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Acute prenatal exposure to ethanol on gestational day 12 elicits opposing deficits in social behaviors and anxiety-like behaviors in Sprague Dawley rats

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Abstract

Our previous research has shown that in Long Evans rats acute prenatal exposure to a high dose of ethanol on gestational day (G) 12 produces social deficits in male offspring and elicits substantial decreases in social preference relative to controls, in late adolescents and adults regardless of sex. In order to generalize the observed detrimental effects of ethanol exposure on G12, pregnant female Sprague Dawley rats were exposed to ethanol or saline and their offspring were assessed in a modified social interaction (SI) test as early adolescents, late adolescents, or young adults. Anxiety-like behavior was also assessed in adults using the elevated plus maze (EPM) or the light/dark box (LDB) test. Age- and sex-dependent social alterations were evident in ethanol-exposed animals. Ethanol-exposed males showed deficits in social investigation at all ages and age-dependent alterations in social preference. Play fighting was not affected in males. In contrast, ethanol-exposed early adolescent females showed no changes in social interactions, whereas older females demonstrated social deficits and social indifference. In adulthood, anxiety-like behavior was decreased in males and females prenatally exposed to ethanol in the EPM, but not the LDB. These findings suggest that social alterations associated with acute exposure to ethanol on G12 are not strain-specific, although they are more pronounced in Long Evans males and Sprague Dawley females. Furthermore, given that anxiety-like behaviors were attenuated in a test-specific manner, this study indicates that early ethanol exposure can have differential effects on different forms of anxiety.

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Keywords

prenatal ethanol; anxiety; social behavior; Sprague Dawley rat; sex differences; fetal alcohol spectrum disorder

1. Introduction

Alcohol use typically begins in early adolescence [1], with early onset of drinking playing an important role in alcohol-related problems later in life [2, 3]. Individuals who engage in episodic heavy drinking at an early age are more likely to become dependent on alcohol [4–6] and to experience a number of long-lasting adverse psychosocial consequences [7]. Alcohol-dependent adolescents have been found to demonstrate enhanced negative emotionality, characterized by anxiety, depression, and high stress reactivity [8].

Emerging evidence points to a relationship between prenatal alcohol exposure, anxiety, and adolescent alcohol use. While affective disorders, including anxiety, are among the most commonly reported problems in children with fetal alcohol spectrum disorder (FASD) [9–11], a number of studies have also shown that, during adolescence, anxiety is associated with alcohol use, abuse, and dependence [12–15]. Although this issue has not been directly addressed in human studies, anxiety disorders, including social anxiety, in individuals with fetal alcohol exposure may potentially play a substantial role in early initiation of drinking during adolescence, with alcohol becoming more appealing for these individuals due to its anxiolytic, calming, and stress-relieving effects.

Prenatal exposure to alcohol is not only associated with anxiety disorders, but also produces alterations in different aspects of social behavior. For instance, children and adolescents with fetal alcohol exposure have difficulties considering the consequences of their actions, understanding social cues, and communicating in social contexts [16–18]. Similarly, older adolescents and adults with FASD experience certain difficulties in interactions with peers [19]. Consequently, alcohol may become appealing to these individuals due to its ability to facilitate interactions with peers [20–22]. Social behavior of laboratory rodents is sensitive to prenatal ethanol exposure as well, with alterations in adolescent-characteristic [23–27] and adult-typical forms of social interactions [23, 28–30] evident following chronic fetal exposure to ethanol. Furthermore, our previous research has shown that in Long Evans rats prenatal exposure to a single, binge-like dose of ethanol on gestational day (G)12 (early neural generation), but not on G7 (gastrulation) or G15 (mid-neuronal generation), reproducibly results in sex- and age of testing-dependent social deficits. Specifically, males, but not females, demonstrate decreases in social investigation, contact behavior, and play fighting when tested in adolescence and adulthood, whereas substantial decreases in social preference and emergence of social indifference or social avoidance are evident in late adolescents and adults regardless of sex [31–33].

In order to generalize the observed social consequences of acute ethanol exposure on G12 and ensure that the observed age- and sex-dependent effects on social behavior are not strain-specific, Sprague Dawley rats were used in the present study. We hypothesized that acute prenatal exposure to ethanol on G12 would produce sex-dependent and age-associated

social alterations in a strain-independent manner. However, given that ethanol can be viewed as a stressor, and stress sensitivity differs between Long Evans and Sprague Dawley females [34], we cannot preclude some differences in the effects of acute prenatal ethanol on social behavior and social preference between the two strains.

