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Pre-pubertal gonadectomy and the social consequences of acute ethanol in adolescent male and female rats

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Abstract

It has previously been shown that pre-pubertal or adult gonadectomy (GX) increases ethanol intake in male rats. This study examined whether this sex-selective increase reflects a GX-induced maintenance in males of more adolescent-typical responsiveness to ethanol characterized by enhanced sensitivity to positive (e.g., socially facilitating) and a decreased sensitivity to adverse (e.g., socially inhibitory) effects of ethanol. Male and female Sprague-Dawley rats were prepubertally GX, sham (SH)-operated, or non-manipulated (NM) at postnatal day (P) 25. During the late adolescent transition into adulthood (P48 -- baseline day), rats were given a saline injection, placed alone into a familiar test apparatus for 30 min and then exposed for 10 min to an unfamiliar partner of the same age and sex. On the following day (P49), similar testing occurred after administration of 0.5, 0.75, 1.0 or 1.25 g/kg ethanol. At baseline, GX males and females displayed higher levels of social activity (especially adolescent-typical play and contact behavior) than SH and NM animals, with GX females displaying greater social activity than GX males. Neither males nor females demonstrated social facilitation at lower ethanol doses, regardless of hormonal status. Whereas the social inhibitory effects of higher doses of ethanol were similar across groups among females, SH males were less sensitive than both GX and NM males to ethanol-induced social inhibition. These results suggest that enhanced ethanol intake in GX males is not related to alterations in sensitivity to ethanol's social inhibitory effects. GX, however, results in retention of adolescent-typical social behaviors, with older GX adolescent rats resembling early adolescents in exhibiting elevated social activity—particularly play and contact behavior.

Keywords

Adolescence; rat; social interactions; gonadal hormones; puberty; ethanol; sex differences

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Introduction

Alcohol consumption is typically initiated during adolescence, with almost half of high school seniors reported consuming alcohol in the preceding 30-day period according to The Monitoring the Future survey (Johnston et al., 2013). Not only is alcohol consumption common during adolescence, it is also consumed at levels greater than those seen in adulthood (SAMHSA, 2013). Alcohol use during adolescence (particularly at heavy and binge-like levels) is concerning given new research showing a strong association between age at first intoxication and risk for problem behaviors (e.g., drug use) (Kuntsche et al., 2013). Given these issues, research has focused on animal models of adolescence to determine possible underlying mechanisms for enhanced ethanol use during this developmental period, given that adolescent rodents have likewise shown similar patterns of elevated ethanol intake (Doremus et al., 2005; Maldonado et al., 2008; Tambour et al., 2008; Vetter et al., 2007).

Adolescence is a transitional period in development. In rodents, early-to-mid adolescence has been defined as postnatal (P) days 28–42, with late-adolescence/emerging adulthood occurring between P42–55 (Vetter-O'Hagen and Spear, 2012b). The neural, behavioral, and hormonal changes associated with adolescence are highly conserved across mammalian species (Spear, 2000, 2010). Some of the behaviors associated with adolescence include increases in risk-taking (Kelley et al., 2004; Laviola et al., 2003; Steinberg, 2004), novelty seeking (Adriani et al., 1998; Kelley et al., 2004), peer-directed social interactions (Montemayor, 1982; Varlinskaya and Spear, 2008), and the initiation and elevated use of alcohol (Doremus et al., 2005; Johnston et al., 2013). The changes that occur in the adolescent brain (e.g., synaptic pruning, myelination, changes in neurotransmitter composition) continue well into adolescence (Stiles and Jernigan, 2010), with this protracted brain development possibly contributing to the differences in alcohol sensitivity observed between adolescents and adults. For instance, whereas adolescents exhibit notable social facilitation in response to low doses of ethanol that is not seen in adults (Trezza et al., 2009; Varlinskaya and Spear, 2002), they conversely are less sensitive than adults to many of the impairing and adverse effects associated with moderate-to-high doses of ethanol (Anderson et al., 2010; Schramm-Sapota et al., 2010). Thus, adolescents may consume more alcohol than adults because of age-specific differences in responsivity to the positive and adverse effects of alcohol, which could lead to continued and sometimes excessive use of alcohol by adolescents.

Another potential contributor to the increased alcohol consumption observed during adolescence may be the puberty-associated hormonal changes that occur during adolescence. Recently, our laboratory has demonstrated that either pre- or post-pubertal gonadectomy in males resulted in patterns of elevated ethanol intake in adulthood that resembled those of adolescent males (Vetter-O'Hagen and Spear, 2011), with this effect being reversed by testosterone treatment (Vetter-O'Hagen et al., 2011). These data support and extend those previously reported, demonstrating elevated ethanol consumption in adult male Long Evans rats after pre-pubertal gonadectomy compared to sham controls (Sherrill et al., 2011). In an attempt to determine whether the increases in ethanol consumption observed after gonadectomy in males were due to alterations in response to the aversive effects of ethanol,

our laboratory utilized the conditioned taste aversion paradigm (Morales and Spear, 2013a). The results from that study, however, did not support the hypothesis that gonadectomized males were less sensitive to the aversive effects of ethanol in adulthood, thus leading them to drink more. Likewise, gonadectomy in males did not influence ethanol-induced social inhibition in adults (Vetter-O'Hagen and Spear, 2012a). Interestingly, while social inhibition after a moderate dose of ethanol (1 g/kg) was uninfluenced by gonadal hormones in that study, it did alter the microstructure of social behavior—more specifically, gonadectomy proportionally increased contact behavior and decreased social investigation in males when compared with their sham controls (Vetter-O'Hagen and Spear, 2012a). These results indicate that gonadectomy in male rats results in patterns of social behaviors that resemble those seen in early adolescent rats (e.g., elevated contact and lower investigation) (Varlinskaya and Spear, 2008).

