assessed the effects of *Srd5a1* genotype, sex, and session (repeated measure) on g/kg ethanol intakes. ANOVAs (factors: *Srd5a1* genotype and sex) were also run for all ethanol bout parameters as well as for BECs. BEC samples collected at the conclusion of session 5 necessitated a staggered start time for the mice, and this had the unintentional consequence of shifting the established drinking routines of the mice. Thus, session 4 data was used for the analysis of bout measures and the temporal distribution of licks to avoid this potential confound introduced during session 5. For the temporal distribution of 10E licks, ANOVAs were implemented with factors *Srd5a1* genotype, sex, and time interval (repeated measure). As with the continuous access preference drinking experiment, pair-wise differences were examined with post hoc tests in the event that a significant 'genotype × sex' interaction was detected. In all cases, data are expressed as the mean ± SEM and statistical significance was set at $p \leq 0.05$.

Results

Continuous access drinking: ethanol and ethanol-saccharin solutions

Baseline water intakes over the 24-h period preceding the initial access session with unsweetened 3E were not different between groups: 5.0 ± 0.4 (male WT), 5.2 ± 0.3 (male KO), 4.7 ± 0.4 (female WT), and 5.0 ± 0.5 ml (female KO). A 3-way ANOVA conducted for unsweetened ethanol consumption revealed a trend towards a genotype × sex interaction [$F(1,25) = 3.58$, $p = 0.07$] and a main effect of concentration [$F(3,75) = 12.99$, $p < 0.001$] for g/kg intake, but no genotype × sex × concentration interaction was apparent. For ethanol preference ratio a significant genotype × sex interaction [$F(1,25) = 4.56$, $p < 0.05$] and main effect of concentration [$F(3,75) = 21.87$, $p < 0.001$] were similarly determined, and the genotype × sex × concentration interaction was significant [$F(3,75) = 2.68$, $p = 0.05$]. Based on the above interactions and our *a priori* hypothesis that genotype differences in ethanol intake would occur in an ethanol concentration-dependent fashion, subsequent analyses were conducted for each ethanol concentration.

A significant main effect of sex was detected for g/kg consumption of 3E [$F(1,25) = 5.27$, $p < 0.05$] and 6E [$F(1,25) = 8.52$, $p < 0.01$]. Overall, female mice consistently consumed 50–65 % greater amounts of 3E ($p < 0.05$) and 6E ($p < 0.001$) than male mice under the continuous access condition (Fig. 1a). A genotype × sex interaction was also realized for g/kg intake of 6E [$F(1,25) = 8.01$, $p < 0.001$] and 10E [$F(1,25) = 6.21$, $p < 0.001$]. Female KO mice consumed significantly more 6E ($p < 0.01$) and 10E ($p < 0.05$) when compared to female WT mice. Female KO mice also exhibited significantly greater ethanol intakes than male KO mice during presentation of 6E ($p < 0.001$) and 10E ($p < 0.01$). Group differences in ethanol preference ratio for unsweetened ethanol solutions (Fig. 1b) mirrored differences observed in g/kg ethanol intake, with significant genotype × sex interactions occurring during access to 6E [$F(1,25) = 5.90$, $p < 0.05$] and 10E [$F(1,25) = 4.59$, $p < 0.05$]. Specifically, female KO mice demonstrated an elevated preference when compared to female WT litter-mates during 6E and 10E presentation ($ps < 0.05$). The ethanol preference

ratios of female KO mice were also significantly greater than those of male KO mice for 6E ($p < 0.01$) and 10E ($p < 0.05$).

Analysis of group differences in g/kg intake of saccharin (0.2 % w/v solution; S) yielded no significant effects of genotype, sex, or a genotype × sex interaction. Average group intakes of S were 0.79 ± 0.09 (male WT), 0.63 ± 0.13 (male KO), 0.69 ± 0.10 (female WT) and 0.81 ± 0.09 g/kg (female KO). However, a 2-way ANOVA determined a significant effect of sex for S preference [$F(1,25) = 4.27$, $p < 0.05$], with males exhibiting a heightened preference for S over females (0.93 ± 0.03 versus 0.86 ± 0.03, respectively).

In general, introduction of S into the ethanol solutions led to increases in ethanol intakes over the 24-h sessions (compare Fig. 1a, c). Similar to the findings with unsweetened ethanol, a 3-way ANOVA conducted for g/kg ethanol consumption in the presence of S demonstrated a trend towards a genotype × sex interaction [$F(1,25) = 3.45$, $p = 0.08$]and a main effect of solution concentration [$F(3,75) = 34.44$, $p < 0.001$] for g/kg ethanol intake. A main effect of sex [$F(1,25) = 3.45$, $p < 0.01$] was also noted for sweetened ethanol intakes. For preference ratios of sweetened ethanol, a trend towards a genotype × sex interaction [$F(1,25) = 3.897$, $p = 0.060$] and a main effect of concentration [$F(3,75) = 106.91$, $p < 0.001$] were observed. Again, due to our *a priori* hypothesis regarding the influence of *Srd5a1* genotype on intakes as being concentration dependent, separate analyses were conducted for each ethanol + S solution.