In addition to the social alterations, prenatal ethanol exposure may result in altered behavioral responsiveness to stressful, anxiety-provoking test situations, including the elevated plus maze (EPM). However, experimental findings are rather inconsistent, with both increases and decreases of anxiety-like behavior on the EPM reported following chronic gestational ethanol exposure [35–44]. Therefore, another objective of the study was to investigate alterations in anxiety-like behaviors resulting from acute prenatal exposure to ethanol.

Specifically, Sprague Dawley pregnant female rats bred in our colony were exposed to ethanol or saline on G12 and their offspring were assessed in a modified social interaction (SI) test on postnatal day (P) 28 (early adolescents), P42 (late adolescents) or P77 (young adults). During adulthood, behavioral responses in classic tests of anxiety-like behaviors (EPM and light/dark box (LDB)) were assessed in the same animals exposed prenatally to ethanol or saline.

2. Materials and Methods

2.1. Subjects

Experimental subjects were the offspring of Sprague Dawley rats that were produced by time mating in our colony at Binghamton University; colony originated from animals purchased from Taconic Inc. (Bloomington, PA). Animals were housed in a temperature-controlled (22°C) vivarium, and maintained on a 12:12 hr light:dark cycle (lights on at 0700 hr) with ad libitum access to food (Purina rat chow) and water. For breeding, adult rats were housed in groups of three females and one male in a large plastic cage during a 4-day period. Vaginal smears were collected every day, with the first day of detectable sperm designated as gestational day (G) 1 [32]. On G12, females were injected intraperitoneally (i.p.) with 2.5 g/kg ethanol (20% v/v ethanol in physiological saline). Two hours later, pregnant females received a second i.p. injection of 1.25 g/kg ethanol. Control females received two i.p. injections of equivalent volumes of saline. Relative to our previous studies with Long Evans rats, the dose of ethanol was decreased from 2.9 g/kg (first injection) and 1.45 g/kg (second injection), as Sprague Dawley females did not give birth with these higher doses (4 out of 4). Non-injected dams provided males and females that were used as social partners. Day of birth was considered P0. Litters were culled to 10 pups within 48 hr of birth on P2, maintaining a 1:1 sex ratio when possible. Pups were left with their dams in standard plastic maternity cages with pine shavings as bedding material. Animals were weaned on P21 and placed into standard plastic cages with same-sex littermates (3 animals per cage in this study, whereas the extra 2 pups of each sex were pair-housed and used for a different study). In all respects, maintenance and treatment of the animals were in accord with guidelines for animal care established by the National Institutes of Health, using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

A total of 16 dams injected with saline (n=8) or ethanol (n=8) on G12 provided 48 male and 48 female experimental subjects (i.e., 24 males and 24 females for each prenatal condition). An additional 48 males and 48 females derived from 16 non-injected females were used as partners for the social testing. Animals were tested socially either on P28 (early adolescence), P42 (late adolescence), or P77 (young adulthood), with animals from a given litter randomly assigned to the age of testing. Equal number of males and females (n = 8 per group) were placed into each prenatal exposure/age condition to allow analysis of sex effects. On P80, animals (8 males and 8 females per each prenatal condition) were tested on an elevated plus maze (EPM), whereas anxiety-like behavior in a light/dark box (LDB) was assessed on P85 (n = 8/sex/prenatal condition). Each experimental subject was tested socially at a given age and then either on the EPM or in the LDB, with the number of animals from each social testing/age condition counter-balanced between the two non-social tests, with only one male and one female subject from a given litter assessed in each test to eliminate the possible confounding of litter with prenatal treatment effect [45, 46]. In the SI test, one male and one female from a given litter was tested at each age.

2.2. Blood Ethanol Concentration

Maternal blood ethanol concentrations (BECs) were determined in trunk blood samples collected at various time points (30, 125, 240, and 480 min) following the first injection of ethanol on G12 (n = 3 pregnant females/time point). Animals were decapitated, and blood samples were collected in heparinized tubes, rapidly frozen, and maintained at -80°C until analysis of BECs. Samples were assessed for BEC via headspace gas chromatography using a Hewlett Packard (HP) 5890 series II Gas Chromatograph (Wilmington, DE). At the time of assay, blood samples were thawed and 25 μl aliquots were 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 gas chromatograph. 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.

2.3. Modified Social Interaction Test

All testing was conducted under dim light in Plexiglas (Binghamton Plate Glass, Binghamton, NY) test apparatuses (30 $\times$ 20 $\times$ 20 cm for adolescents tested at P28 or P42 and 45 $\times$ 30 $\times$ 20 cm for adults tested at P77) containing clean shavings. Each test apparatus was divided into two equally sized compartments by a clear Plexiglas partition that contained an aperture (7 $\times$ 5 cm for adolescents and 9 $\times$ 7 cm for adults) to allow movements of the animals between compartments in a way that only one animal was able to move through the aperture at a time [33, 47, 48].

