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Prenatal exposure to moderate levels of ethanol alters social behavior in adult rats: Relationship to structural plasticity and immediate early gene expression in frontal cortex

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Abstract

The goals of the present study were to characterize the effects of prenatal exposure to moderate levels of ethanol on adult social behavior, and to evaluate fetal-ethanol-related effects on dendritic morphology, structural plasticity and activity-related immediate early gene (IEG) expression in the agranular insular (AID) and prelimbic (Cg3) regions of frontal cortex. Baseline fetal-ethanol-related alterations in social behavior were limited to reductions in social investigation in males. Repeated experience with novel cage-mates resulted in comparable increases in wrestling and social investigation among saccharin- and ethanol-exposed females, whereas social behavioral effects among males were more evident in ethanol-exposed animals. Male ethanol-exposed rats also displayed profound increases in wrestling when social interaction was motivated by 24 hours of isolation. Baseline decreases in dendritic length and spine density in AID were observed in ethanol-exposed rats that were always housed with the same cage-mate. Modest experience-related decreases in dendritic length and spine density in AID were observed in saccharin-exposed rats housed with various cage-mates. In contrast, fetal-ethanol-exposed rats displayed experience-related increases in dendritic length in AID, and no experience-related changes in spine density. The only effect observed in Cg3 was a baseline increase in basilar dendritic length among male ethanol-exposed rats. Robust increases in activity-related IEG expression in AID (*c-fos* and *Arc*) and Cg3 (*c-fos*) were observed following social interaction in saccharin-exposed rats, however, activity-related increases in IEG expression were not observed in fetal-ethanol-exposed rats in either region. The results indicate that deficits in social behavior are among the long-lasting behavioral consequences of moderate ethanol exposure during brain development, and implicate AID, and to a lesser degree Cg3, in fetal-ethanol-related social behavior abnormalities.

Introduction

Heavy consumption of alcohol during pregnancy can result in a constellation of profound morphological, neurological, and behavioral consequences in offspring collectively referred to as Fetal Alcohol Syndrome (FAS; [2,13,38,39,40]). The incidence of FAS is relatively low

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(approximately 0.1%) in the general population [1], however, 5%–20% of pregnant women engage in moderate drinking (1 to 2 drinks per day; [17]), and it has been estimated that 10 to 20 times more children are exposed to moderate levels of alcohol *in utero* that do not result in full-blown FAS [12]. A growing body of data indicates that moderate exposure to alcohol during brain development can cause subtle behavioral and cognitive deficits in the absence of gross morphological effects and profound behavioral and cognitive deficits associated with heavy consumption. These deficits may not become apparent until the child is challenged during the educational years [15,44,81], may increase in severity as the child matures [80], and may persist throughout the life of the individual. Such considerations have recently led to a revised taxonomy of fetal ethanol effects, the Fetal Alcohol Spectrum Disorders (FASD), which include several classifications for lesser-affected children including Partial FAS, Alcohol Related Neurodevelopmental Disorder (ARND) and Alcohol Related Birth Defects (ARBD) (see ref. [36]).

Among the numerous behavioral and cognitive impairments observed in children with FASD are deficits in learning and memory [31,54,88,89], attention [50], and a broad range of behavioral problems that can be classified as social in nature [41,61,69,87]. Social behavior deficits in children with FASD may manifest in a number of clinical problems (e.g., conduct disorder) and likely contribute to or exacerbate deficits in other cognitive and behavioral domains. Animal models have been central to the study of alcohol-related alterations in behavior and cognition and the multifaceted biological mechanisms that underlie these deficits [27]. Several studies have reported abnormal social behaviors in rats and mice exposed to high amounts of alcohol during brain development [19,42,43,49,51,52,57,71] including increased aggression [51,71], increased prepubertal play fighting [71], alterations in the normal pattern of sexually dimorphic play behavior [57], both decreases and increases in responsiveness to social stimuli [42,51,52], and deficits in socially acquired food preferences and social recognition memory [43]. Despite differences in the timing, amount, and duration of alcohol exposure, sex of the animal, and age at which social behavior was measured, these studies consistently reveal ethanol-related abnormalities in social behavior. The long-term consequences of mild to moderate prenatal alcohol exposure on adult social behavior in rodent models of FASD, however, have not been systematically investigated. Developing rational treatment strategies for alcohol-related social behavior abnormalities will critically depend upon an understanding of the affected behavioral processes as well as their neurobiological mechanisms. However, relatively little is known about the neurobiology of alcohol-related abnormalities in social behavior at present.

Research on the neurobiology of alcohol-related behavioral and cognitive deficits has been heavily focused on the cerebellum, hippocampus, and related circuitry. Kelly and Dillingham [42] demonstrated a relationship between abnormal social behavior in female, but not male, rats exposed to alcohol during postnatal days 2–10 and altered DNA and dopamine metabolite (DOPAC) levels in the amygdala. Lawrence et al. [49] measured c-Fos immunoreactivity in adolescent rats exposed to alcohol during early perinatal brain development and found alcohol-related alterations in somatosensory cortex activity that corresponded to tactile stimulation associated with play behavior. Social behaviors engage and require a distributed set of neural circuitry, which, in addition to the amygdala and hippocampal formation, also includes several frontocortical regions. Damage to the orbital prefrontal cortex (OPFC) in primates, and corresponding regions (e.g., agranular insular cortex) in rodents, has consistently been associated with alterations in social behavior including inappropriate aggression, withdrawal, agonistic behaviors [11,45] and altered play behavior [66]. In humans, damage to the prefrontal cortex (including orbital prefrontal cortex) is associated with a range of social behavior deficits [4] and functional neuroimaging studies have linked orbital prefrontal cortex to social cognition in humans [8,18]. The regional specificity of prefrontal circuitry involved in social behavior is indicated by the relative sparing of social behavior following lesions to dorsal frontal cortex

in primates [11] and roughly corresponding medial prefrontal cortex of the rat [45]. Kolb [45] demonstrated persistent alterations in social behavior in male rats after lesions of lateral frontal cortex, including the agranular insular cortex (Zilles' area AID, see Fig. 1), but only transient deficits after medial frontal cortex lesions including prelimbic cortex (Zilles' area Cg3). Recent evidence, however, suggests that deficits in juvenile and adult social behavior are persistent consequences of neonatal medial frontal cortex lesions in the rat [73]. Experience-dependent structural plasticity related to social experience in adult rats has also been demonstrated in AID [32,75], with less robust experience-dependent changes observed in Cg3 [32]. Prenatal exposure to moderate levels of ethanol alters phospholipase C- β 1 and A2 signaling in medial frontal cortex [3], and we have recently demonstrated fetal-ethanol-related decreases in mGluR₅ receptor density in Cg3, indicating a potential deficit in synaptic plasticity within this region. The consequences of moderate alcohol exposure on lateral frontal cortex structure and function, including AID, have yet to be evaluated.

The present study was motivated by the lack of available data on the consequences of exposure to moderate levels of ethanol during brain development on social behavior and frontocortical regions critically involved in social behavior. The specific goals of the present study were to characterize the effects of moderate prenatal ethanol exposure during brain development on adult 1) social behavior, 2) behavioral adaptation to social experience, 3) dendritic length, branching, and spine density in AID and Cg3, 4) experience-dependent structural plasticity in AID and Cg3 related to social experience, and 5) immediate early gene expression in AID and Cg3 as a marker of neural activity elicited by social interaction.

Experiment 1

In Experiment 1 male and female rats were exposed to moderate levels of ethanol (EtOH) or saccharin (SAC) during prenatal development. In adulthood (postnatal day 90) half the rats from each diet condition were paired-housed with a novel, but familiar, cage-mate every 48 hours for 40 days, and the other half were pair-housed with the same cage-mate for 40 days [75]. This manipulation increases social investigation and leads to alterations in dendritic length and spine density in AID [32,75] while causing little or no changes in Cg3 neurons. Several social and non-social behaviors were quantified during the 10 minutes immediately following the introduction of the cage-mate. Effects of prenatal diet condition and the social experience manipulation on dendritic length, branching, and spine density in AID and Cg3 neurons were evaluated.