A 2-way ANOVA detected significant genotype × sex interactions for g/kg ethanol intake during the presentation of 6E + S [$F(1,25) = 8.80$, $p < 0.01$] and 10E + S [$F(1,25) = 6.45$, $p < 0.05$], as well as main effects of sex on ethanol intake with the 6E + S [$F(1,25) = 13.30$, $p < 0.001$] and 10E + S [$F(1,25) = 9.89$, $p < 0.01$] solutions. Male KO mice consumed significantly less g/kg ethanol during access to 6E + S ($p < 0.01$) and 10E + S ($p < 0.05$) when compared to male WT mice (Fig. 1c). Male KO mice also exhibited ethanol intakes that were approximately 45 % lower than those observed in the female KO mice during presentation of the 6E + S and 10E + S solutions ($ps < 0.001$). For preference ratios, differences were limited to a main effect of sex with access to 3E + S [$F(1,25) = 5.62$, $p < 0.05$] and a genotype × sex interaction when 6E + S was made available [$F(1,25) = 8.03$, $p < 0.01$]. Specifically, male KO mice demonstrated significantly lower preference ratios for 6E + S when compared to male WT ($p < 0.01$) and female KO ($p < 0.05$) mice (Fig. 1d).

Limited access drinking: ethanol

The most profound impact of *Srd5a1* deletion on continuous access ethanol drinking was observed commensurate with presentation of 6E and 10E solutions. Because of our laboratory's extensive experience with limited access procedures using 10E, this ethanol concentration was selected for further study. ANOVA revealed no significant effect of cohort on the g/kg consumed, and hence data from the three passes of mice were collapsed in all subsequent analyses. The lickometer circuits permitted a reliable evaluation of ethanol consumption on a sub-second time scale, as demonstrated by the positive correlation between the number of 10E licks and the volume of 10E depleted from the sipper throughout the 2-h session ($r = 0.98$; $p < 0.001$; $n = 46$). Baseline water consumption (total ml) during the 72-h immediately prior to the inaugural 10E access session was not different between

groups: 13.8 ± 0.9 (male WT), 15.2 ± 0.8 (male KO), 14.7 ± 0.7 (female WT) and 15.4 ± 1.3 ml (female KO). This finding was consistent with the absence of group differences in water baseline intakes preceding the assessment of continuous ethanol access (see above). Average body weights throughout the limited access experiment were 25.3 ± 1.0 (male WT), 28.6 ± 1.0 (male KO), 20.0 ± 0.9 (female WT), and 19.9 ± 1.0 g (female KO). Although body weights were lower in female versus male mice [$F(1,42) = 50.07$, $p \leq 0.001$], the genotype × sex interaction did not meet statistical significance.

Deletion of the *Srd5a1* gene facilitated the acquisition of 10E consumption across sessions in male mice whereas it attenuated acquisition in female mice (Fig. 2a). A 3-way repeated measures (RM) ANOVA determined main effects of session [$F(4,168) = 3.13$, $p < 0.05$] and sex [$F(1,42) = 4.79$, $p < 0.05$], as well as a significant session × genotype × sex interaction [$F(4,168) = 7.85$, $p < 0.001$] for g/kg ethanol intake. Male KO mice demonstrated significant increases in ethanol intake during sessions 3 and 5 (each $p < 0.05$) and session 4 ($p < 0.01$) versus session 1, and their levels of consumption were significantly elevated compared to WT males on sessions 4–5 (each $p < 0.05$). Female WT, but not female KO, mice similarly acquired ethanol self-administration over sessions (Fig. 2a), with significantly greater g/kg intake occurring on sessions 2 and 4 (each $p < 0.05$) and session 5 ($p < 0.01$) when compared to the first session. Further, female WT mice consistently consumed greater amounts of ethanol than male WT mice ($p < 0.05$ for session 3; $p < 0.01$ for all other sessions).

Parallel changes in ethanol preference ratio were limited to male mice (Fig. 2b). A 3-way RM ANOVA revealed a significant genotype × sex interaction [$F(1,33) = 4.22$, $p < 0.05$], but no session × genotype × sex interaction. Subsequent analyses determined that male KO mice expressed significantly greater preference for 10E than their WT counterparts on sessions 4–5 (each $p < 0.05$). Thus, deletion of *Srd5a1* modulated acquisition of limited access 10E consumption and preference in a sex-dependent fashion. Although mean BECs evaluated at the conclusion of session 5 (Fig. 3a) closely mirrored mean g/kg ethanol intakes, a significant genotype or sex difference in BEC was not found. However, a significant correlation between BEC and g/kg ethanol intake was observed (Fig. 3b; $r = 0.81$, $p < 0.001$, $n = 46$).