On test day, animals were taken from their home cage and placed individually in the testing apparatus for 30 min. A social partner of the same age and sex was then introduced for a 10-min test period. Partners were always unfamiliar with both the test apparatus and the experimental animal, were not socially deprived prior to the test, and were experimentally naïve [49–51]. Weight differences between test subjects and their partners were minimized as much as possible, with this weight difference not exceeding 10 g at P28, 20 g at P42, and

30 g at P77, and test subjects always being heavier than their partners. In order to differentiate experimental animals from their social partners during the test, each experimental animal was marked with a vertical black line on the back.

During the 10-min test session, the behavior of the animals was recorded by a video camera, with real time being directly stamped onto the video record for later scoring (Easy Reader II Recorder; Telcom Research TCG 550, Burlington, Ontario). All testing procedures were conducted between 0900 and 1100 hr under dim light (15–20 lx). All experimental animals were returned to their home cages, with each animal in a cage tested only once in the SI test.

As in our previous studies, the frequencies of social investigation, contact behavior, and play fighting were analyzed from video recordings [31–33, 49–52] by a trained experimenter without knowledge of the experimental condition of any given animal. The frequencies, rather than time, were scored and analyzed, given that elementary behavioral acts and postures (i.e., ethogram) demonstrated by experimental subjects are discrete and extremely short lasting, especially during adolescence. Therefore, this analysis allows us to provide better comparisons between adolescent and adult rats. Social investigation was defined as the sniffing of any part of the body of the partner. Contact behavior defined as crawling over and under the partner and social grooming. Play fighting was scored as the sum of the frequencies of the following behaviors: pouncing or playful nape attack (experimental subject lunges at the partner with its forepaws extended outward); following and chasing (experimental animal rapidly pursues the partner); and pinning (the experimental subject stands over the exposed ventral area of the partner, pressing it against the floor). Play fighting can be distinguished from serious fighting in the laboratory rat by the target of the attack — during play fighting, snout or oral contact is directed towards the partner's nape, whereas during serious fighting the partner's rump is the object of the attack [53]. Aggressive behavior (serious fighting) was not analyzed in these experiments, since subjects did not exhibit serious attacks or threats.

Modification of the social interaction test [47], allowing experimental animal to freely move toward or away from a non-manipulated social partner in a 2-compartment testing apparatus, permitted assessment of social motivation via a preference/avoidance coefficient. The number of crossovers demonstrated by the experimental subject towards, as well as away from, the social partner was measured separately, and a coefficient of preference/avoidance was then calculated [coefficient (%) = (crossovers to the partner – crossovers away from the partner)/(total number of crosses both to and away from the partner) × 100]. Social preference was defined by significantly positive values of the coefficient, whereas social avoidance was associated with negative values that differed significantly from 0 [47]. The values of the coefficient that did not significantly differ from 0 reflected social indifference. Total number of crossovers (movements between compartments) was used as an index of general locomotor activity under these circumstances.

2.4. Elevated Plus Maze

On P80, anxiety-like behavior was assessed using a standard EPM apparatus, as previously described [54] with incandescent lighting (~90 lux at junction). The maze consisted of two open arms and two closed arms (each arm was 48.3 cm long and 12.7 cm wide). The open

arms had small plastic edges (1.3 cm high) located along each side and end of the open arms to prevent the animals from slipping off the edge. The closed arms were surrounded by walls 29.2 cm tall. The maze was elevated 50.0 cm above the floor. On testing day, animals were socially isolated in an unfamiliar holding cage for 60 min in a novel room, as this has been shown to increase exploration in the open arms [55, 56]. At the beginning of testing, each subject was placed on the center platform facing an open arm and was allowed to freely move in the maze for 5 min. During testing, the experimenter was not in the room. Between animals, the apparatus was cleaned with 50% ethanol and thoroughly dried. All sessions were video recorded and then scored by an experimenter blind to the experimental condition of each subject. Measures scored included time in open and closed arms, as well as entries into open and closed arms. An animal was considered to have entered an arm when all four paws were placed in the arm. An animal was considered to have exited an arm when at least two front paws were placed outside the arm. Percentage of time spent on the open arms and percentage of the open arm entries were used as reliable measures of anxiety-like behavior on the EPM [56, 57]. Closed arm entries were used as indices of activity [55, 58, 59].