Method

Subjects—Subjects were 128 (64 male, 64 female) Long-Evans rats (Harlan Industries, Indianapolis, IN). Half of the rats were bred at the University of New Mexico Health Sciences Center Animal Resource Facility (HSC-ARF; breeding protocol described below). The other 64 rats were acquired directly from Harlan and served as cage-mates for the 64 animals that were exposed to EtOH or SAC *in utero*. Housing methods are described below. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico.

Apparatus and Procedures

Voluntary Drinking Paradigm: All breeding procedures were conducted in the University of New Mexico HSC-ARF. Three to four-month-old Long-Evans rat breeders (Harlan Industries, Indianapolis, IN) were single-housed in plastic cages at 22°C and kept on a reverse 12-hour dark/12-hour light schedule (lights on from 2100 to 0900 hours) with Purina Breeder Block rat chow and tap water *ad libitum*. After one week of acclimation to the animal facility, all female rats were provided 0.066% saccharin in tap water for four hours each day from 1000

to 1400 hours. On Days 1 and 2, the saccharin water contained 0% ethanol, on Days 3 and 4, saccharin water contained 2.5% ethanol (v/v). On Day 5 and thereafter, saccharin water contained 5% ethanol (v/v). Daily four-hour consumption of ethanol was monitored for at least two weeks and then mean daily ethanol consumption was determined for each female breeder. At the end of two weeks of daily ethanol consumption, females that drank less than one standard deviation below the mean of the entire group were removed from the study. The remainder of the females were assigned to either a saccharin control or 5% ethanol drinking group and matched such that the mean pre-pregnancy ethanol consumption by each group was similar.

Subsequently, females were placed with proven male breeders until pregnant as evidenced by the presence of a vaginal plug. Female rats did not consume ethanol during the breeding procedure. Beginning on Gestational Day 1, rat dams were provided saccharin water containing either 0% or 5% ethanol for four hours a day, beginning precisely at 1000 hours (1 hour following the onset of the dark cycle). The volume of 0% ethanol saccharin water provided to the controls was matched to the mean volume of 5% ethanol in saccharin water consumed by the ethanol-drinking group, which remained highly consistent at 16mL per four-hour drinking period over multiple breeding rounds. Rat chow was available *ad libitum* during both the drinking and non-drinking periods. Because the saccharin control group does not involve yoking caloric intake to that of ethanol-drinking dams, as in a pair-feeding procedure, and chow was available *ad libitum*, maternal weight gain during pregnancy and offspring birth-weight were quantified in order to detect any potential differences in ethanol-exposed and non-exposed dams and pups. Daily four-hour ethanol consumption was recorded for each dam. At birth, all litters were weighed and culled to 10 pups. Offspring were weaned at 24 days of age and transferred from the HSC-ARF to the Psychology ARF where they were group-housed (2 males or 3 females per cage) until postnatal day 90.

Maternal Serum Ethanol Levels: Serum ethanol concentrations were determined in a separate set of 12 rat dams that completed the same voluntary drinking paradigm as described above. Blood samples were obtained under isoflurane anesthesia at the end of the four-hour ethanol consumption period on each of three alternate days during the third week of gestation. One hundred μ L of whole blood was collected from the tail vein and immediately mixed with 0.2 mL of 6.6% perchloric acid, frozen and stored at -20°C until assayed. Serum ethanol standards were created by mixing rat whole blood from untreated rats with known amounts of ethanol ranging from 0 to 240 mg ethanol/dL and then mixing 100 μ L aliquots of each standard with perchloric acid and storing the standards frozen with the samples. Blood ethanol samples were assayed using a modification of the method of Lundquist and colleagues [53].

Social Experience and Behavioral Analysis: At postnatal day 90, male and female rats from the ethanol and saccharin exposure conditions were housed in same-sex pairs in standard plastic hanging cages with a cage-mate of comparable weight that was not exposed to either prenatal diet treatment. Efforts were made to limit the differences between weights of cage-mates in all experimental conditions. At 48 hour intervals (3 h after the onset of the dark cycle) over a period of 40 days, the animals were removed from their cages and moved to clean cages with fresh bedding, food, water, and either the same cage-mate (Control condition) or a new cage-mate (Social condition). The pool of possible cage-mates was constrained such that animals serving as cage-mates were always housed with animals from the same prenatal diet condition and sex. Because there were 8 animals in each combination of sex, prenatal diet condition and social experience condition, 8 cage-mates were assigned to each combination. The sequence of cage-mates for rats in the Social condition was determined randomly without replacement such that the fetal-ethanol and saccharin animals were housed with each of the possible cage-mates at least once before being housed with a particular cage-mate again. Over the course of the 40 days, each fetal-ethanol and saccharin animal would have been housed with each possible cage-mate 2–3 times. We note that stage of estrous was not considered as a factor here

because 1) the manipulation was repeated over 40 days, and cage-mate changes occur more frequently (every other day) than the estrous cycle, thus, averaging the data over a large number of cage-changes (see below) ensures that all stages of estrous are represented in the behavioral data, 2) pilot data failed to detect significant effects of estrous on dendritic morphology in the regions of interest, 3) a systematic consideration of estrous would require a substantially more complicated experimental design to evaluate stage of estrous for both cage-mates, and 4) we wanted to avoid introduction of additional handling procedures that might interact with the other treatments. A clear demonstration of differential effects of fetal ethanol exposure on social behavior or any of the neurobiological measures employed in the present paper would motivate future studies that include consideration of estrous. Immediately after the cages were changed for an individual pair of animals, their behavior was video-taped (under dark conditions) for a period of 10 minutes for subsequent analysis. On the day following the final cage change the rats were euthanized and the brains processed for Golgi-Cox staining (see below). This social experience treatment has been shown to result in alterations of dendritic morphology and spine density in the frontocortical regions of interest [32,75].

Videos were digitized and transferred to a computer for analysis using custom software developed in our laboratory. The duration and frequency (over 10 min) of the following behaviors were quantified: anogenital sniffing, other sniffing of the partners body (body sniffing), sniffing/digging in the bedding, rearing, wrestling (including pinning), boxing, self-grooming, allogrooming (of the partner), and crawling over/under the partner [6]. These behaviors were selected to provide measurements of social behaviors directed toward the cage-mate (e.g., sniffing, wrestling), self-directed behaviors (e.g., grooming), and behaviors directed toward the environment (e.g., digging). Social behaviors included measures of investigation (e.g., anogenital sniffing), aggression (e.g., wrestling), and agonistic behaviors (e.g., crawling over/under). Behaviors classified as body sniffing were mainly directed toward the flank of the cage-mate. Examples of each of the behaviors measured in Experiments 1 and 2 are illustrated in the Supplementary Video. Instances of play behavior (chasing, attempting to contact the nape of the neck) occur less frequently in adult rats than in juvenile rats, and were rare in our observations, thus, these behaviors are not reported. Behaviors were quantified for rats that were exposed to either saccharin or ethanol prenatally. Behaviors of the rats that served only as cage-mates were not analyzed. All behavioral coding was performed by a rater blind to prenatal diet condition. For analysis, averages for each of the behavioral measures were computed over the final 20 days (last half) of the social housing manipulation. This was done for several reasons: 1) the overarching goal was to simplify presentation of the behavioral results, 2) behavioral data were highly consistent over the final half of the social housing manipulation, 3) during the initial days of the manipulation cage-mates in both the control and social conditions are novel, therefore, there are not robust differences in behavior between these conditions and the intended effect of the social housing manipulation is not evident, 4) prior work using this manipulation [32,75] indicates that the emergence of clear behavioral and dendritic morphology differences require approximately 5–9 cage-mate changes (10–18 days) to emerge, and 5) the behavioral data during the initial 18 days of the manipulation are highly variable making it difficult to detect effects of both the housing or prenatal diet conditions.