Since gonadectomy did not impact the adverse effects of ethanol in the conditioned taste aversion or social inhibition paradigms, perhaps alterations (i.e., decreases) in sensitivity to the adverse effects of ethanol do not contribute to the elevated ethanol consumption seen in castrated males, at least under these circumstances. Therefore, the present study examined whether this sex-selective increase in ethanol intake reflects gonadectomy-induced maintenance of more adolescent-typical ethanol responsiveness into late adolescence in terms of enhanced sensitivity to positive (e.g., socially facilitating) effects of ethanol rather than a decreased sensitivity to the aversive (e.g., socially inhibitory) effects of ethanol. We have previously shown that physical and hormonal indicators of puberty in male and female rats occurs after P30 (Vetter-O'Hagen and Spear, 2012b); thus, rats in the current study were gonadectomized on P25—an age that ensures pre-pubertal status in both sexes. Although we previously examined the influence of gonadal hormones on ethanol-induced social inhibition in adulthood (Vetter-O'Hagen and Spear, 2012a), the current study utilized a dose-response curve to examine not only the social inhibitory effects of ethanol, but also the possibility that a lack of gonadal hormones may allow for the continued ontogenetic expression of ethanol-induced social facilitation at an age where this effect is not typically evident (i.e., P49; late adolescence) (Varlinskaya and Spear, 2004, 2006). This would support the idea that perhaps castration in males leads to elevated ethanol intake in adulthood because these animals retain sensitivity to the positive (e.g., socially facilitating) effects of ethanol. Testing rats at P49 also allowed for exploring whether removal of gonads prior to puberty results in maintenance of immature patterns of social behavior into late adolescence, an age normally characterized by more adult-typical patterns of social behavior and ethanol sensitivity. Although our laboratory has not generally observed notable effects of ovariectomy in females, females were included in the current study given the tendency for some of the effects observed on social behavior after gonadectomy to be driven by females (Vetter-O'Hagen and Spear, 2012a).

Materials and Methods

Subjects

Two hundred and forty male and female Sprague-Dawley rats derived from our breeding facility served as experimental subjects for the current experiment (an equal number served

as partners). On the day after birth (postnatal day [P] 1), litters were culled to 8–10 pups, maintaining a relatively equal sex ratio whenever possible. On P21, pups were weaned and pairhoused with a littermate of the same sex and given ad libitum access to food (Purina Lab chow, Lowell, MA) and water in a temperature-controlled vivarium with a 14:10 hr light-dark cycle (lights on at 0700 hr). At all times animals were treated in accordance with guidelines for animal care established by the National Institutes of Health under protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

Experimental Design

The current experiment investigated the impact of gonadal hormones on sensitivity to alterations in social behavior following ethanol administration in male and female rats. Thus, the experimental design was a 2 sex X 3 surgical condition (gonadectomy [GX], sham-operated [SH], non-manipulated [NM]) X 4 ethanol dose (0.5, 0.75, 1.0, 1.25) mixed factorial, with ten subjects placed into each condition.

Surgery

On P25, experimental animals were anesthetized using isoflurane (3.5% initially) and maintained at surgical levels of anesthesia throughout the surgery via nose cone supplementation (3% repeated as necessary). For castration in males, each testis was removed, a suture made in each tunic and in the inguinal ring (to prevent possible herniation), and the incision closed with Vetbond tissue adhesive (3M, St. Paul, MN). For ovariectomies, an incision was made on the dorsal side of the animal, caudal to the last rib and through the skin perpendicular to the midline. On each side of this incision, an opening was made in the muscle wall via blunt dissection, with the oviduct on each side sutured proximal to the ovary, the ovary excised, and the muscle wall sutured. For SH surgeries, animals were anesthetized and an incision was made; however, the reproductive tissue was not manipulated nor the gonads removed. At the time of surgery and then again in the afternoon, animals received a subcutaneous injection of the anti-inflammatory agent, ketofen (5 mg/kg); this two-injection procedure was repeated the following day. Following surgery, animals were given a subcutaneous injection of saline and were returned to their home cages, with a wire-mesh divider used to separate each housing pair for a recovery period of approximately 72 hours. On the fourth day, the divider was removed, and subjects were left undisturbed until testing in late adolescence (i.e., P47).

Drugs

Ethanol was administered intraperitoneally (i.p.) as a 12.6% v/v solution in 0.9% saline at doses of 0.5, 0.75, 1.0, and 1.25 g/kg. Saline was administered at an equivalent volume to the ethanol dose designated to each experimental subject. All solutions were administered at room temperature.