2.5. Light-Dark Box

On P85, we extended our assessment of generalized anxiety-like behaviors using the LDB. Animals that had been tested on the EPM were not used. The apparatus consisted of two chambers ($34 \times 24 \times 24$ cm) joined together length-wise [60]. There was an aperture (8×8 cm) between the two chambers that the rat could use to move between chambers. The chambers were made of white and black opaque Plexiglas. The black chamber was covered with a black lid (the dark box), while the white chamber was covered with a transparent lid and illuminated by ambient room lighting to ~450 lux (the light box). During the test, an individual rat was placed in the center of the light box facing away from the aperture connecting the two chambers and allowed to freely explore both chambers for a 5-minute period. Between animals, the apparatus was cleaned with 50% ethanol and thoroughly dried. All sessions were video recorded, and the latency to enter the dark chamber, time spent in the light chamber, the latency to re-enter the light chamber (i.e., time to return to the light side after initial entry to the dark side), and number of transitions between the chambers were scored by an experimenter without knowledge of the experimental condition of any given animal.

Data Analyses

For the SI test, levels of social investigation, contact behavior, play fighting, social preference, and overall number of crossovers were assessed using separate 2 (prenatal exposure: saline, ethanol) $\times$ 3 (age: P28, P42, P77) $\times$ 2 (sex) analyses of variance (ANOVAs). For the EPM and LDB tests, data were analyzed using separate 2 (prenatal exposure: saline, ethanol) $\times$ 2 (sex) ANOVAs. Where significant main effects or interactions with sex were evident, planned ANOVAs within each sex were conducted to explore consequences of prenatal ethanol exposure. These analyses were followed by post-hoc tests in order to determine the locus of significant main effects and interactions within each sex using Fisher planned LSD tests which were used to avoid inflating the possibilities of Type II errors (see [61]). These planned tests were focused on comparisons between animals exposed prenatally to ethanol and saline-exposed animals at each age (social interactions)

and sex. One sample t-tests were used to assess whether values of the coefficients differed significantly from 0 within each age/sex/prenatal exposure group. Significance was set at $p < 0.05$, and all data are expressed as mean $\pm$ standard error ($M \pm SEM$).

3. Results

3.1. Blood Ethanol Concentration

BECs in trunk blood were comparable at the first and the third time points and reached 308.0 ± 3.5 mg/dl when assessed 30 min following the first ethanol injection and 304.7 ± 14.2 mg/dl at 240 min post-injection. BECs peaked at the 125-min time point (i.e., 5 min following the second injection) and reached 394.7 ± 36.1 mg/dl. By 480 min post-injection, BECs were relatively low, although still detectable (53.8 ± 3.8 mg/dl).

3.2. Litter data

Number of pups delivered by each female, male/female ratio, and average pup weight on P2 were compared among non-exposed females and females exposed to saline or ethanol on G12 (Table 1). Number of pups per litter and sex ratio did not differ as a function of prenatal exposure. However, a significant main effect of prenatal exposure, $F(2, 21) = 6.00$, $p < 0.05$, was evident for average pup weight, with saline-exposed pups having higher body weights than their non-exposed counterparts.

3.3. Social Interaction Test

The overall ANOVA of social investigation revealed a significant prenatal exposure $\times$ age $\times$ sex interaction, $F(2, 84) = 3.42$, $p < 0.05$, suggesting sex-related differences in the consequences of prenatal exposure to ethanol (Fig. 1). Indeed, in males, social investigation differed only as a function of prenatal exposure, $F(1, 42) = 162.64$, $p < 0.0001$, with significant decreases in social investigation evident in ethanol-exposed males relative to their saline-exposed counterparts at P28, P42, and P77. In females, however, the analysis of social investigation revealed a significant prenatal exposure $\times$ age interaction, $F(2, 42) = 9.04$, $p < 0.001$, with only older females exposed to ethanol prenatally and tested at P42 or P77 demonstrating substantial decreases in social investigation relative to age-matched saline-exposed controls. Social investigation of females on P28 was not affected by prenatal ethanol exposure.

The analysis of contact behavior showed a significant prenatal exposure $\times$ age $\times$ sex interaction, $F(2, 84) = 3.80$, $p < 0.05$ (Fig. 2). In males, contact behavior differed as a function of prenatal exposure only, $F(1, 42) = 12.92$, $p < 0.001$, with males exposed to ethanol prenatally demonstrating less contact behavior than their saline-exposed counterparts. In females, the ANOVA revealed a significant prenatal exposure $\times$ age interaction, $F(2, 42) = 3.53$, $p < 0.05$, with only P42 females showing significant prenatal ethanol-associated decreases in contact behavior relative to saline-exposed age-matched controls.