Golgi-Cox Staining and Analysis: Rats were deeply anesthetized with an overdose of sodium pentobarbital and perfused transcardially with 0.9% (wt/vol) saline, resulting in exsanguination. The brains were extracted, weighed, and immersed in GolgiCox solution [26] for 14 days and subsequently immersed in 30% (wt/vol) sucrose for at least 3 days. The brains were then cut into coronal sections (200 μ m thick) on a vibrating microtome, mounted on slides, and stained according to the procedures described by Gibb and Kolb [25].

Layer II/III neurons of agranular insular cortex (Zilles' area AID, [97]) and Layer III neurons of Zilles' area Cg3 were selected for analysis (see Fig. 1). An Olympus light microscope (Model BX51) equipped with a drawing attachment was used for analysis. The brain regions of interest were first identified at low power (100X magnification), and five neurons from each hemisphere were traced in each region at a higher power (250X final magnification) using the camera lucida technique. Selection was limited to neurons which were not obscured by stain precipitate, blood vessels, astrocytes, or other artifacts, and had intact dendritic fields that were well impregnated and visible within a single section.

Dendritic branching was measured by counting bifurcations on each dendrite [14]; the main apical branch was designated as a zero-order branch and basilar dendritic segments prior to the first bifurcation from the cell body were designated as first-order branches. Each subsequent bifurcation for a particular dendritic branch increased the branch order by 1. The total number of branches were quantified separately for apical and basilar dendritic fields. Dendritic length was measured using a Sholl analysis of ring intersections [74]. A series of concentric rings at 20 μ m intervals (calibrated to the final magnification of 250 $\times$) was transferred to a transparency which was centered over the cell body. The total number of intersections between rings and each dendritic branch and rings was counted. Spine density was measured by tracing the length of a dendritic terminal tip (>20 μ m in length) at high power (2000 $\times$ final magnification), computing the length of the dendritic segment, and counting the number of spines along the segment. Spine density was quantified on five terminal segments for each hemisphere on third-order (or greater) branches for apical dendrites and on fourth-order (or greater) branches for basilar dendrites.

Results

Due to poor Golgi-Cox staining, 4 rats were excluded from the final analyses and 3 additional rats either died or were removed from the study during the course of the social experience treatment due to fighting. This left 15 animals in the Ethanol-Social group and 14 animals in each of the other groups. All test statistics reported here were significant at $p < .05$ unless otherwise noted. Statistical analyses were performed using SPSS version 16.01. For all analyses, Sex, prenatal Diet condition (saccharin, ethanol) and Social condition (control, social) were between-subjects factors in analyses of variance (ANOVAs). Within each subsection in the Results section, we follow the convention of reporting significant planned comparisons of social conditions within levels of the diet condition for males and females first, followed by effects involving the prenatal diet condition, and, lastly, presentation of other effects not involving diet condition.

Voluntary Drinking Paradigm

Effects of daily four-hour ethanol consumption on female dams (weight gain, ethanol consumption, and serum ethanol concentration) and offspring (litter size and pup birthweight) are provided in Table 1. Rat dams stably consumed an average of 2.82 ± 0.13 grams of ethanol/kg body weight over the four-hour interval each day (approximately 16 mLs of 5% ethanol in 0.066% saccharin water). This pattern and level of ethanol consumption produced a mean maternal serum ethanol concentration of 84 ± 5.5 mg/dL during the third week of gestation. Ethanol consumption did not affect maternal weight gain during pregnancy and other experiments examining the effects of this voluntary consumption pattern of offspring indicate no effects on litter size or offspring birthweight [90].

Brain Weight

An ANOVA for brain weight revealed a significant Sex difference [$M_{MALE} = 2.19$ g, $M_{FEMALE} = 1.95$ g; $F(1, 49) = 123.03$]. No other main effects, interactions or planned comparisons were significant.

Behavior

Behavioral data (total time and frequency) for combinations of Diet, Sex and Social conditions of Experiment 1 are shown in Table 2. The effects of social experience and prenatal ethanol exposure on social behavior in male and female rats are summarized in Table 3. Planned comparisons revealed two overall effects of social experience in saccharin rats. There was a decrease in rearing [control > social; $F_{TIME}(1,26) = 5.24$] and an increase in wrestling [social > control; $F_{TIME}(1, 26) = 8.47$]. In female saccharin rats, social experience resulted in increased anogenital sniffing, body sniffing, wrestling, and crawling over/under [all $ps < .05$]. In contrast, social experience only resulted in significant reductions in rearing in male saccharin rats. For fetal-ethanol rats, there were significant effects for body sniffing [social > control; $F_{TIME}(1, 27) = 26.29$, $F_{FREQ}(1, 27) = 27.72$], anogenital sniffing [social > control; $F_{TIME}(1, 27) = 9.45$, $F_{FREQ}(1, 27) = 10.47$], wrestling [social > control; $F_{TIME}(1, 27) = 9.67$, $F_{FREQ}(1, 27) = 11.49$], boxing [social > control; $F_{TIME}(1, 27) = 5.24$, $F_{FREQ}(1, 27) = 7.67$], and rearing [control > social; $F_{TIME}(1, 27) = 5.10$]. The effect for allogrooming approached significance [social > control; $F_{FREQ}(1, 27) = 3.88$, $p = .059$]. Effects of social experience in female fetal-ethanol rats were similar to those of female saccharin rats, with significant increases in anogenital sniffing, body sniffing, and wrestling [all $ps < .05$]. Significant reductions in rearing and a lack of experience-related increases in crawling over/under distinguish female ethanol rats from saccharin rats. In contrast to saccharin males, social experience significantly increased anogenital sniffing, body sniffing, boxing, and grooming in male ethanol rats [all $ps < .05$], and an increase in wrestling approached significance [$ps = .06$].

There were significant Diet $\times$ Social interactions for body sniffing [$F_{TIME}(1, 49) = 5.86$, $F_{FREQ}(1, 49) = 4.28$]. The relevant simple effects were described in the prior paragraph. There was also a Sex $\times$ Diet interaction for anogenital sniffing [$F_{TIME}(1, 49) = 6.10$, $F_{FREQ}(1, 49) = 9.83$]. Analyses of simple effects revealed that fetal-ethanol males engaged in significantly fewer instances of anogenital sniffing than saccharin males [$F(1, 26) = 4.42$], which was largely attributable to significant reductions in the control housing condition [$F(1, 12) = 4.95$]. A non-significant effect in the opposite direction was observed in females. A similar pattern of numerical differences was observed for time engaged in anogenital sniffing for males and females, however, neither of the simple effects reached significance. There were also significant Diet $\times$ Sex $\times$ Social interactions for body sniffing [$F_{TIME}(1, 49) = 4.12$, $F_{FREQ}(1, 49) = 4.99$] and grooming [$F_{FREQ}(1, 49) = 5.52$]. Analyses of simple effects revealed that fetal-ethanol males in the social condition engaged in more body sniffing than saccharin males [$F_{TIME}(1, 12) = 5.10$], whereas a non-significant numerical trend in the opposite direction was observed for males in the control condition. No simple effects of Diet for body sniffing were observed for females in either social experience condition. Analyses of simple main effects for the significant grooming interaction revealed a significant effect of social experience in fetal-ethanol males [$F_{FREQ}(1, 13) = 7.12$]. None of the other simple effects for females or saccharin males were significant.