Social-Interaction Apparatus

Each social testing apparatus was made of clear Plexiglas (Binghamton Plate Glass, Binghamton, NY) and measured 45 × 30 × 20 cm. The apparatus was divided on the long axis into two equal sized compartments by a wall containing an aperture (9 × 7 cm) that

allowed movement between the compartments. The apparatus was separated by a solid wall that allowed testing of two pairs of animals of the same sex at the same time with little to no interference between the pairs. A white noise generator was located in the room to attenuate any extraneous sound. Before animals were placed into the social testing apparatuses, the walls/floors were wiped clean with a 6% hydrogen peroxide solution and fresh shavings were added.

Behavioral Measures

Each social test session was conducted without an experimenter present in the room and was videotaped with a camera (Sony Handycam) for later scoring. The behaviors of each experimental animal during the 10-min test session were later scored by two experimenters blind to the experimental condition of each animal (90% inter-experimenter agreement). The following behaviors were scored in terms of frequency: play (pouncing or playful nape attack, chasing and pinning); social investigation (sniffing any body part of the partner); and contact (crawling over or under the partner and social grooming). Frequency of each was summed together to obtain an overall social activity score. An index of social motivation was determined using a social preference/avoidance coefficient: $\text{Coefficient (\%)} = (\# \text{ of crossovers toward the compartment containing the partner} - \# \text{ of crossovers away from the partner}) / (\# \text{ of crossovers toward the partner} + \# \text{ of crossovers away from the partner}) \times 100$, with positive scores indicating a relative preference and negative scores indicating relative avoidance of the partner. An index of motor activity was calculated by summing the number of crossovers between compartments made by the experimental animal during the test.

Procedure

Experimental subjects received either GX or SH surgery or were left undisturbed (NM) until testing. Animals to be used as partners did not receive any manipulation prior to testing and were partnered with only one experimental animal. On P47, experimental and partner animals were weighed and individually habituated to the social testing apparatus for 30 min. On the following day (P48), each experimental animal was weighed, marked with a vertical line across the back to differentiate it from the partner animal, and injected with saline. Immediately after saline injection, each experimental animal was placed into the apparatus for 30 min, and then an unfamiliar partner of the same age and sex (weight difference within 10g) was added for a test duration of 10 min. Experimental and partner animals were then returned to their home cage until testing the following day. On P49, experimental animals underwent the same procedure as on the previous day, except they were challenged with 0.5, 0.75, 1.0 or 1.25 g/kg ethanol immediately prior to the 30 min habituation period in the test apparatus. After the 10 min social interaction test, experimental animals were decapitated and blood was collected for assay of testosterone, estradiol, progesterone, and corticosterone (CORT), with brains collected for determination of brain ethanol concentrations (BrECs).

Hormone assays

Plasma collected from whole blood was stored and maintained at -80°C until time of assay. Testosterone and estradiol levels were used to confirm removal of the gonadal tissue in males and females, respectively. For analysis of testosterone and progesterone, plasma

samples were thawed and assessed via radioimmunoassay (RIA) using a ^{125}I double antibody kit from MP Biomedicals (Solon, OH), with specificities of 100% for each hormone assayed. Testosterone assay sensitivity was 0.03 ng / ml, with inter- and intra-assay coefficients of variation of 10.1 and 6.6%, respectively. Progesterone assay sensitivity was 0.11 ng/ml, with inter-assay coefficients of 7.9% and intra-assay coefficients of 5.9%. Samples and standards for each hormone assay were run in duplicate, using a Packard Cobra II Autogamma Counter (PerkinElmer, Boston, MA), with disintegrations per minute averaged against a standard curve. 17 β -Estradiols were assayed using an enzyme linked immunosorbent assay (ELISA) kit (EnzoPlasma). A liquid-liquid extraction was performed on all samples using ethyl ether, anhydrous, with the organic phase being desiccated in a vacufuge and then subsequently reconstituted with assay buffer. The sensitivity of the assay was 14.0 pg/ml and intra-assay precision for controls containing 391.0, 127.2, and 70.6 pg/ml was 2.1, 7.1, and 5.7% respectively. Inter-assay precision of controls containing 380.8, 123.3, and 65.1 pg/ml was 8.3, 9.3, and 14.2% respectively. CORT levels were determined by RIA using competitive binding tritium-based kits obtained from MP Biomedicals, Inc. with 100% specificity for rat CORT. The sensitivity of the competitive binding assay was 25 pg/tube, with inter-assay coefficients of 10.4% and intra-assay coefficients of 6.1%. Standards and samples were assayed in duplicate, with disintegrations per minute averaged against a standard curve plotted with each assay and input into GraphPad Prism 5.0 software for calculation of concentration in each sample.

For BrEC, samples were assessed via headspace gas chromatography using a Hewlett Packard (HP) 5890 series II Gas Chromatograph (GC) (Wilmington, DE). Brain samples were thawed at time of analysis and 2 ml of cold distilled water added per gram of brain tissue before the sample was homogenized at 5 °C and placed in airtight vials. Vials were placed in a HP 7694E Auto-Sampler, which heated each individual vial for 8 min, and then extracted and injected a 1.0 ml sample of the gas headspace into the GC. Ethanol concentrations in each sample were determined using HP Chemstation software which compares the peak area under the curve in each sample with those of standard curves derived from reference standard solutions.