In order to gain a more complete understanding of the drinking routines that were established following repeated opportunities to consume 10E, analyses of total session licks and bout patterns were performed using the lickometer data output during session 4 (Table 1). Significant genotype × sex interactions were detected for both 10E licks [$F(1,42) = 8.63$,$p < 0.01$] and water licks [$F(1,42) = 4.71$, $p < 0.05$]. Consistent with the group differences in g/kg intake during session 4 (refer to Fig. 2a), male KO mice exhibited a 2.8-fold increase in 10E licks ($p < 0.01$) when compared to male WT mice (Table 1). A difference in 10E licks was also observed between male and female WT mice, with female WT mice demonstrating a significantly greater amount of 10E licks ($p < 0.05$). No pair-wise comparisons for water licks met statistical significance, but the overall interaction seemed to be driven by trends of sex within KO subjects ($p = 0.08$) and genotype within females ($p = 0.08$). Further, no difference in total fluid intake (sum of 10E and water licks) between groups was noted, suggesting that acquisition of ethanol intake over sessions was

accompanied by parallel reductions in water consumption (data not shown). In regards to bout patterns, genotype × sex interactions were determined for both the latency to 1st bout [$F(1,30) = 17.44$, $p < 0.001$] and bout frequency [$F(1,42) = 8.89$, $p < 0.01$]. Specifically, male KO mice displayed a 77 % shorter latency to the 1st bout ($p < 0.001$) and a fivefold increase in bout frequency ($p < 0.05$) when compared to male WT mice (Table 1). Although female KO mice showed a 43 % reduction in bout frequency and a 3.5-fold increase in latency to 1st bout when compared to their WT counterparts (Table 1), these differences did not reach significance. No group differences were noted for bout size, lick rate or inter-bout interval. Thus, augmented ethanol intake in male KO mice during session 4 (Fig. 2a; Table 1) was primarily attributable to genotype differences in bout frequency.

A 3-way RM ANOVA of the temporal distribution of licks was conducted (Fig. 4; absolute values per 20-min interval), and revealed a main effect of interval [$F(5,210) = 2.95$, $p < 0.05$] and an overall genotype × sex interaction [$F(1,42) = 8.63$, $p < 0.01$]. Based on our *a priori* hypothesis that the onset of limited access ethanol consumption would be particularly sensitive to *Srd5a1* deletion, subsequent pair-wise comparisons within each 20-min interval were conducted. Consistent with this hypothesis, significant genotype × sex interactions [$Fs(1,42) > 4.79$, $ps < 0.05$] were realized within the 0–20, 20–40, 40–60 and 100–120 min intervals. Specifically, male KO mice exhibited greater 10E lick counts than male WT mice during minutes 0–20 ($p < 0.05$), 40–60 ($p < 0.05$) and 100–120 ($p < 0.01$) of the session. The robust onset of licks early in the session (0–20 min; Fig. 4) was consistent with the observation of a significantly reduced latency to 1st bout in male KO mice (see Table 1). In contrast, female KO mice made significantly fewer 10E licks during the 20–40 min interval when compared to female WT mice ($p < 0.05$). Differences between male and female WT mice were observed during minutes 20–40, 40–60 and 100–120 (all ps < 0.05), with female mice consistently licking more than males (Fig. 4).

Discussion

Summary of findings

In the current work, we examined the effects of *Srd5a1* deletion in male and female mice on ethanol intake under continuous and limited access conditions so that interpretation of the results would consider sex-dependency and drinking contingency. Under continuous access conditions, intake of unsweetened 6E and 10E solutions was significantly increased in KO versus WT female mice, while intake of sweetened 6E and 10E was significantly decreased in KO versus WT male mice. One possible limitation of these findings was the low sample size of the male KO group available for the continuous access study, and it is uncertain whether the addition of more animals to this group would have altered the outcome. In contrast, there was an opposite effect of *Srd5a1* genotype on the acquisition of limited access intake; unsweetened 10E intake was significantly increased in male KO versus WT mice and non-significantly decreased in female KO versus WT mice. These results clearly demonstrate that deletion of the *Srd5a1* gene produced marked genotype and sex differences in ethanol consumption, but ethanol access conditions significantly influenced the direction of the genotype effect. Additionally, the stability in overall fluid consumption indicated that

the *Srd5a1* genotype was not driving increases or decreases in overall consumption, but rather specifically shifting consumption toward one or the other available fluids.