The main effects of Social experience, Sex, and their interaction are not critical to the major goals of the present study, but are presented in the interest of completeness (see Table 2). There were main effects of the Social experience treatment for rearing [control > social; $F_{TIME}(1, 49) = 12.86$], body sniffing [social > control; $F_{TIME}(1, 49) = 17.86$, $F_{FREQ}(1, 49) = 19.99$], anogenital sniffing [social > control; $F_{TIME}(1, 49) = 11.92$, $F_{FREQ}(1, 49) = 13.99$], allogrooming [social > control; $F_{FREQ}(1, 49) = 5.06$], wrestling [social > control; $F_{TIME}(1, 49) = 16.03$, $F_{FREQ}(1, 49) = 14.92$], and boxing [social > control; $F_{TIME}(1, 49) = 6.67$, $F_{FREQ}(1, 49) = 8.88$]. Sex differences were detected for rearing [female > male; $F_{TIME}(1, 49) = 14.17$, $F_{FREQ}(1, 49) = 8.24$] and boxing [male > female; $F_{TIME}(1, 49) = 12.46$, $F_{FREQ}(1, 49) = 18.41$]. Significant Sex $\times$ Social interactions were detected for digging [$F_{FREQ}(1, 49) = 4.63$], anogenital sniffing [$F_{TIME}(1, 49) = 5.14$, $F_{FREQ}(1, 49) = 8.46$], crawling over/under

[$F_{FREQ}(1, 49) = 4.25$], and boxing [$F_{TIME}(1, 49) = 4.63$]. Analyses of simple effects revealed that social experience reduced digging in males [$F_{TIME}(1, 26) = 4.74$, $F_{FREQ}(1, 26) = 4.24$], but not females. Anogenital sniffing [$F_{TIME}(1, 27) = 31.32$, $F_{FREQ}(1, 27) = 37.64$] and crawling over/under were increased in females [$F_{TIME}(1, 27) = 4.23$, $F_{FREQ}(1, 27) = 4.34$], but not males. Social experience increased boxing in males [$F_{TIME}(1, 26) = 5.37$] and females [$F_{FREQ}(1, 27) = 6.72$], but the numerical effect was considerably larger in males.

Dendritic Length, Branching, and Spine Density

Separate ANOVAs were conducted for branch order, length, and spine density for apical and basilar dendritic fields. All values in the text and figures are expressed as the percentage of saccharin-control condition means for each measure. Where necessary, means for data not represented in figures are reported in the text.

Dendritic Length

Agranular insular cortex (AID): Mean apical and basilar dendritic length for combinations of Social and Diet conditions are shown in Figs. 2A and 2B. There was a significant Diet $\times$ Social interaction for basilar dendritic length [$F(1, 49) = 6.70$]. Analyses of simple main effects revealed a significant social experience effect for fetal-ethanol rats [social > control; $F(1, 27) = 5.04$], but a non-significant social experience effect in the opposite direction for saccharin rats. Inspection of the means for basilar dendritic length shown in Fig. 2B suggests that ethanol rats in the Control social condition had lower basilar dendritic length than saccharin rats, however, this effect only approached significance [$F(1, 26) = 3.95$, $p = .057$]. There was also a significant Sex difference for basilar dendritic length [$M_{MALE} = 102.87$, $M_{FEMALE} = 93.23$; $F(1, 49) = 13.62$]. None of the other main effects, interactions or planned comparisons were significant.

Prelimbic Cortex (Cg3): Mean apical and basilar dendritic lengths from Cg3 for combinations of Social and Diet conditions are shown in Figs. 2C and 2D. The only significant effect was the Diet $\times$ Sex interaction for basilar dendritic length [data not shown; $F(1, 49) = 5.13$]. Analyses of simple effects revealed a significant Diet effect for males [$M_{SAC} = 99.90$, $M_{EtOH} = 107.45$; $F(1, 49) = 4.06$], but not females [$M_{SAC} = 102.94$, $M_{EtOH} = 99.16$]. None of the other main effects, interactions or planned comparisons were significant.

Branch Number—There were no significant main effects, interactions or planned comparisons for apical or basilar branch counts in either AID or Cg3 (data not shown).

Spine Density

Agranular Insular Cortex (AID): Apical and basilar spine density means for combinations of Social and Diet conditions are shown in Figs. 3A and 3B. There was a main effect of Diet for apical spines [saccharin > ethanol; $F(1, 49) = 4.06$] and a significant Diet $\times$ Sex $\times$ Social interaction for basilar spines [$F(1, 49) = 4.71$]. Inspection of individual means for Diet, Sex, and Social conditions [see Fig. 4] suggests the interaction was attributable to differential effects of social experience among female and male saccharin rats, whereas no effects of social housing or sex differences were apparent for fetal-ethanol rats. A significant follow-up Sex $\times$ Social interaction for saccharin animals [$F(1, 24) = 7.03$], but not for fetal-ethanol animals, was consistent with this impression. This approach revealed an effect of social housing in saccharin males that only approached significance [control > social; $F(1, 49) = 4.55$, $p = .056$], whereas a non-significant social housing effect in the opposite direction was observed in saccharin females. In contrast, there were no effects of the social manipulation for fetal-ethanol males or females. Simple effect analyses indicated that differential patterns of Diet effects for combinations of Sex and Social housing provided a better characterization of the three-way

interaction; There was a significant Diet effect for female rats in the social condition [saccharin > ethanol; $F(1, 14) = 7.73$], but not for the control housing condition. In contrast, there was a similar trend toward a Diet effect for male rats in the control housing condition [saccharin > ethanol; $F(1, 13) = 3.82$, $p = .072$], whereas spine density was comparable for saccharin and ethanol-exposed male rats in the social condition. Thus, where significant Diet effects or trends were present, they were in the direction of lower spine density in fetal-ethanol animals. None of the other main effects, interactions, or planned comparisons were significant.

Prelimbic Cortex (Cg3): Apical and basilar spine density means from Cg3 for combinations of Social and Diet conditions are shown in Figs. 3C and 3D. There were no significant main effects, interactions or planned comparisons for apical or basilar spine density.

Discussion

Several major findings from Experiment 1 are relevant to our primary goals. The sole significant ethanol-related effect on social behavior in the Control housing (baseline) condition was a reduction in social investigation (anogenital sniffing) in male ethanol-exposed rats. There were also significant reductions in apical dendritic spines on AID neurons of male and female fetal-ethanol-exposed rats, and a similar trend for baseline reductions in basilar spine density on AID neurons in ethanol-exposed males. There was also a trend toward baseline reductions in basilar dendritic length on AID neurons in male and female fetal-ethanol-exposed rats that approached significance. Although these effects were relatively modest, and in some cases only approached significance, they stand in clear contrast to the fetal-ethanol-related effects in the Control housing condition observed in Cg3, where the only observed ethanol-related effect was a significant *increase* in basilar dendritic length on Cg3 neurons in male rats. The apparent complementary effect between ventrolateral and medial frontal cortex regions has been noted elsewhere [16], and may be important to consider in future research. Generally, the baseline dendritic morphology data indicate fetal-ethanol-related reductions in AID dendritic space, and where evident, increases in Cg3 dendritic space.

Social experience had several effects on social behavior in saccharin-exposed rats that differed for males and female. Whereas social experience only resulted in reduced rearing behavior in saccharin males, there were robust increases in anogenital sniffing, body sniffing, wrestling, and crawling over/under in saccharin females. In saccharin-exposed animals, social experience was associated with a reduction in basilar spine density on AID neurons in male rats that approached significance, whereas a non-significant increase was observed in female rats. There were no other remarkable effects of social experience on dendritic morphology in AID or Cg3 in saccharin-exposed animals, although there was a non-significant reduction in basilar dendritic length on AID neurons associated with social experience in male and female saccharin-exposed rats.

Social experience resulted in a broad range of robust behavioral changes in fetal-ethanol rats including increased social investigation (anogenital sniffing and body sniffing), increased wrestling and boxing, and decreased environment-directed behavior (rearing). The behavior effects of social experience observed in fetal-ethanol female were similar to those of saccharin females. In contrast, the range of behavioral effects affected by social experience in fetal-ethanol males was considerably broader than observed in saccharin males. Increases in body (flank) sniffing for fetal-ethanol rats in the social condition (particularly among males) was among the most robust effects that differentiated fetal-ethanol rats from saccharin controls. Although the significance of this observation is not entirely certain, body sniffing may represent a form of social investigation that requires less effort and, therefore, may occur more frequently than other forms of investigation. Sniffing of the flank may also be related to aggressive behaviors as there are odors emitted from glands distributed across the flank of rats [10] that

convey information about social status. Collectively, the behavioral data from Experiment 1 indicate that alterations in social behavior are consequences of prenatal exposure to moderate levels of ethanol that persist into adulthood.