Data analysis

Data were checked for outliers and any data points meeting the criteria of ± 2 standard deviations from the mean were removed prior to statistical analysis. Collectively, the outliers for social behavior and motor activity resulted in the removal of <10% of the animals, with no more than two animals removed from any experimental group.

Due to the significant differences across groups in social and locomotor activity that emerged on saline baseline day (see results and Figures 2 and Figures 3), comparisons between the saline and ethanol challenge days were analyzed separately by sex for each surgical condition. Social behavior and motor activity on day 1 (i.e., on saline challenge baseline day), body weight (g), hormone levels, and BrECs were analyzed via factorial ANOVAs, whereas comparisons between baseline and ethanol challenge days were analyzed via repeated measures ANOVAs in females and males (Figure 5). Given that significant effects on locomotor activity (indexed via chamber crossovers) emerged

following ethanol (see Results; Figure 5, top panels), along with significant correlations between social behavior and locomotor activity evident across surgical conditions and sexes, all social behavior data were also analyzed via ANCOVAs using crossovers as a covariate to confirm that the observed changes in social behavior were not due to general activity suppressing effects of ethanol emerging at higher doses. Significant effects and interactions were explored further using Tukey's post hoc tests. Data are expressed as mean ($\pm$ SEM).

Results

Body weight

The factorial ANOVA for body weight (g) yielded a surgical condition X sex interaction [$F(2,234) = 109.77$, $p < 0.00001$]. Tukey's test revealed that GX females weighed significantly more than NM and SH females whereas an opposite pattern emerged in males, with GX males weighing significantly less than NM and SH males. There were no significant differences in body weight between NM and SH at either sex, and as expected, males weighed more than females (Figure 1).

Saline baseline (Day 1)

Baseline activity (crossovers)—Total number of crossovers on the saline baseline day revealed a main effect of surgical condition [$F(2,221) = 22.75$, $p < 0.00001$], with GX animals (48.33 ± 1.36) exhibiting significantly more crossovers than SH and NM animals (36.53 ± 1.07 and 39.19 ± 1.44 , respectively), regardless of sex.

Baseline levels of social behavior—The factorial ANOVA for overall social activity after a saline challenge revealed a main effect of surgical condition [$F(2,223) = 26.77$, $p < 0.00001$] and a surgical condition X sex interaction [$F(2,223) = 4.04$, $p < 0.05$]. GX females displayed significantly higher levels of overall social activity than GX males and than NM and SH females. GX males had significantly higher levels of overall social activity than SH males (Figure 2).

The ANOVA for play yielded main effects of sex and surgical condition [$F(1,223) = 10.10$, $p < 0.01$ and [$F(2,223) = 21.01$, $p < 0.00001$, respectively], as well as an interaction between the two variables [$F(2,223) = 4.99$, $p < 0.01$]. GX females exhibited significantly higher levels of play behavior than all other groups whereas the frequency of play in GX males was higher than SH males (Figure 3, top). The ANOVA for social investigation yielded a main effect of sex [$F(1,223) = 4.94$, $p < 0.05$], with males having significantly higher levels of social investigation than females (Figure 3, center). The ANOVA for contact behavior yielded a main effect of surgical condition [$F(2,223) = 40.74$, $p < 0.00001$], with GX animals displaying significantly more social contact than NM and SH animals, regardless of sex (Figure 3, bottom).

Analysis of the social preference/avoidance coefficient after a saline injection revealed a sex X surgical condition status interaction [$F(2,221) = 13.15$, $p < 0.00001$], GX females exhibited significantly greater social preference scores than SH females whereas an opposite pattern emerged in males, with GX males having significantly lower social preference coefficients than NM and SH males (Figure 4).

Ethanol test day (Baseline vs. test day analyses)

Total number of crosses following challenge with ethanol—The ANOVA for crossovers at ethanol test revealed that significant reductions in locomotor activity emerged at lower doses in GX females and males than in their NM counterparts, and than in SH females (see Figure 5, top panels).

Social activity following ethanol challenge—In females, the 1.0 and 1.25 g/kg doses of ethanol significantly reduced overall social behavior in NM, SH, and GX animals (Figure 5, bottom panels), with similar patterns emerging for play, social investigation and contact (data not shown). In males, levels of overall social behavior (Figure 5, bottom panels), play, investigation and contact behavior (data not shown) were reduced after challenge with 1.0 and 1.25 g/kg ethanol in NM and GX males; however, this effect only emerged after 1.25 g/kg in SH males. In contrast to the ethanol effects seen with the other measures of social behavior, the social preference/avoidance coefficient revealed no significant differences (data not shown).

A summary of the ANCOVA data using crossovers as the covariate is provided in Table 1 for overall social activity, each social behavior, and the social preference/avoidance coefficient. In females from all surgical conditions, 1 and 1.25 g/kg ethanol significantly reduced overall social activity compared to corresponding baseline days. Reductions at these doses were also seen for each component social behavior among SH and GX females, but only for social investigation in NM females. Among NM females, no decreases in play were seen at any dose, with only the highest dose decreasing contact behavior. GX and NM males showed disruptions in overall social activity and each component social behavior at doses of 1 and 1.25 g/kg ethanol; SH males in contrast were generally less sensitive to ethanol, showing a decrease in overall social activity at only the highest dose and no significant decreases in investigation or contact at any dose.