The overall ANOVA of play fighting revealed significant main effects of prenatal exposure, $F(1, 84) = 6.34$, $p < 0.05$, age; $F(2, 84) = 28.04$, $p < 0.0001$; and sex, $F(1, 84) = 4.37$, $p <$

0.05 (Fig. 3). In males, play fighting differed as a function of age only, $F(2, 42)$, $p < 0.0001$. As expected, there was a gradual decrease in play fighting with age regardless of prenatal exposure. In females, play fighting also decreased with age, $F(2, 42) = 16.67$, $p < 0.0001$, and was significantly lower in ethanol-exposed females than saline-exposed controls [main effect of prenatal exposure, $F(1, 42) = 11.19$, $p < 0.005$]. This effect of prenatal exposure was driven predominantly by females tested at P42 (Fig. 3, right panel).

The overall ANOVA of the preference/avoidance coefficient revealed a significant 3-way interaction, $F(2, 84) = 9.03$, $p < 0.001$ (Fig. 4), suggesting sex differences in the responsiveness to prenatal ethanol. All saline-exposed animals (both males and females) showed significant social preference, regardless of age, with all coefficients being positive and significantly ($p < 0.05$) different from 0 (as indexed by one-sample t-tests performed for each of the saline groups). However, in males, the analysis of the coefficient revealed a significant prenatal exposure x age interaction, $F(2, 42) = 3.41$, $p < 0.05$, whereby a significant prenatal ethanol-associated decrease of the coefficient suggests a switch from social preference to social indifference at P28 ($t = 1.53$, $p = 0.17$) and P42 ($t = -0.17$, $p = 0.87$) (indifference since the coefficients at these ages in prenatal exposed did not differ from 0). However, ethanol-exposed males demonstrated social preference at P77, with the positive coefficient being significantly different from 0 ($t = 4.12$, $p < 0.01$). In females, prenatal ethanol-induced alteration in the social preference differed as a function of age [prenatal exposure x age, $F(2, 42) = 8.03$, $p < 0.001$]. In contrast to P28 ethanol-exposed males, female counterparts exhibited social preference, indicated by the positive coefficient being significantly different from 0 ($t = 8.00$, $p < 0.0001$). However, at P42 and P77 females, ethanol-exposed females showed a significant switch to social indifference, indexed by non-significant differences from 0 ($t = 1.37$, $p = 0.21$ and $t = 0.46$, $p = 0.66$, respectively).

The analysis of general locomotor activity indexed via crossovers revealed a significant prenatal exposure x age x sex interaction, $F(2, 84) = 3.11$, $p < 0.05$ (Fig. 5). In males, the ANOVA of total number of crossovers revealed a significant prenatal exposure x age interaction, $F(2, 42) = 6.16$, $p < 0.005$, with only P28 males demonstrating significant prenatal ethanol-associated increases in locomotor activity relative to saline-exposed age-matched controls. In females, overall number of crossovers differed as a function of prenatal exposure and age as well, $F(2, 42) = 3.30$, $p < 0.05$. However, ethanol-exposed females showed significant decreases in general locomotor activity under social circumstances relative to age-matched saline-exposed controls only at P42.

3.4. Elevated Plus Maze

The ANOVA of percent open arm entries revealed significant main effects of sex, $F(1, 28) = 4.69$, $p < 0.05$, and prenatal exposure, $F(1, 28) = 4.39$, $p < 0.05$. Females showed significantly more open arm entries than males, whereas animals prenatally exposed to ethanol demonstrated more open arm entries than their saline-exposed counterparts – an effect of prenatal exposure driven predominantly by males (Fig. 6, left panel). Percent open arm time differed as a function of sex only, $F(1, 28) = 13.24$, $p < 0.005$, with females spending significantly more time on the open arms than males (Fig. 6, middle panel).

Activity on the EPM, as indexed by number of closed arm entries, was higher in females than males [main effect of sex, $F(1, 28) = 10.10$, $p < 0.005$].

3.5. Light-Dark Box

Behavior in the LDB was not affected by prenatal ethanol exposure. The latency for entering the dark box did not differ as a function of prenatal exposure and sex (overall mean = 17.7 sec, SEM = 2.5 sec). In contrast, females (mean = 80.2 sec, SEM = 16.2) had significantly shorter latencies for re-entering the light box relative to males (mean = 147.5 sec, SEM = 27.3 sec) regardless of prenatal exposure condition [main effect of sex, $F(1, 28) = 4.58$, $p < 0.05$]. Neither time spent in the light box (overall mean = 68.3 sec, SEM = 7.0 sec), nor the number of transitions between the boxes (overall mean = 4.6, SEM = 1.0) differed as a function of prenatal exposure or sex.