Contribution of steroids to genotype effects

Because earlier work from our laboratory and others indicated that manipulation of endogenous ALLO levels can alter ethanol intake (see “Introduction” section), our original hypothesis was that a genotype and/or sex difference in ethanol consumption in the *Srd5a1* mutant mice would be based on differences in endogenous levels of ALLO between KO versus WT mice, either basally or following ethanol exposure. Our prior work in intact male and female C57BL/6J mice indicated that whole brain ALLO levels were higher in female versus male mice under basal conditions and that limited access ethanol consumption significantly increased ALLO levels only in the male mice (Finn et al. 2004). Continuous access ethanol intake also increased cortical ALLO levels in male Y_1R/LacZ transgenic mice on an FVB background (Eva et al. 2008). Further, finasteride administration reduced both endogenous (see review by Finn et al. 2006) and ethanol-induced ALLO levels following either systemic injection (e.g., Kaufman et al. 2010; VanDoren et al. 2000) or oral consumption (Eva et al. 2008). However, it is uncertain whether basal ALLO levels differ in *Srd5a1* KO versus WT mice, as recent work reported that basal hippocampal ALLO levels did not differ in gonadectomized male and female KO and WT mice (Osborne and Frye 2009), nor did basal ALLO levels in plasma, cortex, hippocampus, or midbrain differ in intact, regularly cycling female KO and WT mice during diestrus (Koonce et al. 2012; Koonce and Frye 2014). Although we did not examine ALLO levels in the current experiments because of individual differences in the amount of ethanol consumed, we did not observe a difference in plasma ALLO levels in intact *Srd5a1* KO versus WT male and female mice following a saline injection, as reported in our companion study for this special issue (Tanchuck-Nipper et al. 2014), suggesting that the female mice in that study were in diestrus at the time of euthanasia. However, it should be noted that endogenous ALLO levels were reported to be significantly higher in intact *Srd5a1* KO and WT female mice during proestrus versus diestrus, but levels during proestrus were lower in the KO mice (Koonce et al. 2012; Koonce and Frye 2014). Based on all of the above and because intact WT and KO female mice are reported to exhibit regular 4–5 day estrous cycles, we presume that endogenous ALLO levels in the present study fluctuated in the female KO and WT mice across the estrous cycle, that levels were higher in proestrus females versus male mice, and that levels during proestrus were lower in the KO versus WT female mice. Taken in conjunction with the ethanol consumption data, it is unlikely that fluctuations in endogenous ALLO levels contributed to the genotype and sex differences in continuous or limited access ethanol intake. It remains to be determined whether the effects of *Srd5a1* deletion on ethanol intake can be explained by changes in the endogenous levels of other 5α-reduced neurosteroids, such as the metabolites of deoxycorticosterone and testosterone.

Another possible steroid mechanism underlying changes in drinking behavior in KO mice could be the accumulation of steroid precursors (progesterone, deoxycorticosterone and testosterone) or the shunting of precursors to alternate biosynthetic pathways. In the earliest work characterizing the *Srd5a1* null mutant model, it was found that no genotype difference in plasma progesterone levels of KO and WT female mice existed throughout pregnancy

(Mahendroo et al. 1996), suggesting the absence of progesterone accumulation. More recent evidence indicates that estradiol is higher in intact *Srd5a1* KO versus WT females during proestrus and diestrus (Koonce et al. 2012). It is possible that the elevated estradiol levels in the KO females contributed to their increased ethanol intake versus WT mice in the continuous access procedure, as estradiol is known to facilitate drug self-administration (see Carroll et al. 2004; Carroll and Anker 2010; Becker and Hu 2008; Ford et al. 2004). Lastly, exposure to corticosterone (CORT) in drinking water produced heightened levels of this steroid during the dark cycle and lower levels during the light cycle, and this exaggerated rhythmicity was found to blunt ethanol's interoceptive effects and to produce a transient increase in ethanol self-administration (Besheer et al. 2012, 2013). Although we observed lower CORT levels in KO versus WT mice during the light phase in our companion study (Tanchuck-Nipper et al. 2014), it is not known whether the circadian rhythm of CORT secretion is altered in *Srd5a1* KO mice. Taken together, the available evidence suggests that alterations in estradiol or CORT may have contributed to the observed effects of genotype on drinking. Additional work is clearly needed to more fully characterize the effect of *Srd5a1* gene deletion on basal concentrations of 5α-reduced metabolites, steroid precursors, and steroids within alternate pathways.

Sex differences in ethanol intake

Higher ethanol intake in female versus male rodents is well documented (see Finn et al. 2010) and a growing body of literature has documented marked sex differences in drug self-administration patterns at every stage of drug exposure history (Carroll et al. 2004; Becker and Hu 2008; Lynch et al. 2002; Wiren et al. 2006). Curiously, under the continuous access condition WT mice did not demonstrate sex differences in g/kg ethanol intake regardless of ethanol concentration offered (Fig. 1a). This was in contrast to the large disparity between sexes noted in the KO mice, with intakes of unsweetened ethanol in males falling within the range reported for ethanol non-preferring 129 substrains (e.g., Belknap et al. 1993; Bachmanov et al. 1996, 2002; Logue et al. 1998; Tordoff et al. 2002; Yoneyama et al. 2008) whereas ethanol intakes in females was comparable to amounts reported in ethanol-preferring C57BL/6 mice (e.g., Belknap et al. 1993; Phillips et al. 1994; Bachmanov et al. 1996, 2002; Logue et al. 1998; Tordoff et al. 2002; Yoneyama et al. 2008). The magnitude of this sex difference in ethanol intake in KO mice exceeded those historically reported for the C57BL/6 and 129 mouse strains (e.g., Bachmanov et al. 2002; Yoneyama et al. 2008). Unexpectedly, sex differences in ethanol intake and the resultant influence of KO differed considerably under the limited access condition. In this case, marked sex differences were apparent in WT mice, but male and female KO mice consumed comparable amounts of 10E (Fig. 2a). It was also notable that male KO mice exhibited drinking patterns (e.g., latency to 1st bout and bout frequency) that most highly resembled female WT mice (Table 1). Despite the disparities between continuous and limited access conditions (see discussion below), these findings do collectively underscore the likely contribution of the interaction between genotype and neurosteroids and sex steroids to the biological basis of sex differences in alcohol use.