The effects of prenatal ethanol exposure on structural plasticity related to social experience were of two types. First, fetal-ethanol-exposed rats displayed some capacity for structural plasticity related to the social housing treatment, as is evident in the increase in AID basilar dendritic length for fetal-ethanol rats in the social condition. This change, however, was in the opposite direction of the effects observed in saccharin animals and, thus, deviates from the normal pattern of social-experience-related structural plasticity observed in this region. Second, both male and female fetal-ethanol-exposed rats failed to show experience-dependent changes in AID basilar spine density, whereas a sexually dimorphic effect was observed in saccharin animals. As with saccharin-exposed rats, there were no significant social-experience-related effects observed for Cg3 neurons in ethanol-exposed rats. Importantly, the effects of fetal ethanol exposure on behavior noted above appear to correspond well with ethanol-related dendritic changes in AID, and occur against a background of no major changes in gross brain weight or generalized changes in dendritic morphology. Overall, these findings implicate AID in the neurobiology of ethanol-related social behavior abnormalities.

It is important to emphasize that the effects of prenatal ethanol exposure on social behavior and dendritic morphology observed following social experience were the result of a chronic social housing manipulation. The goal of the present experiment was to evaluate effects of fetal-ethanol exposure on behavioral adaptation and structural plasticity in frontal cortex following chronic social experience. Consistent with our previous observations, alterations in social behavior in both the saccharin-exposed and ethanol-exposed animals took approximately 10–18 days (5–9 cage-mates) to emerge compared to control-housed animals, and pilot work indicated that significant alterations in frontocortical dendritic morphology require at least three to four weeks to emerge compared to control-housed animals. It is, of course, possible that the effects of fetal-ethanol exposure with respect to social behavior and frontal cortex function may be demonstrable following more acute treatments. That fetal-ethanol effects were observed for behavioral and dendritic measures in AID following chronic social experience suggests that individual, acute social experiences may differentially recruit frontocortical circuitry involved in social behavior in fetal-ethanol-exposed animals. Experiment 2 was undertaken, in part, to evaluate this hypothesis.

Experiment 2

The fetal-ethanol-related decreases in dendritic length and spine density in AID observed in Experiment 1 suggests fewer excitatory synapses in this region, which may contribute to abnormal social behavior in fetal-ethanol rats. One implication is that AID neurons may be less responsive to stimulation related to social experience in fetal ethanol rats. To address this possibility, expression of immediate early genes (IEGs) related to social interaction was measured in AID and Cg3. Several immediate early genes (IEGs) are activated when neurons fire action potentials, thus, IEG expression serves as a marker of neural activity. Quantification of IEGs can be performed with regional specificity allowing the relative experience-related engagement of brain regions to be compared. Such approaches, along with measuring proteins associated with IEGs, have proven useful in identifying brain regions implicated in alcohol-related behavioral deficits [37,49,60]. In Experiment 2, rats were exposed to ethanol during prenatal development as described in Experiment 1. In adulthood, rats were isolated from their cage-mate for 24 hours after which they either remained in their cage or interacted with their cage-mate for 10 minutes. The brains were processed for fluorescence *in situ* hybridization (FISH) and effects of fetal ethanol exposure on social behavior and expression of two IEGs, *c-fos* [5,58,59] and *Arc* [29,30], in AID (layer II/III) and Cg3 (layer III) were evaluated. In

addition to addressing the question of whether social-experience-related activity in AID and Cg3 is altered in fetal-ethanol rats, the use of an acute social isolation manipulation to motivate social behavior, rather than cage-mate novelty as in Experiment 1, will provide data pertinent to the range of situations in which social behavior abnormalities are observed in moderately exposed fetal-ethanol rats.

Method

Subjects—Subjects were 64 (32 female, 32 male) Long Evans rats acquired from the breeding protocol described in Experiment 1. After weaning rats were transferred from the HSC-ARF to the University of New Mexico Psychology ARF. Animals were housed in same-sex, same-diet-condition pairs on a 12h:12h reverse light:dark schedule with lights on at 1900h. Food and rat chow were available *ad libitum*.

Apparatus and Procedures

Behavior: A total of 24 rats served as caged controls and 40 rats were assigned to the social interaction condition. An equal number of rats for each sex and prenatal diet condition were assigned to each of the two Social conditions. Two brains were not analyzed (1 female ethanol rat in the Social condition, and 1 Male saccharin rat from the caged Control condition), leaving 62 animals in Experiment 2.

All behavioral testing was performed when animals were 90–120 days old in a room nearby to the animal colony. Prior to testing, animals were placed in pairs, with their cage-mate, into the test apparatus for 30 min on each of three successive days in order to habituate the animals to the test environment. The apparatus was a chamber (95cm × 47cm × 43cm) with a clear Plexiglas front and mirrors in the back to aid with behavioral analyses. The behavioral testing room was always kept dark and quiet. Following the final habituation day, all animals were housed in isolation for 24 h after which they were transported to the testing room in their individual cages. Animals in the social condition were placed in the test chamber with their cage-mate for 10 min. The apparatus was illuminated with infrared lights and behavior was video-taped with an infrared camera. After each session, the chamber was cleaned and fresh bedding was placed on the floor. Upon completion of the social interaction period, animals were returned to their individual cages and remained in the testing room for a 20 min holding period. Animals in the caged control condition were transferred individually to the testing room, but remained in their individual cages for a 20 min holding period. At the end of the holding period, animals were rapidly anesthetized with isoflurane and decapitated. The brains were quickly extracted and frozen in isopentane (2-methylbutane) equilibrated in a dry-ice/ethanol slurry and stored at –80°C until processed for FISH (see below).

Behavioral analyses was performed by a rater blind to prenatal diet condition. The frequency and duration of 10 behaviors, described in Experiment 1, were quantified for each individual animal in the Social condition during the 10 min social interaction period.

Fluorescence *in situ* hybridization: Brains were sliced into 20µm thick coronal sections which were mounted, air-dried, and stored at –80°C until processed. Purified plasmid DNA for probe synthesis of the genes *Arc* and *c-fos* were kindly provided by Dr. John Guzowski. Digoxigenin-labeled *c-fos* riboprobe and Fluorescein-labeled *Arc* riboprobes were generated using a commercial transcription kit (MaxiScript; Ambion) and digoxigenin or fluorescein RNA-labeling mixes (Roche Molecular Biochemicals) as described in ref. [29]. Riboprobes were then purified using the mini quick RNA columns (Roche Molecular Biochemicals). Fluorescence *in-situ* hybridization was carried out as described in ref. [29]. Sections were fixed in 4% buffered paraformaldehyde, treated with 0.5% acetic anhydride/1.5% triethanolamine, dehydrated in ethanol, incubated in chloroform, rehydrated in ethanol, and equilibrated in

2xSSC. The RNA probe solution was diluted in 1x hybridization solution, heat denatured, cooled, and added to each slide. Slides were incubated for 16 h in a 56°C hybridization oven and washed to a final stringency of 0.5xSSC at 56°C. Digoxigenin and fluorescein-labeled *c-fos* and *Arc* probes were detected with anti-digoxigenin and anti-fluorescein horseradish peroxidase (Roche Molecular Biochemicals) and a commercial cyanine-3 and fluorescein isothiocyanate (FITC) substrate kit (TSA Amp Kit; Perkin Elmer), respectively. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes). The slides were then cover-slipped using VectaShield (Vector Labs) and visualized.