Hormone levels

In females, the ANOVA for estradiol levels revealed a main effect of hormonal status [$F(2,117) = 4.66$, $p < 0.01$], with GX females having significantly lower estradiol levels than SH and NM females (see Table 2a). Although there was a trend ($p = 0.08$) for ethanol to increase estradiol levels in a step-wise fashion, this effect did not reach statistical significance (Table 2b). Testosterone levels were significantly lower in GX than SH and NM males (main effect of hormonal status in ANOVA of testosterone levels: $F(2,117) = 58.10$, $p < 0.000001$) (Table 2a), and there was no effect of ethanol on testosterone levels (Table 2b).

The analysis of progesterone revealed a main effect of sex [$F(1,234) = 115.27$, $p < 0.000001$], hormonal status [$F(2,234) = 29.08$, $p < 0.000001$], dose [$F(3,234) = 6.35$, $p < 0.001$] and an interaction between the sex and hormonal status [$F(2,234) = 24.75$, $p < 0.000001$]. As expected, there were no differences among males of any hormonal status and GX females had significantly lower progesterone levels than SH and NM females. Overall, SH and NM females had significantly higher progesterone levels than their male counterparts, with no difference observed between GX males and females (Table 2a). Post-hoc analyses collapsed

across sex and hormonal status to explore the main effect of dose revealed that higher doses of ethanol (1.0 and 1.25 g/kg) significantly elevated progesterone levels, with significantly higher progesterone levels seen following administration of 1.25 g/kg ethanol than 0.5 and 0.75 g/kg, and at 1.0 g/kg relative to the 0.5 g/kg group only (Table 2b).

Analysis of corticosterone levels likewise revealed a main effect of sex [$F(1,234) = 154.15$, $p < 0.000001$], hormonal status [$F(2,234) = 3.64$, $p < 0.05$], ethanol dose [$F(3,234) = 24.40$] and an interaction between sex and hormonal status [$F(2,234) = 16.04$, $p < 0.000001$]. As expected, females in all groups had significantly higher corticosterone levels than their male counterparts. GX females had significantly lower corticosterone levels than SH and NM females, whereas an opposite pattern emerged in males: GX males had higher corticosterone levels than SH males, with a similar trend seen relative to NM males (Table 2a). Ethanol challenge resulted in an increase in corticosterone levels, with 1.25 g/kg resulting in significantly higher corticosterone levels compared to all other ethanol challenge doses. Similarly, 1 g/kg resulted in significantly elevated corticosterone levels compared to the lower ethanol challenge doses (Table 2b).

Brain ethanol concentrations (BrECs)

The analysis of BrECs revealed a main effect of sex [$F(1,216) = 9.52$, $p < 0.01$], with males having slightly but significantly higher BrECs (50.85 ± 2.77) than females (47.07 ± 2.76). A main effect of dose [$F(3,216) = 729.97$, $p < 0.00000001$] emerged, indicating a significant dose-dependent increase in BrECs, regardless of sex or hormonal status (Table 3).

Discussion

The present study revealed no effects of gonadectomy on the social consequences of an acute ethanol challenge in terms of sensitivity to ethanol-induced social facilitation or inhibition in late adolescent male and female rats. Pre-pubertal gonadectomy, however, did alter baseline social behavior, with saline-challenged animals demonstrating an immature social behavioral profile in late adolescence, indexed via increases in play fighting and contact behavior, regardless of sex. Effects of early manipulation (i.e., sham surgery) were also observed in males, with the sham control group demonstrating decreased sensitivity to ethanol-induced social inhibition.

To the extent that the low dose facilitation of social activity seen early in adolescence declines developmentally due to rises in gonadal hormones, it would have been expected that as late adolescents, previously gonadectomized animals would continue to show low-dose ethanol facilitation of social activity (i.e., play fighting and contact). However, this was not observed, as there were no signs of social facilitatory effects seen after any ethanol dose during late adolescence in any of the experimental groups. Furthermore, regardless of surgical condition, males and females generally exhibited decreased social behavior following the higher ethanol doses tested (i.e., 1 and 1.25 g/kg). Gonadectomy also had no effect on the social preference/avoidance coefficient, likely because this measure was found to be insensitive to ethanol under the present test circumstances. The lack of ethanol-induced social avoidance in the current study may be attributed to the 2-day, repeated measures test procedure used—a procedural difference that appears to affect the social preference/

avoidance coefficient differently than other social behaviors when compared to between subjects, single day test (Morales and Spear, 2013b; Morales et al., 2013).

The ethanol challenge data seen in the present study support the existing literature demonstrating that during the late-adolescent/adult transition, social facilitation is not seen after low doses of ethanol, whereas moderate ethanol doses induce social inhibition under non-stressful/non-anxiety-provoking test circumstances (Varlinskaya and Spear, 2006; Varlinskaya et al., 2013). These data are also reminiscent of the overall lack of gonadectomy effects on ethanol-induced social suppression previously reported in adult rats (Vetter-O'Hagen and Spear, 2012a). Together, these data support the suggestion that gonadal hormones may not have a notable impact on responses to ethanol in a social context. Although there was no effect of surgical condition on typical ethanol-induced social inhibition observed at moderate doses in either sex, our data do show that females exposed to surgical manipulations were more sensitive to ethanol-induced inhibition of social behaviors (i.e., play and contact) relative to non-manipulated controls, with this social inhibition evident at lower ethanol doses in gonadectomized females and sham controls than in their non-manipulated counterparts. Given that these effects were apparent regardless of ovarian hormonal status and occurred in both surgical conditions, it may reflect an effect of the stress presumably induced by the surgical manipulation itself or some other aspect of the surgical procedure (e.g., exposure to isoflurane).