Murine *Srd5a1* gene and background strain

Recent data in an F2 hybrid from the C57BL/6ByJ and 129P3/J strains indicated that the high ethanol intake and preference of the C57 mice were inherited in the F2 generation as additive or dominant traits (Bachmanov et al. 2002). When gene mapping was conducted in these F2 mice for consumption of 3 and 10 % ethanol solutions, one of the six suggestive linkages that were identified for 10 % ethanol preference was on murine chromosome 13, in a region where *Srd5a1* maps, and it explained 5.3 % of the phenotypic variance in 10 % ethanol preference (Bachmanov et al. 2002). Relevant to the present paper, there were epistatic interactions between loci on murine chromosome 13 and other chromosomal regions to influence 3 and 10 % ethanol intake and preference. Thus, we cannot rule out the possibility that chromosomal loci identified in the F2 cross by Bachmanov et al. (2002) have the potential to modify the effects of a targeted mutation at *Srd5a1*, which is located on murine chromosome 13, and impact alcohol intake and preference.

In their review of alcohol-related gene modifications on ethanol intake, Crabbe and colleagues (2006) posited that most studies to date have examined self-administration in a context devoid of self-intoxication, as rodents tend to drink ethanol in a manner that circumvents the accumulation of BECs that would generate inebriation (see Dole and Gentry 1984). The range of BECs observed at the conclusion of the 2-h access period (Fig. 3; all group means ≤ 0.5 mg/ml) would indicate the absence of intoxication in the majority of mice studied. The genetic background of mice in the current work (i.e., mixed C57BL/6J × 129/SvJ) may partly explain the absence of intoxication, as our laboratory has conducted an identical limited access procedure in male C57BL/6J mice and regularly demonstrated BECs in the range of 0.8–1.0 mg/ml (for example, Ford et al. 2005a). Further, a strain comparison of continuous access to a 10E solution that employed an identical procedure as conducted for this work, revealed that C57BL/6J mice consumed eightfold greater g/kg ethanol than 129S1/SvlmJ mice (16 versus 2 g/kg/day; Yoneyama et al. 2008).

In the examination of the acquisition of ethanol intake, it is noteworthy that 5 of 12 male *Srd5a1* WT mice tested exhibited no ethanol bouts and an additional five subjects had only one observable ethanol bout. This minimum level of drinking in male WT mice may have hampered the ability to detect statistically significant differences in bout size, lick rate and inter-bout interval. The low ethanol drinking in male WT mice may be due in part to the contribution of the 129 mouse strain to the background of these mice, as we have previously reported that 129 mice drink relatively small quantities of 10E when compared to C57BL/6J mice (Yoneyama et al. 2008). A potential future direction would be to back-cross the *Srd5a1* genetically-modified mice onto the ethanol-preferring C57BL/6J strain over multiple generations, and then re-examine the influence of the *Srd5a1* gene in mice that exhibit the ability to drink high amounts of ethanol and self-intoxicate.

Continuous versus limited ethanol access

Curiously, *Srd5a1* genotype exerted an opposite effect on the acquisition of limited access 10E intake, when compared with the results under continuous access conditions; limited access 10E intake was significantly increased in male KO versus WT mice and non-significantly decreased in female KO versus WT mice. In a study of *Gad2* KO mice

(Blednov et al. 2010) the influence of genotype on ethanol consumption hinged upon both the genetic background of the mice as well as the self-administration procedure employed (i.e., limited versus continuous access). However, in this earlier work, a genotype effect was observed with continuous access to ethanol, but no effect was observed under multiple variations of a limited access scenario. That makes the current findings with *Srd5a1* KO mice unique, in that the gene deletion caused bidirectional changes in ethanol intake as a factor of drinking session length. Our earlier assessment of ethanol drinking patterns in male C57BL/6J mice demonstrated that the majority of 10E intake occurred during the circadian dark phase, with peaks in intake typically occurring approximately 3 h into the dark phase (Finn et al. 2010). The limited access sessions conducted in this work were intentionally run starting 2-h into the dark phase to increase the likelihood that mice would consume levels of ethanol associated with intoxication, much like the drinking-in-dark procedure developed by Rhodes and colleagues (Rhodes et al. 2005, 2007). In contrast, even though continuous access to ethanol as a 2-bottle preference test is commonly employed, mice seldom experience the intoxicating effects of ethanol under this condition (Crabbe et al. 2006; Dole and Gentry 1984). One explanation for the current findings is that *Srd5a1* genotype differentially influences ethanol intakes depending on whether the mice approached levels of intoxication when drinking, which would have been more likely during limited access sessions. Another possible explanation is that the limited access sessions were in close temporal proximity to the diurnal modulation of the hypothalamic– pituitary–adrenal axis, in which a surge of glucocorticoid and other steroid precursors are released from the adrenals of rodents at the end of the light phase and beginning of the dark phase. Thus, the limited access sessions corresponded to a time of day of peak steroid metabolism and production of 5α-reduced steroids and CORT (see steroid discussion above). Regardless of the potential explanation, if the pattern of drinking was shifted in the KO in the present study, then different genotype results might be detected when the examination of ethanol intake was limited to a 2-h dark period versus an examination of ethanol intake across 24-h.