Fluorescence images were acquired for each signal on an Olympus microscope (Model BX51) equipped with a fluorescence lamp and digital camera (Olympus Model DP70) attached to a personal computer. Separate color images were acquired for DAPI, *c-fos*, and *Arc* signals in each region at 40× magnification using Olympus DP Controller software. Both left and right hemisphere images were acquired. Exposure time was held constant at 143 msec for *c-fos* and *Arc* signals. For each subject, images were acquired from three separate sections from separate FISH runs conducted on different dates. All image collection and subsequent measurement was performed by an experimenter blind to group membership using software developed in our laboratory. The DAPI images for each section were used to select regions of interest and background regions. Two background regions in which no tissue was present were selected for each section (one adjacent to the left hemisphere and one adjacent to the right hemisphere). Selection of AID regions of interest primarily included Layer II/III neurons. Selection of Cg3 neurons primarily included layer III neurons. The software computed the average *c-fos* and *Arc* signal intensity for the selected AID and Cg3 regions in both hemispheres as well as in the background regions for each section. The proportional difference between regions of interest and background were computed [(ROI signal - Background signal)/Background signal.] The average *c-fos* and *Arc* values for each region, averaged across hemispheres and all three sections were analyzed.

Results

Behavior—Means (and SEMs) for the frequency and total time male and female animals from both diet conditions engaged in each behavior measured during the social interaction period are shown in Table 4. Separate ANOVAs were conducted for each dependent measure obtained for rats in the Social condition with Sex and prenatal Diet condition as factors. There were significant Diet effects for wrestling [EtOH > SAC; $F_{TIME}(1, 35) = 6.15$, $F_{FREQ}(1, 35) = 10.71$]. None of the other Diet effects were significant. There were significant Sex × Diet interactions for wrestling [$F_{TIME}(1, 35) = 6.78$, $F_{FREQ}(1, 35) = 10.99$], and the interaction for boxing approached significance [$F_{TIME}(1, 35) = 3.64$, $p = .065$, $F_{FREQ}(1, 35) = 3.71$, $p = .062$]. Among males there were significant Diet effects for wrestling [EtOH > SAC; $F_{TIME}(1, 18) = 6.86$, $F_{FREQ}(1, 18) = 11.48$], and there were trends toward Diet effects for boxing [EtOH > SAC; $F_{TIME}(1, 18) = 3.52$, $p = .077$, $F_{FREQ}(1, 18) = 3.75$, $p = .069$]. Females rarely engaged in wrestling or boxing, and Diet effects for these measures in females were non-significant. All other Sex × Diet interactions were non-significant.

There were significant Sex effects for body sniffing [male > female; $F_{TIME}(1, 35) = 4.35$, $F_{FREQ}(1, 35) = 6.09$], anogenital sniffing [female > male; $F_{TIME}(1, 35) = 4.45$, $F_{FREQ}(1, 35) = 6.00$], wrestling [male > female; $F_{TIME}(1, 35) = 10.40$, $F_{FREQ}(1, 35) = 13.30$], and boxing [male > female; $F_{TIME}(1, 35) = 8.99$, $F_{FREQ}(1, 35) = 8.84$]. There were also Sex differences in the number of instances of digging and sniffing in the bedding [female > male; $F(1, 35) = 9.58$].

Immediate early gene expression—Separate ANOVAs were conducted for each brain region and IEG with prenatal Diet condition, Sex, and Social condition (Social interaction vs.

caged Control) as factors. Planned comparisons of Social and Control data were conducted within levels of the Diet condition. Representative sections showing *c-fos* and *Arc* expression in AID for saccharin animals in the control and social conditions are shown in Fig. 5. Mean *c-fos* and *Arc* values expressed as percentage of SAC-Control means are shown in Fig. 6 for combinations of Diet condition and Social condition.

Agranular insular cortex (AID): There was a significant Diet $\times$ Social interaction for *c-fos* [$F(1, 54) = 4.04$], however, the interaction failed to reach significance for *Arc* [$F(1, 54) = 3.25$, $p = .077$]. Planned comparisons revealed significant effects of social experience in saccharin animals for *c-fos* expression [social > control; $F(1, 29) = 11.31$] and *Arc* expression [social > control; $F(1, 29) = 7.90$]. In contrast, no effects of social experience were observed in fetal-ethanol animals for *c-fos* or *Arc*.

There was a main effect of Social interaction for *c-fos* [social > control; $F(1, 49) = 4.76$]. All other effects were non-significant.

Prelimbic cortex (Cg3): The Diet $\times$ Social interaction for *c-fos* approached significance [$F(1, 54) = 3.71$, $p = .059$]. Planned comparisons revealed significant effects of social experience in saccharin animals for *c-fos* expression [social > control; $F(1, 29) = 4.23$], however, no differences in *Arc* expression were detected. No effects of social experience were observed in fetal-ethanol animals for *c-fos* or *Arc*.

Discussion

The results of Experiment 2 further indicate that social behavior abnormalities are long-lasting consequences of prenatal exposure to moderate levels of ethanol, and provide additional evidence that these effects can be linked to frontal cortex. Saccharin-exposed rats displayed social-experience-related increases *c-fos* expression in AID and Cg3, and increases in *Arc* expression in AID. A further distinction between AID and Cg3 is evident in that *c-fos* expression in AID was more robust than in Cg3, and more robust than *Arc* expression in either region. Male fetal-ethanol rats displayed robust increases in wrestling during a single social interaction after 1 day of isolation, but fetal-ethanol rats did not display social-experience-related increases in *c-fos* or *Arc* expression in either AID or Cg3. Collectively, the results demonstrate that AID and Cg3 are not recruited normally during social interaction in fetal-ethanol rats. Although not statistically significant, it is important to note that social-experience-related *c-fos* expression in AID in saccharin-exposed rats was more robust in males (44%) than females (26%), whereas all other effects were comparable for male and female saccharin-exposed animals. This pattern suggests that ethanol-related effects on activity-related IEG expression in AID were more pronounced for male rats, and this observation corresponds well with the pattern of wrestling observed in male fetal-ethanol rats. These observations are also consistent with the sexually dimorphic behavioral and structural plasticity effects observed in Experiment 1, and strengthen the general conclusion that the social behavior deficits observed in fetal-ethanol rats are related to functional changes in frontocortical regions, including AID and Cg3.

General Discussion

Rats exposed to moderate levels of ethanol during prenatal brain development displayed subtle but reliable changes in social behavior in adulthood. These changes were generally of two types, one related to altered social investigation and the other related to wrestling which may reflect increased play fighting or aggressive behaviors. Fetal-ethanol-exposed animals housed in standard conditions displayed decreased investigation and increased wrestling. When housed with various social partners, fetal-ethanol rats displayed abnormal increases in certain forms

of social investigation and wrestling. When social interaction was motivated by prior isolation rather than cage-mate novelty, male, but not female, fetal-ethanol animals also displayed robust increases in wrestling. Collectively, the behavioral data from both experiments reported here indicate that altered social behavior can be included among the long-term consequences of exposure to moderate levels of ethanol during brain development in the rat.

It is important to recognize that increases in wrestling could reflect increases in serious fighting or increases in play fighting, as wrestling may be present in both [65,78]. Serious fighting can be distinguished from play fighting with regard to the target of the attack (e.g., nape of the neck versus the rump, see ref. [65], as well as other measures associated with serious fighting, such as biting, piloerection, and tail rattling. In the behavioral observations for both experiments, only three unambiguous instances of serious fighting based on these additional indicators were observed. All three instances were observed in ethanol-exposed rats in the Social housing condition of Experiment 1 (1 female and 2 males), however, the vast majority of wrestling observed in both experiments was not accompanied by behaviors indicative of serious fighting. It is possible that increased wrestling in the absence of these observations may, nonetheless, indicate increases in aggressive behavior, but the absence of clear indicators of aggression (e.g., biting) makes it difficult to unambiguously distinguish play fighting from serious fighting in the present study. Another possibility is that ethanol-exposed animals engage in greater levels of adult play fighting, which could indicate a reduction in the rate of maturation of play behavior following moderate prenatal ethanol exposure. Determining whether the increases in wrestling observed following moderate fetal ethanol exposure reflect increased play fighting, serious fighting, or some combination thereof will require additional research. What is clear in the present findings is that aspects of social behavior related to wrestling and related behaviors (e.g., boxing) are altered following moderate prenatal ethanol exposure.