4. Discussion

Similar to our previous findings with Long Evans rats [31, 32], the results of the present study that used Sprague Dawley rats as experimental subjects clearly demonstrate that ethanol exposure on G12 resulted in age- and sex-dependent social alterations. In general, ethanol-exposed males showed social deficits at all ages, whereas decreases in social preference (i.e. emergence of social indifference) decreased with age and was no longer evident on P77. In contrast, ethanol-exposed early adolescent females did not show changes in social behavior or social preference relative to their age-matched saline-exposed controls, whereas older females tested at P42 or P77 demonstrated substantial decreases in social behavior and social preference following prenatal ethanol exposure on G12. In adulthood, when animals were tested under non-social circumstances, anxiety-like behavior assessed in the EPM showed that prenatal ethanol exposure significantly decreased open arm entries in both sexes, indicative of decreased anxiety-like behavior. However, this effect was not observed in the LDB.

Consistent with our previous data [32], i.p. injections of pregnant females with ethanol on G12 had no adverse effects on offspring body weight or sex ratio (see Table 1). In contrast, mean body weight of saline-injected pups was slightly but significantly higher relative to pups born from non-injected females. This effect of prenatal saline injections may be related to a lower (although non-significantly lower) number of pups born from saline-injected mothers. This is consistent with findings that demonstrate a negative correlation between the litter size and body weight of the pups [62].

Interestingly, more detailed comparisons between our earlier research and the present study revealed not only certain similarities but also some differences in social alterations induced by an acute binge-like exposure of ethanol on G12. The observed similarities included substantial decreases in social investigation in ethanol-exposed males tested during adolescence and adulthood. Males of both strains demonstrated decreases in contact behavior, as well as significant decreases in social preference indexed via emergence of social indifference. However, Sprague Dawley males showed these alterations during adolescence, but not in adulthood, whereas Long Evans ethanol-exposed males demonstrated similar alterations regardless of age (see [32]). Surprisingly, in the present

study, play fighting was not affected in males, whereas prenatal ethanol-associated significant decreases in this form of social interaction were evident throughout ontogeny in our previous study. Although not affected by prenatal ethanol in males, play fighting was significantly decreased in ethanol-exposed Sprague-Dawley females tested on P42 and P77. The resistance of play fighting to the adverse effects of prenatal ethanol in Sprague Dawley males may be associated, at least in part, to the fact that this strain demonstrates the most pronounced play fighting, including frequency of playful nape attacks as well as pinning [63]. In contrast to our previous findings [31, 32], the present study clearly demonstrates that social investigation and play fighting reflect behaviorally distinctive and differentially regulated forms of interactive social behavior that have different ontogenetic patterns [51, 52, 64] and differential responsiveness to adverse, anxiety-provoking and stressful situations. For instance, play fighting, but not social investigation, is drastically enhanced by social isolation throughout the entire adolescent period [51, 52], whereas social investigation is exclusively decreased by prior history of exposure to non-social stressors [59, 65]. These two forms of social behavior are differentially responsive to acute prenatal ethanol in Sprague Dawley rats, regardless of sex (Figs. 1 and 3).

The age of an occurrence of social alterations in females was common among the strains, with decreases in social preference becoming evident during late adolescence and adulthood. However, the most striking differences between the two studies and, hence, the two strains, was evident in the effects of prenatal ethanol exposure on other forms of social behavior in the female offspring. The most pronounced decreases in social investigation, contact behavior, and play fighting were demonstrated by ethanol-exposed Sprague Dawley females tested at P42 (Figs. 1 – 3), whereas no alterations in these forms of social behavior were reported previously for adolescent and adult Long Evans females exposed to ethanol on G12 [32].

There are several potential reasons for the observed differences between the current findings and our previous studies in Long Evans rats. One difference is that our previous studies used pregnant females shipped from a vendor, while in the present study all animals were bred in-house. It is likely that the level of prenatal stress differed dramatically between the studies. Indeed, during shipping animals are exposed to uncontrolled stress. The stressors associated with shipping include, but are not limited to, exposure to noise, temperature and humidity fluctuations, noxious odors, and fluid deprivation. Many of these factors are used as independent variables in stress research. Stress (including stress during shipping) at any point in development (including prenatal period) exerts long-lasting or even permanent effects on subsequent neurobehavioral functioning that may mask, amplify or otherwise interact with variables being experimentally manipulated [66–69]. Therefore, in our previous studies with Long Evans rats, the effects of prenatal ethanol exposure on G12 were combined to a certain extent with prenatal stress very early in gestation, with this combination resulting in a more robust effect in male offspring.

The doses of ethanol administered to the dams also differed between the studies and were lower for Sprague Dawley dams, since these pregnant females injected with the higher doses (administered previously to Long Evans pregnant rats) did not produce any litters. These results might suggest that under certain conditions, sensitivity to ethanol is strain-dependent.