Implications

Given that the limited access condition more likely modeled levels of alcohol intake that were physiologically relevant, the most notable effect of *Srd5a1* deletion was the enhanced acquisition of ethanol intake over sessions in male mice (Fig. 2a). Earlier studies in human subjects found that men and women possessing a minor C-allele of *Srd5a1* (rs248793) expressed both a higher ratio of dihydrotestosterone to testosterone (Ellis et al. 2005) and a reduced risk for alcohol dependence (Milivojevic et al. 2011), suggesting that a heightened level of 5α-reduced neurosteroid production may be protective against the development of dependence. In this context, deletion of *Srd5a1* or a decline in enzyme function might be expected to reduce the biosynthesis of 5α-reduced neurosteroids and exacerbate the risk of alcohol dependence. This conclusion would be consistent with the exaggerated acquisition observed in male, but not female, KO mice. Overall, deletion of the *Srd5a1* gene produced marked genotype and sex differences in ethanol consumption, but the ethanol access condition significantly impacted the direction of the genotype effect. Sex differences in the ability of *Srd5a1* genotype to modulate ethanol drinking behavior may reflect an interaction between developmental compensations to deletion of the *Srd5a1* gene with alterations in estradiol or CORT levels. Future studies implementing conditional knock-out or site-

directed knockdown models for *Srd5a1* could also assist in untangling this complex genotype × sex interaction on ethanol self-administration.

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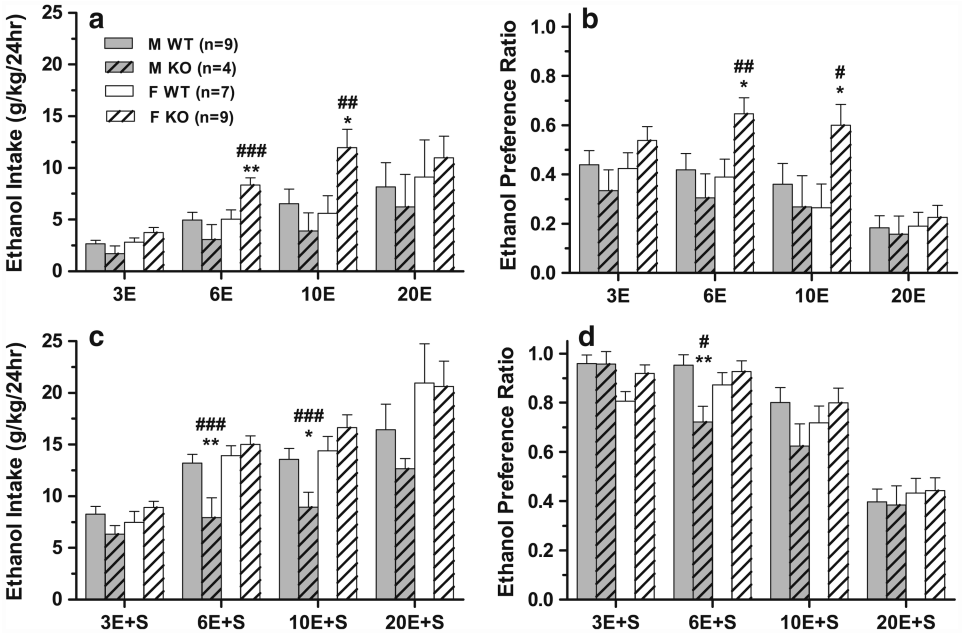

Fig. 1.