Alterations in social behavior similar to those described here have also been observed following acute ethanol exposure [91,93,92,94]. High doses of ethanol (e.g., 1g/kg) result in social inhibition, characterized by decreased social investigation and play fighting in adolescent (e.g., P28, P48) and adult rats [91,93]. In contrast, lower doses of ethanol (e.g., .5g/kg) facilitate social investigation and play fighting [92,94]. In the present study, modest evidence for social inhibition was observed in male ethanol-exposed rats under baseline conditions (Control housing condition of Experiment 1), whereas cage-mate novelty or social isolation facilitated social interaction (in the form of increased sniffing) and/or presumptive play fighting (in the form of increased wrestling). Given these similarities, it will be important to evaluate whether the behavioral consequences of prenatal and acute ethanol exposure are additive or if they interact, and if similar mechanisms are involved in producing social behavioral alterations following prenatal or acute ethanol exposure. It is important to recognize that the specific social behavioral alterations characterized here may be related to distinct ethanol-related changes in the brain. This point was highlighted in the recent work of Varlinskaya and Spear [94], who reported a clear dissociation between social investigation (e.g., sniffing) and play fighting with respect to μ -opioid receptors. Systemic administration of μ -opioid antagonists blocked ethanol-related enhancement of play fighting, but did not suppress ethanol-related enhancements of social investigation. It is interesting to note that we observed an increase in wrestling, but not investigation, among male ethanol-exposed rats in Experiment 2. If acute ethanol exposure during early adolescence and prenatal ethanol exposure enhance play behavior via similar mechanisms, and if the increases in wrestling observed here represent play rather than serious fighting, then μ -opioid antagonists should attenuate the fetal-ethanol-related increases in wrestling and related behaviors. Given that the findings of Varlinskaya and Spear [94] were limited to 28-day-old rats, it will be important for future research efforts to systematically evaluate the contributions of μ -opioid receptors to specific aspects of social behavioral alterations observed in fetal ethanol rats throughout development.

In the present study, fetal ethanol exposure was associated with reduced dendritic length and spine density in agranular insular cortex (AID), increased dendritic length in Cg3, and abnormal experience-dependent structural plasticity in AID. Specifically, fetal-ethanol rats showed experience-dependent increases in dendritic length in AID, but showed no experience-dependent changes in AID spine density as was observed in saccharin-exposed animals. Experience-dependent alterations in dendritic structure are associated with normal experience [46,48], but are also associated with pathological states including FAS [21] and a broad range of other neurodevelopmental [67] and neurological disorders [22]. Previous studies have shown decreased dendritic spine density in somatosensory cortex in animals exposed to high levels of ethanol *in utero* [20,23,72], and hippocampal CA1 spines in fetal-ethanol rats do not undergo normal experience-dependent increases [7]. A major difference between heavy exposure, including binge, models and mild to moderate exposure paradigms such as the one employed here is that the former tend to result in gross craniofacial abnormalities [82], gross morphological changes in brain [64] and profound behavioral effects. In contrast, moderate exposure paradigms tend to result in subtle, but reliable, deficits in behavior [84] and synaptic plasticity [83,90] that are observed in the absence of clear physical abnormalities or structural changes in the brain. Although ultrastructural changes have been documented following lesser ethanol exposure [3,90], to our knowledge, the present data are the first demonstrations of clear changes in dendritic structure and structural plasticity associated with lesser ethanol exposure, as well as being the first demonstrations of ethanol-related alterations in the structure and function of agranular insular cortex neurons. Importantly, and in keeping with the pattern noted above in which moderate exposure results in functional consequences that are more profound than structural consequences, the observed structural effects were far less robust than the behavioral effects (Experiments 1 and 2) and functional (IEG expression) effects observed in Experiment 2.

Although the functional implications of the ethanol-related effects on dendritic length and spine density were not thoroughly examined here, these observations clearly implicate AID in the ethanol-related behavioral effects noted above, particularly considering that no experience-related effects in ethanol-exposed rats were observed in dendritic length or spine density of prelimbic cortex neurons. The lack of normal activity-related IEG expression in AID, and to a lesser degree prelimbic cortex, in fetal-ethanol-exposed rats further establishes the potential importance of frontocortical regions in fetal-ethanol-related behavioral deficits.

As with the behavioral observations noted above, the effects of prenatal exposure to moderate levels of alcohol on dendritic morphology, structural plasticity and IEG expression were more apparent in males than females. Given that numerous other studies have identified sexually dimorphic effects of prenatal ethanol exposure [28,33,42,56,63,95,98], future studies aimed at addressing the neural mechanisms responsible for fetal-ethanol-related effects in the social domain will benefit from examining both sexes, as evaluating sexual dimorphisms could provide a means of constraining hypotheses and interpretations regarding the putative mechanisms of ethanol-related social behavior abnormalities. An important refinement for subsequent studies utilizing the methods employed will be to systematically evaluate potential effects related to stage of estrous. Although the effects of prenatal ethanol exposure observed here had modest effects in females compared to males, possible interactions between stage of estrous and ethanol exposure with respect to behavioral and neurobiological measures were not investigated in the present study. Future studies that include estrous as a factor will be more complicated due to the need to consider stage of estrous and diet condition for both cage-mates, however, evaluating these factors will more thoroughly inform the conditions in which effects of prenatal ethanol exposure does or does affect social behavior in females.

One issue that potentially complicates interpretation of the present findings concerns a discrepancy with some of our previous observations regarding structural plasticity in AID

associated with social experience. In our previous studies [32,75] we found that experience with a variety of social partners increased social investigation and wrestling, particularly in male rats. These behavioral changes were accompanied by dendritic changes in AID, specifically increases in dendritic length and decreases in spine density [32,75]. Consistent with our previous findings, experience-dependent changes in dendritic spine density were observed in saccharin-exposed animals, however, the experience-dependent decreases in dendritic length observed in saccharin-exposed animals housed with various social partners is inconsistent with our previous observations. There are several factors that potentially contributed to these differences. In our previous work all cage-mate changes occurred in the light phase, whereas in the present study all cage-mate changes were done in the dark phase. Because the majority of intense social interaction and wrestling tend to occur within a 10–15 minute period after the animals are placed in the cage, it is possible that the differences in lighting conditions influenced social behavior and associated structural plasticity. Indeed, the increases in anogenital sniffing, wrestling and boxing related to the social housing manipulation were less dramatic in the present study compared to our previous observations. This is partially due to the fact that saccharin-exposed rats in the control housing condition engaged in more social interaction, wrestling and boxing under dark conditions than we observed under light conditions in our previous studies. Other potential factors involved in the aforementioned discrepancy were the source of the rats and the prenatal treatment. The rats used in our previous studies and the rats used in the present study were of the same strain (Long Evans), however, they were bred and born at different sites and were derived from different stock (Charles River and Harlan, respectively). Further, all rat dams in the saccharin and ethanol groups of the present study consumed ethanol prior to becoming pregnant which may also have contributed to the aforementioned differences. It will be important to determine whether the effects observed here hold generality and apply to a broad range of strains of rats and species of rodents, as well as a broad range of manipulations involving social experience. Although the present effects observed in control rats did not entirely match our previous findings, the behavioral, dendritic, and functional data obtained in ethanol-exposed rats were clearly different from the findings in saccharin-exposed animals. It is also important to reiterate that the dendritic spine density measures obtained in the present study did match our previous observations. Experience-dependent changes in spine density were obtained in saccharin-exposed animals, but no such changes were observed in ethanol-exposed animals.