Despite the fact that gonadectomy did not influence ethanol-induced changes of social behaviors, it did alter social behaviors at baseline, with gonadectomized females displaying significantly higher levels of play fighting than their sham and non-manipulated counterparts. Likewise, in males, gonadectomy resulted in higher levels of play fighting relative to sham males. Analogous effects emerged for contact behavior for each sex. Similar findings have been reported recently (Vetter-O'Hagen and Spear, 2012a), with gonadectomy altering the microstructure of social behaviors in adulthood (i.e., rats spent proportionally less time in social investigation and more time in contact behavior), resembling a social behavioral profile of early adolescents (Panksepp, 1981; Vanderschuren et al., 1997; Varlinskaya et al., 2013). It may be that the rise in gonadal hormones during puberty plays an inhibitory role in these social behaviors, and thus removing the gonads results in opposite behavioral patterns. Support for this hypothesis comes from another study showing that "curiosity" behavior, which is akin to social investigation in the current study (i.e., sniffing and following), was found to be lower when tested in a familiar environment in young adult (P60) male rats castrated pre-pubertally than in intact males (Primus and Kellogg, 1989). In contrast, another study showed that pre-pubertal castration in male rats reduced the expression of rough-and-tumble play (Cooke and Woolley, 2009) that is normally observed in intact mid-adolescent (P31–35) males (Vanderschuren et al., 1997; Varlinskaya et al., 1999), with no differences emerging throughout the remainder of the testing procedure (P41–45). Although we cannot directly compare results across laboratories, it is possible that procedural differences may have contributed to these disparate findings. For example, the rats in the Cooke and Woolley (2009) study were tested multiple times a week in their home-cage under red light, whereas animals in the current study were tested twice (saline injection, ethanol injection), in another environment (social interaction apparatus in a different room) and under dim lighting (<30 lux). Thus, the effects

of early gonadectomy on social interactions in males and females are not entirely clear. These changes appear to be dependent on methodological differences, including age at castration in males (Beatty et al., 1981), and should be investigated further.

The role of gonadal hormones on play behavior in male and female rats has been studied for decades. For instance, a series of lesion studies conducted in the 1980s demonstrated that the amygdala was differentially involved in modulation of play fighting in males and females, whereas this sex-dependent effect was not observed following lesions of other brain areas (Beatty and Costello, 1983; Meaney et al., 1981). The amygdala contains receptors for gonadal hormones (Sheridan, 1979; Shughrue et al., 1997), suggesting that sex steroids may affect the actions of cells in this nucleus. Neurophysiological differences between gonadectomized and intact animals may contribute to the baseline behavioral differences between these hormonal status conditions. For instance, neurons in the medial amygdala—a brain region shown to be involved in social recognition (Ferguson et al., 2001)—of intact male rats had lower dendritic spine density than GX male neurons (Cooke and Woolley, 2009). However, this effect does not seem to be age-specific, given that adult (~P90) GX rats showed significant decreases in dendritic spine density in the posterodorsal medial amygdala compared to their respective same-sex, intact, counterparts (de Castilhos et al., 2008). These authors also reported that, in females, injections of estradiol benzoate (EB) or EB and progesterone significantly increased spine density in medial amygdala neurons relative to neurons in GX females (de Castilhos et al., 2008). Likewise, estradiol has also been shown to promote the development of dendritic branches in medial amygdala neurons compared to controls (Lorenzo et al., 1992). These results suggest that in some brain regions, gonadal hormones may modulate neural activity associated with certain behaviors, including social (play) behaviors.

Our laboratory has previously observed robust effects of early surgical manipulation in studies examining the impact of gonadal hormones on ethanol-related behaviors (Vetter-O'Hagen and Spear, 2011, 2012a). For instance, males given pre-pubertal sham surgery (P23) consumed more ethanol in adulthood relative to non-manipulated controls and adult sham animals (Vetter-O'Hagen and Spear, 2011). Male and female rats that were sham-operated also were found to exhibit reduced exploration of a novel object when compared to adult sham animals, and to not show the social avoidance after a moderate dose (1 g/kg) of ethanol (Vetter-O'Hagen and Spear, 2012a) that is normally evident in adult rats (Varlinskaya and Spear, 2002). In the current experiment, we observed that pre-pubertal sham surgery in males attenuated later sensitivity to the social inhibitory effects of ethanol, with inhibition of overall social activity evident only at 1.25 g/kg ethanol and no significant inhibition of contact or social investigation at any dose. Thus, again stress or some other aspect of the surgical manipulation process itself during the juvenile developmental period was found to attenuate later ethanol sensitivity. To the extent that these effects of ethanol normally serve as feedback cues to moderate ethanol intake, such attenuations in ethanol sensitivity may be related to the elevated ethanol intake observed in these animals in adulthood (Vetter-O'Hagen and Spear, 2011). Indeed, the literature does show that a variety of early life stressors enhances ethanol consumption in adulthood, with this effect seen in rodents (Butler et al., 2014; Chappell et al., 2013; Schenk et al., 1990), non-human primates (Higley et al., 1991), and humans (Enoch, 2011; Sinha, 2001). While much of the research