Consumption of and preference for ethanol solutions under a continuous access condition in male (M) and female (F) *Srd5a1* knockout (KO) and wildtype (WT) mice. Values for g/kg ethanol intake and ethanol preference ratio represent the 4-day mean ± SEM during serial presentation of 3 % v/v ethanol (3E), 6E, 10E and 20E solutions. Ethanol concentrations were first presented unsweetened (*panels A–B*) and then in combination with 0.2 % w/v saccharin (+S) (*panels C–D*). Sample sizes per group are shown in panel A. **p*<0.05 and ***p*<0.01 versus same-sex WT; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus respective genotype of opposite sex. Post-hoc pair-wise comparisons were conducted via the Fisher LSD method

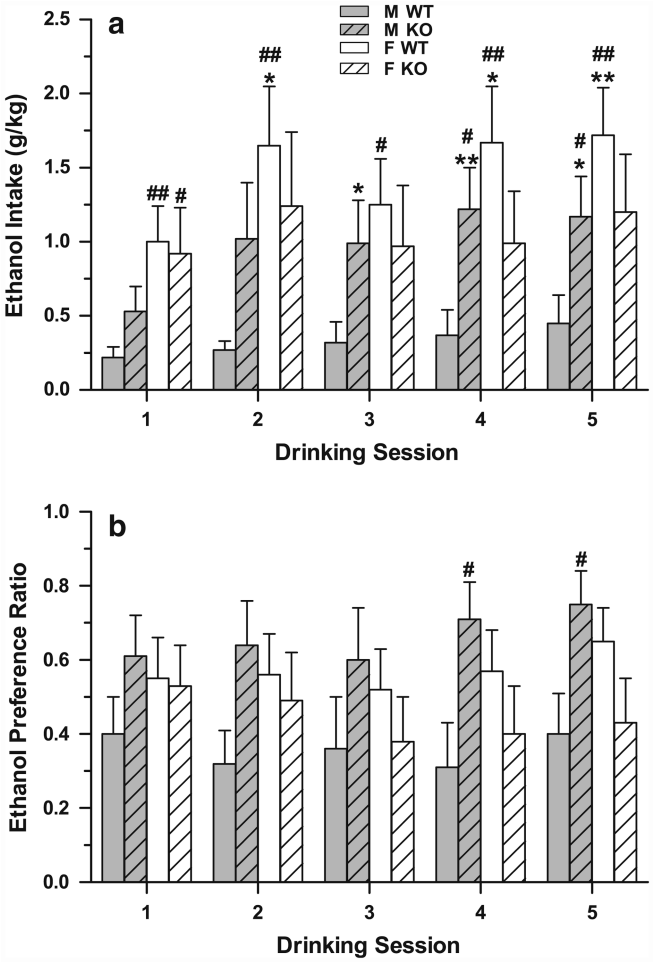

Fig. 2.

Acquisition of ethanol self-administration during limited access sessions in male (M) and female (F) *Srd5a1* knockout (KO) and wildtype (WT) mice. Daily mean ± SEM values for g/kg ethanol intake (*panel A*) and ethanol preference ratio (*panel B*) are depicted following 2-h/day access to a 10 % v/v ethanol solution. Sample size per group was $n = 11$, except $n = 13$ for F WT mice. *$p < 0.05$ and **$p < 0.01$ versus respective session 1 value; #$p < 0.05$ and $p < 0.01$ versus M WT group. Post-hoc pair-wise comparisons were conducted via the Fisher LSD method

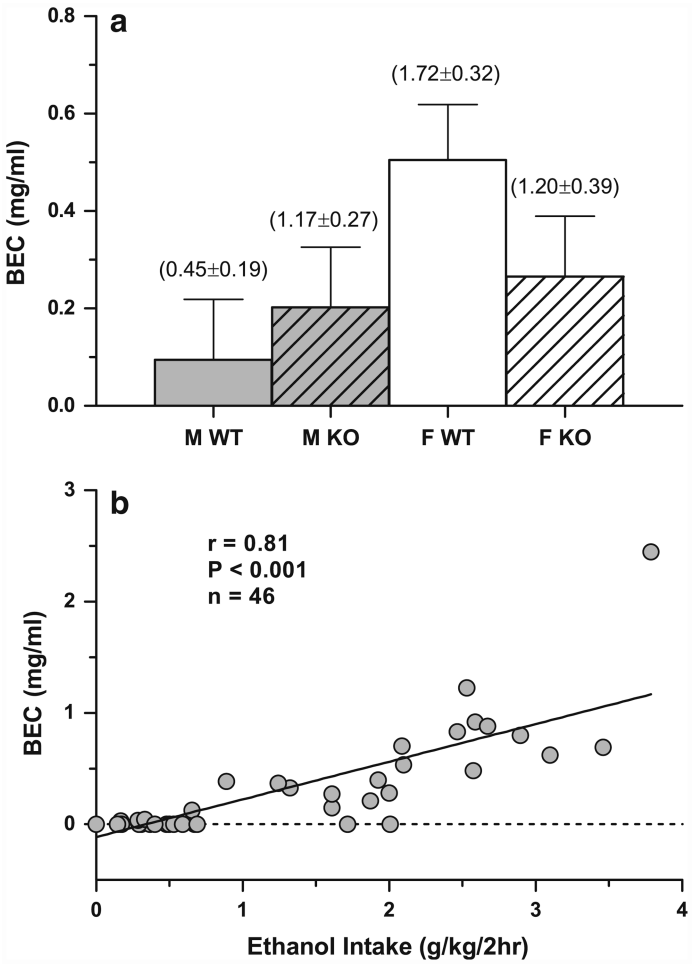

Fig. 3.