Indeed, Sprague Dawley dams, although injected with lower doses of ethanol, not only demonstrated higher BECs (present study) than their Long Evans counterparts (see [32], but also took longer to clear ethanol from their systems relative to Long Evans females. Therefore, pharmacokinetic factors might play a critical role in some differences between Sprague Dawley and Long Evans pregnant females in terms of social consequences of prenatal exposure to acute ethanol on G12. There is no doubt that direct comparisons of ethanol sensitivity in Long Evans and Sprague Dawley females on G12 are needed, given that strain-related differences in ethanol pharmacokinetics were seen in different studies performed in different laboratories. In contrast to pregnant females, no differences in ethanol sensitivity were evident between Long Evans and Sprague Dawley males [70]. Furthermore, Horowitz et al. found no differences in BECs between the two strains, although Long Evans males ingested more ethanol than their Sprague Dawley counterparts [71]. Taken together, these findings suggest that the proposed strain differences in ethanol sensitivity are sex-dependent and may be related to females being pregnant as well.

The results of the present study confirmed that the social consequences of prenatal ethanol exposure differed in males and females [31, 32]. Social alterations following prenatal ethanol and indexed via substantial decreases in social investigation and social preference relative to saline-exposed controls were more pronounced in adolescent males, with their adult counterparts demonstrating decreased social investigation only. In contrast, ethanol-exposed early adolescent females did not show any signs of social alterations, with these alterations becoming apparent during and after puberty in ethanol-exposed females. This pattern of experimental findings suggests some protective role of male gonadal hormones against social anxiety-like alterations associated with prenatal ethanol. In contrast, an increase in female gonadal hormones is likely to contribute to the emergence of social anxiety associated with prenatal ethanol exposure, as indexed by significant decreases in social investigation and social preference in ethanol-exposed females at P42 and P77 relative to their saline-exposed counterparts. Recent studies have demonstrated the sensitivity of the hypothalamic-pituitary-gonadal (HPG) axis to prenatal ethanol exposure, particularly in females, and the mechanisms underlying the long-lasting neurobehavioral outcomes [72, 73]. Moreover, to the extent that social indifference is associated with the hypothalamus-pituitary-adrenal (HPA) axis hyper-responsiveness to social stimuli [74], gonadal steroids, through alterations in the HPG axis, may play an indirect role in the emergence of social indifference through modulation of the HPA axis.

One of the more surprising findings was that, in contrast to social behavior, adult males and females showed significantly decreased anxiety-like responses when tested in the EPM, but not the LDB. It is important to note that the EPM was validated as a test of anxiety-like behavior for rats [56, 75], and interpretation of the anxiety/fear state elicited by the EPM is still controversial. It has been suggested that behavior on the EPM does not reflect generalized anxiety, but rather situation-dependent anxiety and/or fear [76], suggesting that the EPM can be viewed as an aversive task for experimental subjects. Nevertheless, we specifically found that using this aversion-inducing task, saline-exposed controls demonstrated more anxiety-like behavior than their ethanol-exposed counterparts. While this was unexpected, much of the literature is inconsistent regarding the effects of prenatal ethanol exposure on anxiety-like behaviors. Many studies have found increased [35, 37-39,

⁴⁴], while some studies have shown decreased anxiety-like behaviors following prenatal ethanol exposure [40, 41, 43]. A recent study by Wiczorek and colleagues found increased anxiety-like responses in the LDB, but decreases in the EPM [42]. It has also been shown that only if ethanol exposed offspring are stressed prior to anxiety testing will they exhibit increased anxiety-like responses [36, 77]. That the effects on social and anxiety-like behaviors opposed each other is also not entirely novel as Cullen et al. also reported test-dependent behavioral differences following low-dose ethanol exposure through the entire gestation [35]. However, in that study, ethanol-exposed animals demonstrated higher frequency and duration of sniffing in the SI test, with the same animals demonstrating significantly lower percent open arm time relative to their control counterparts. From this, it is clear that expression of these behaviors is very complex and influenced by many factors. While the mechanisms affected by prenatal ethanol exposure that drive different forms of anxiety-like responses across studies is unknown, one major contribution is the amount and timing of ethanol exposure [78]. For example, exposure during the neonatal period (3rd trimester-equivalent) increased anxiety-like behavior only following a brief binge-like [79], but not an extended moderate [80], ethanol exposure in Sprague Dawley rats. Importantly, similar reports in humans suggest that the odds of developing an anxiety disorder following prenatal alcohol exposure is also dependent on timing and amount of alcohol consumption [81]. Another important consideration regarding the mixed findings on anxiety-like responses is that many brain structures and networks involved in these behaviors are forming at different points during early development [82], making it difficult to interpret findings across different ethanol exposures. Regardless, future studies are warranted to examine in more detail this relationship as it pertains to expression of anxiety-like behaviors and underlying mechanisms.