on early stress altering ethanol intake or sensitivity in adulthood has focused predominantly on males, there have been some studies that included females (Lopez et al., 2011). For instance, in rats, early stress (i.e., post-weaning isolation) was shown to have no effect on a conditioned taste aversion to ethanol in adult female rats (Smith et al., 1998).

As expected, removal of the gonads results in significantly lower levels of estradiol and testosterone in female and male rats, respectively. Despite significant reductions in estradiol levels after GX, the decline was modest. It was not surprising that estradiol levels were still notable after gonadectomy because of other (non-ovarian) origins of estrogen (Nelson and Bulun, 2001; Takahashi, 1961). Although complete removal of the ovaries was not confirmed, we are confident that females were properly GX given that progesterone levels were more robustly reduced in GX females and this hormonal status condition also demonstrated significantly greater body weight than SH and NM counterparts., replicating results from our laboratory (Morales and Spear, 2013a). The progesterone finding was expected given progesterone's role in female reproductive function (Graham and Clarke, 1997). Not surprisingly, progesterone levels were also higher in females relative to males, with progesterone levels in males not differing as a function of surgical condition. Females of all surgical conditions demonstrated significantly higher corticosterone levels than their male counterparts, a sex difference reliably seen across studies (Handa et al., 1994; Morales and Spear, 2013a; Willey et al., 2012). Corticosterone levels were significantly higher in gonadectomized than sham-operated males whereas an opposite effect emerged in females, supporting previous results in male and female rats (Seale et al., 2004a). The higher levels of corticosterone seen in GX males than intact controls is consistent with prior findings (Morales and Spear, 2013a) and has been shown to be reversed by hormone replacement (Seale et al., 2004b). Although not significant, there were clear trends for SH males also to have lower corticosterone levels than NM and GX males ($p=0.08$ and $p=0.07$, respectively). Similar trends emerged in earlier work (Morales and Spear, 2013a), an effect that reached significance in that study in SH versus GX males. It is possible that the stress of SH surgery altered their HPA axis in a manner different to that of intact males, with the removal of the gonads generally precluding this surgery effect. Unfortunately, it is unclear what, if any, effects pre-pubertal surgical stress may have on corticosterone levels given that most studies of gonadectomy do not include a NM group to assess potential consequences of the surgical process per se. The data are also mixed on how juvenile/adolescent stress alters later basal corticosterone levels, with reports of no effect (Barha et al., 2011; Isgor et al., 2004; McCormick et al., 2008) contrasting with findings of higher corticosterone levels (Jacobson-Pick and Richter-Levin, 2010) following prior stress in male rodents. Given that our laboratory has found consistent effects of early surgical manipulation, future studies may benefit from the inclusion of both a SH-operated and NM control group to further explore and understand the role that surgical manipulation per se may have on subsequent behavioral and hormonal outcomes.

It was not surprising that ethanol elevated levels of corticosterone given that administration of ethanol has consistently been shown to increase corticosterone levels (Silveri and Spear, 2004; Willey et al., 2012). We were somewhat surprised to find that this ethanol effect did not interact with sex or hormonal status, given that typically females and GX males show a greater corticosterone response to ethanol than intact males in adulthood (Ogilvie and

Rivier, 1997; Rivier, 1993). It is possible that these sex differences may be more pronounced in adult-tested animals than in the late adolescent animals assessed in this study. Progesterone levels also were increased after acute ethanol challenge in both sexes. This finding is consistent with previous work in females (Budec et al., 2002), and extends this effect to males (where this effect has been little explored).

Taken together, these results are consistent with the suggestion that the social consequences of an acute ethanol challenge are not influenced by the presence or absence of gonadal hormones in late adolescent rats. Although not involved in responsiveness to the social consequences of acute ethanol, gonadectomy did significantly alter social behaviors at baseline, supporting previous findings from our laboratory that pre-pubertal removal of the gonads results in an immature social behavioral profile. We also replicated the important finding that surgical manipulations during juvenile development have long-lasting consequences, including alterations in ethanol sensitivity later in life.

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Highlights

- Gonadectomy (GX) increased basal levels of social interactions in males and females
- Neither GX males nor females showed low-dose EtOH facilitation of social behavior
- Higher doses of EtOH reduced social behavior in both sexes, regardless of GX status
- Sham males displayed an insensitivity to EtOH-induced social inhibition

Figure 1.

Body weight (g) in adolescent (P47) male and female rats. GX females weighed significantly more than NM and SH females whereas GX males weighed significantly less than both NM and SH males. Overall, males weighed more than females (# denotes a sex effect; + denotes a hormonal status effect).

Figure 2.

Overall social activity (frequency) after saline challenge. GX females had significantly higher levels of social activity relative to SH and NM females, and GX males. Frequency of social activity was significantly higher in GX males than SH males (# denotes a sex effect; + denotes a hormonal status effect).