Blood ethanol concentrations (BECs) following 2-h ethanol intake in male (M) and female (F) *Srd5a1* knockout (KO) and wildtype (WT) mice. The mean ± SEM values for BEC sampled immediately following session 5 are depicted in *panel A.* The corresponding mean ± SEM g/kg consumed during the same 2-h session is reported in parentheses above each bar. Sample sizes per group are the same as stated in Fig. 2. In *panel B,* the solid line depicts the linear regression of data points representing the g/kg ethanol intake and BEC for individual mice (r = 0.81, $p < 0.001$, $n = 46$)

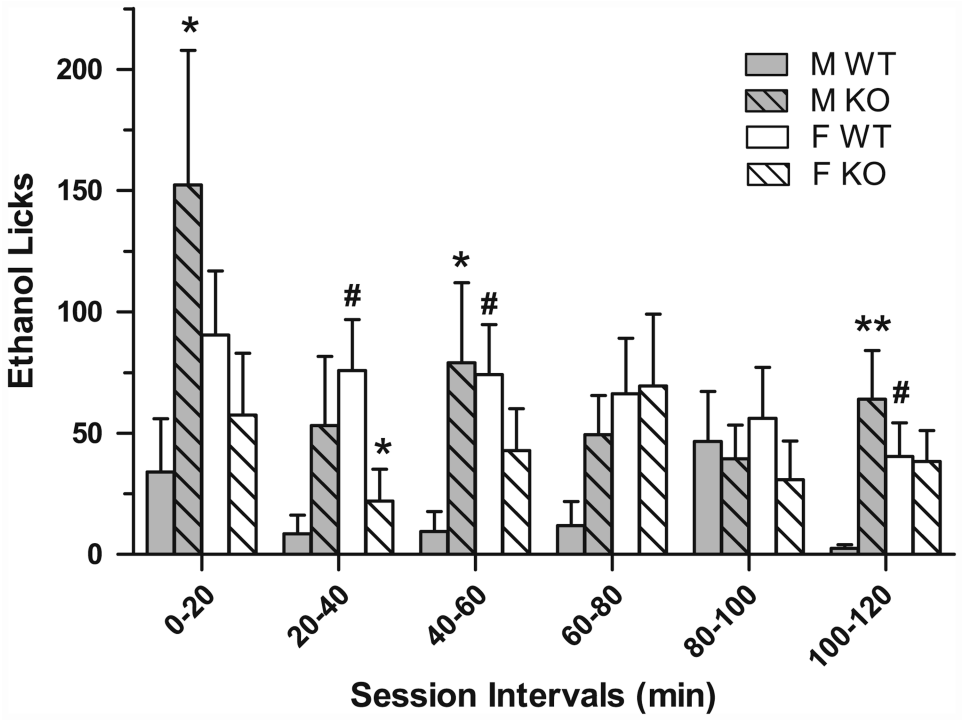

Fig. 4.

Temporal distribution of ethanol licks upon acquisition of limited access ethanol intake in male (M) and female (F) *Srd5a1* knockout (KO) and wildtype (WT) mice. Values depict the mean ± SEM of licks on the ethanol sipper within each 20-min interval throughout session 4 (i.e., asymptote reached in Fig. 2a). Sample sizes per group are the same as reported in Fig. 2. *$p < 0.05$ and **$p < 0.01$ versus same-sex WT; $^{\#}p < 0.05$ versus M WT group. Post-hoc pair-wise comparisons were conducted via the Fisher LSD method

Table 1

Limited access licks and drinking patterns in male and female *Srd5a1* knockout (KO) and wildtype (WT) mice

	Male		Female	
	WT	**KO**	**WT**	**KO**
Lick counts				
10E	113 ± 84	437 ± 84**	403 ± 78$^{\#}$	261 ± 84
Water	371 ± 93	204 ± 93	208 ± 86	441 ± 93
10E Bout patterns				
Latency to 1st Bout (min)	70 ± 11	16 ± 9***	10 ± 10$^{\#\#\#}$	35 ± 10
Bout frequency	1.1 ± 1.1	5.0 ± 1.1*	5.6 ± 1.0$^{\#\#}$	3.2 ± 1.1
Bout size (licks)	65 ± 13	87 ± 10	66 ± 11	66 ± 12
Rate (licks/min)	348 ± 64	450 ± 53	287 ± 56	277 ± 59
Inter-Bout interval (min)	30.8 ± 6.3	22.8 ± 3.0	13.9 ± 3.0	21.4 ± 3.7

Values represent the mean ± SEM of drinking measures from session 4. Sample sizes per group are the same as stated in Fig. 2. Post-hoc pair-wise comparisons were conducted via the Fisher LSD method

$^{*}p < 0.05$,

$^{**}p < 0.01$ and

$^{***}p < 0.001$ versus WT within sex;

$^{\#}p \leq 0.05$,

$^{\#\#}p \leq 0.01$ and

$^{\#\#\#}p \leq 0.001$ versus respective genotype between sex