Although the EPM is widely used as a test of anxiety-like behavior in laboratory rodents [83], the social interaction test has been used extensively for the assessment of anxiety-like behavior in rats as well [84–86]. In the SI test, the social behavior of animals is suppressed under anxiety-provoking circumstances (e.g., in an unfamiliar test environment or under bright light conditions). This suppressed social behavior can be restored with anxiolytic compounds [86]. In the traditional SI test, a pair of rats is placed into a testing arena, and overall time spent in social interactions is used as a dependent variable [84]. In prior work, however, we have found specific forms of social behavior to be especially sensitive to anxiogenic manipulations and anxiolytic compounds, namely social investigation and social preference/avoidance. For instance, both social investigation and the preference coefficient were reduced by testing rats in an unfamiliar environment [49] or following repeated restraint stress [59, 65], with these anxiogenic effects reversed by acute ethanol challenge under both circumstances [49, 65]. Similarly, the GABA_A receptor partial agonist, L-838,417, reversed social avoidance and increased social investigation in an unfamiliar, anxiogenic environment and reversed stress-associated decreases in social preference under familiar test circumstances [87]. Given these earlier findings, the results of the present study suggest that decreases in social investigation and/or social preference demonstrated by ethanol-exposed animals may reflect anxiety-like alterations evident under social circumstances in a familiar and hence non-anxiety-provoking test situation. To the extent that decreases in social investigation and social preference reflect anxiety-like alterations

associated with prenatal ethanol exposure on G12, these social alterations should be sensitive to anxiolytic compounds, including ethanol. Clearly, more studies are needed to pharmacologically validate social alterations induced by acute prenatal ethanol exposure as a model of social anxiety.

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Highlights

- Ethanol exposure in mid-gestation alters social behavior in Sprague Dawley rats.
- Ethanol-exposed male offspring show deficits from early adolescence through adulthood.
- Ethanol-exposed females show deficits from late adolescence through adulthood.
- Non-social anxiety-like behavior was decreased by prenatal ethanol exposure.

Figure 1.

The impact of exposure to ethanol on G12 on social investigation of male (left panel) and female (right panel) rats tested as early adolescents (P28), late adolescents (P42) or adults (P77). Asterisks (*) indicate significant ($p < 0.05$) differences between age-matched ethanol- and saline-exposed females, whereas a significant difference between ethanol-exposed males and saline controls regardless of age is indicated with (#). $n = 8$ per group.

Figure 2.

The impact of exposure to ethanol on G12 on contact behavior of male (left panel) and female (right panel) rats tested as early adolescents (P28), late adolescents (P42) or adults (P77). An asterisk (*) indicates a significant ($p < 0.05$) difference between age-matched ethanol- and saline-exposed females, whereas a significant difference between ethanol-exposed males and saline controls regardless of age is indicated with (#). $n = 8$ per group.

Figure 3.

The impact of exposure to ethanol on G12 on play fighting of male (left panel) and female (right panel) rats tested as early adolescents (P28), late adolescents (P42) or adults (P77). A significant difference between ethanol-exposed females and their saline controls regardless of age is indicated with (#). $n = 8$ per group.

Figure 4.

The impact of exposure to ethanol on G12 on social preference/avoidance of male (left panel) and female (right panel) rats tested as early adolescents (P28), late adolescents (P42) or adults (P77). Asterisks (*) indicate significant ($p < 0.05$) differences between age- and sex-matched ethanol- and saline-exposed rats. $n = 8$ per group.

Figure 5.

The impact of exposure to ethanol on G12 on general locomotor activity under social test circumstances, indexed via overall number of crossovers (movements between compartments) of male (left panel) and female (left panel) rats tested as early adolescents (P28), late adolescents (P42) or adults (P77). Asterisks (*) indicate significant ($p < 0.05$) differences between age- and sex-matched ethanol- and saline-exposed rats. $n = 8$ per group.

Figure 6.

The impact of exposure to ethanol on G12 on percent open arm entries (left panel), percent open arm time (central panel), and closed arm entries (right panel) of adult males and females. A significant difference between ethanol-exposed rats and saline controls, with data collapsed across sex is indicated with (\$).

Table 1

Litter Data.

Prenatal Exposure	Number of pups/litter	Sex ratio (% males/litter)	Average pup weight on P2 (g)
No Exposure	13.3 $\pm$ 1.0	48.8 $\pm$ 5.4	7.9 $\pm$ 0.3
Saline	11.5 $\pm$ 0.5	52.9 $\pm$ 6.7	9.7 $\pm$ 0.4 *
Ethanol	13.4 $\pm$ 1.3	48.8 $\pm$ 5.4	8.6 $\pm$ 0.5

Data shown as mean $\pm$ standard error of the mean.

* denotes significant difference from non-exposed animals. n = 8 per group.