Figure 3.

Frequency of play (left), social investigation (center) and social contact (right) behavior after saline. GX females had significantly higher levels of play behavior than SH and NM females, and GX males. Males had significantly higher levels of social investigation relative to females. GX females and males displayed significantly more social contact behavior than SH and NM animals (# denotes a sex effect; + denotes a hormonal status effect).

Figure 4. Social preference/avoidance coefficient after a saline challenge (i.e., baseline) in females and males. GX resulted in females with a significantly higher coefficient than NM and SH females, whereas GX males had a significantly lower coefficient than NM and SH males (+ denotes a hormonal status effect).

Figure 5.

Total crossovers (top panels) and overall social activity (bottom panels), in female and male rats, as indicated. Locomotor activity and overall social activity was generally suppressed at higher ethanol doses (* denotes a significant difference between saline baseline day and ethanol challenge test day).

Table 1

Significant Day x Dose interactions, with dose effects indicated

Dependent Variable	NM					SH					GX				
	0.5	0.75	1.0	1.25	F	0.5	0.75	1.0	1.25	F	0.5	0.75	1.0	1.25	F
Overall social	---	---	↑	↑	18.86	---	---	↑	↑	6.86	---	---	↑	↑	10.56
♀	---	---	↑	↑	0.90	---	---	---	↑	4.12	---	---	↑	↑	24.29
♂															
Play	---	---	↑	↑	4.13	---	---	↑	↑	6.64	---	---	↑	↑	3.57
♀	---	---	↑	↑	5.91	---	---	---	↑	18.22	---	---	↑	↑	5.95
♂															
Investigation	---	---	↑	↑	12.38	---	---	↑	↑	NS	---	---	↑	↑	7.27
♀	---	---	↑	↑	8.49	---	---	---	↑	13.57	---	---	↑	↑	25.91
♂															
Contact	---	---	↑	↑	23.38	---	---	↑	↑	7.43	---	---	↑	↑	12.28
♀	---	---	↑	↑	6.89	---	---	---	↑	NS	---	---	↑	↑	13.93
♂															
Coefficient	---	---	---	---	NS	---	---	---	---	NS	---	---	---	---	NS
♀	---	---	---	---	NS	---	---	---	---	NS	---	---	---	---	NS
♂															
Motor	---	---	↑	↑	7.15	---	---	↑	↑	6.17	---	↑	↑	↑	9.31
Activity	---	---	↑	↑	10.82	↑	↑	↑	↑	36.96	↑	↑	↑	↑	5.06
♀															
♂															

Bold indicates a main effect of day

Table 2

a. Hormone levels (mean±SEM) in female and male rats.

Hormonal Status	Females			Males		
	Estradiol (pg/ml)	Progesterone (ng/ml)	Corticosterone (ng/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)	Corticosterone (ng/ml)
NM	45.19±3.61	64.51±7.16 ^c	510.93±42.63 ^c	1.29±0.13	12.45±2.40	190.20±23.21
SH	46.77±3.57	53.01±5.51 ^c	537.37±41.76 ^c	1.32±0.11	6.49±1.58	117.13±18.26
GX	33.76±2.51 ^a	11.16±1.66 ^a	331.70±30.51 ^{ac}	0.00±0.00 ^a	8.53±1.14	221.80±25.18 ^b

b. Hormone levels (mean±SEM) after ethanol challenge

Ethanol dose (g/kg)	Progesterone (ng/ml) [*]	Corticosterone (ng/ml) [*]	Estradiol (pg/ml) ^{**}	Testosterone (ng/ml) ^{***}
0.5	16.73±2.89	215.40±26.24	34.76±2.30	0.88±0.15
0.75	22.83±4.63	233.88±26.98	41.29±4.06	1.00±0.19
1.0	29.70±5.13 [‡]	372.85±34.22 [‡]	42.85±4.31	0.84±0.15
1.25	34.85±4.41 [‡]	450.62±33.48 [‡]	48.74±4.29	0.76±0.15

^a GX animals differed significantly from same-sex SH and NM counterparts

^b GX animals differed significantly from same-sex SH only

^c Sex difference for the corresponding hormonal status

^{*} Data collapsed across sex

^{**} Data collected in females only

^{***} Data collected in males only

[‡] 1 and 1.25 g/kg elevated hormone levels compared to lower ethanol doses

Table 3

Brain Ethanol Concentrations (BrECs) (mean±SEM) in NM, SH, and GX female and male rats

Ethanol dose (g/kg)	Female (mg/dl)			Male (mg/dl) *		
	NM	SH	GX	NM	SH	GX
0.5	10.30±1.37	7.51±1.33	7.43±1.16	12.75±2.33	15.56±3.25	11.92±2.84
0.75	33.43±1.01	33.24±1.26	34.57±1.56	38.93±2.22	38.00±2.52	34.25±2.88
1.0	60.22±2.80	59.74±1.66	59.82±1.83	69.31±2.58	57.54±5.58	65.37±2.38
1.25	89.24±2.46	81.06±5.69	88.28±4.30	91.79±3.57	85.25±5.66	89.54±1.87

* ♂ > ♀