The decreases in baseline AID dendritic length and spine density observed in fetal-ethanol-exposed rats indicates reductions in excitatory synapses [34,62], which may partially account for the reduced activity-related IEG expression in ethanol-exposed animals as well as the observed behavioral effects. An important line of future research will involve systematically examining the range of functional and behavioral consequences of these changes, and attempting to identify the underlying mechanisms. For example, the behavioral and IEG expression data indicate that there are clear functional consequences of prenatal ethanol exposure that can be directly linked to AID. Reductions in excitatory synapses should also result in other quantifiable physiological effects including reduced spontaneous activity, as well as diminished evoked responses. One obvious expectation is that there should be reduced glutamatergic transmission and fewer glutamate receptors (e.g., AMPA, NMDA) in the agranular insular cortex of ethanol-exposed animals. We have observed fetal-ethanol-related decreases in mGluR₅ receptor density in Cg3 [90] in the absence of changes in overall AMPA and NMDA receptors, suggesting that we may expect greater ethanol-related reductions in mGluR₅ in AID than in AMPARs and NMDARs. We have, however, not yet performed these measurements in AID.

It will also be important to evaluate a range of other potential behavioral and functional consequences of ethanol-related alterations of AID circuitry. The primate OPFC and the corresponding lateral frontal cortex of the rat, including agranular insular cortex are involved

in other general functions that may be related to social behavior, but may manifest in deficits that are not purely social in nature. In addition to gustatory and olfactory processes, the latter of which would be of clear importance for social behavior in many animals, damage to OPFC in primates and AID in rodents results in response perseveration which can be made apparent when response-outcome contingencies are modified [9,55,70]. The moving platform variant of the Morris water task [79,96], in which the escape platform is routinely moved to a novel spatial location, is a commonly used procedure which lends itself well to measurement of response perseveration. Normal rats rapidly learn, within a single trial, to navigate directly to the new escape location and stop navigating to old escape locations. Kolb [47] found that rats with lateral frontal cortex lesions that included AID persisted in visiting an old location when the platform was moved, whereas animals with medial frontal cortex lesions that included prelimbic cortex showed a more general spatial learning deficit. Response perseveration has been observed in rats exposed to high levels of ethanol during brain development [68,85,86] and reversal learning/response perseveration deficits are among the many consequences observed in children with FAS [44]. Further, impairments in the moving platform variant of the Morris water task have been observed in rats exposed to moderate levels of ethanol [84], whereas normal performance was observed when the platform remained in a fixed location. Although alcohol-related deficits in the hippocampal long-term potentiation were observed in the same ethanol exposure paradigm [83], the pattern of spatial learning deficits may also be linked to functional alterations in AID. Future efforts to characterize the more general response perseveration deficits, their functional implications, and their mechanisms in animals exposed to moderate levels of ethanol are needed.

One of the major goals of the present study was to determine whether agranular insular cortex and prelimbic cortex are implicated in fetal-ethanol-related social behavior deficits. In pursuing this hypothesis, it was not our intention to imply that the regions under investigation were the only ones potentially implicated in the observed behavioral effects. Indeed, a distributed set of neural circuitry has been implicated in social behavior, and previous studies employing different ethanol exposure paradigms and behavioral approaches than those used here have revealed ethanol-related changes in amygdala [42] and somatosensory cortex [49], which are implicated in normal sensory, cognitive, and behavioral processes involved in social behavior. In addition to the regions normally linked with social behavior (amygdala, hippocampus, frontal cortex, nucleus accumbens), it will also be important to examine other regions as well. For example, Sirevaag and Greenough [76,77] reported structural plasticity in posterior brain regions in response to social housing, thus, social experience may have widespread effects in neocortex. Importantly, our findings indicate a dissociation between Cg3 and AID with respect to structural plasticity, thus, the social housing manipulation used here does not result in generalized structural changes throughout the brain. Nonetheless, whether fetal-ethanol-related changes in structural plasticity are apparent in the posterior brain regions should be evaluated.

During inspection of individual fluorescent images for analysis it was also apparent that other frontal regions adjacent to AID showed clear activity-related IEG expression, including the lateral and ventral orbital areas (Zilles' VO and LO; see Fig. 1), as well as regions ventral to the rhinal fissure, including piriform cortex (Zilles' Pir). In contrast, activity-related IEG expression was not obviously apparent in regions dorsal to Cg3 and AID, including Fr1, Fr2, and Fr3. We are currently carrying out additional analyses to determine whether this apparent experience-dependent dissociation between ventral and dorsal aspects of prefrontal cortex is also affected by prenatal alcohol exposure. It will also be important to examine ethanol-related alterations in regions that are connected with agranular insular cortex and prelimbic cortex. There are prominent projections from hippocampus and associated rhinal cortices to prelimbic cortex [35], and AID receives prominent projections from piriform cortex, contralateral AID and the basolateral amygdala [24]. If the number of AID excitatory synapses is reduced in fetal-ethanol-exposed animals, as discussed above, evoked responses elicited by stimulation of the

aforementioned afferent pathways should be reduced. The pathways from piriform cortex and basolateral amygdala are particularly important targets for future research on ethanol-related alterations in circuitry involved in social behavior.

The present findings indicate that there are long-term consequences of moderate fetal ethanol exposure on adult social behaviors, thus, another critical issue that needs to be addressed in future research concerns the developmental processes and mechanisms that lead to abnormal ethanol-related social behavior in adulthood. Understanding how the fetal-ethanol-related effects reported here were established will be critical for developing treatments. In the present experiment there was no explicit attempt to alter experience of fetal-ethanol rats during development, however, based on previous findings [49,51] it seems reasonable to expect that fetal-ethanol-related effects on social behavior would be present very early in life. This could dramatically alter experiences throughout development which could further contribute to the behavioral, structural, and functional effects reported here. Characterizing the developmental trajectories of the effects reported here, and evaluating factors that attenuate or enhance the effects observed in adulthood throughout the lifespan represent important goals for future work.

In summary, the present findings indicate that alterations in social behavior are important consequences of prenatal exposure to moderate levels of ethanol that persist well into adulthood. Further, the effects of prenatal alcohol exposure on dendritic morphology, structural plasticity, and immediate early gene expression indicate that agranular insular cortex, and to a lesser degree, prelimbic cortex are implicated in fetal-ethanol-related social behavior deficits. These regions, among the many other regions involved in social behavior, can be identified as clear targets for future research efforts. Considerable future work will be needed to address the neurobiological mechanisms of the effects described here, and several immediate goals for investigating these mechanisms have been suggested. Collectively, these pursuits should improve our understanding of the basic mechanisms underlying fetal-ethanol-related social behavior deficits so that rational treatment strategies can be developed and evaluated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NIAAA/NIH AA015356 to DH.

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Figure 1.

Coronal sections (adapted from Zilles, 1985) showing regions of agranular insular cortex (AID) and prelimbic cortex (Cg3) from which neurons were sampled for Experiment 1 and for which immediate early gene expression was measured in Experiment 2. Distance anterior to bregma is provided beneath each section. Abbreviations for other areas shown here, but not analyzed in the present study: Acb = Nucleus accumbens, aci = anterior commissure (intrabulbar), AO = Anterior olfactory nucleus, Cl = Claustrum, DPC = Dorsal peduncular cortex, Fr1-3 = Frontal cortex, IL = Infralimbic cortex, lo = Lateral olfactory tract, LO = Lateral orbital area, MO = Medial orbital area, Par1 = Parietal cortex, Pir = Piriform cortex, TT = Taenia tecta, Tu = Olfactory tubercle, VLO = Ventrolateral orbital area, VO = Ventral orbital area

Figure 2.

Mean (+ SEM) dendritic length expressed as the percentage of SAC-Control mean: A) AID apical length, B) AID basilar length C) Cg3 apical length, and D) Cg3 basilar length. * indicates a significant Social experience effect within Diet condition at $p < .05$. The Diet effect for rats in the Control condition (#) approached significance, $p = .057$.

Figure 3.

Mean (+ SEM) spine density expressed as the percentage of SAC-Control mean: A) AID apical spines, B) AID basilar spines, C) Cg3 apical spines, and D) Cg3 basilar spines. * indicates a significant Diet effect at $p < .05$. There was a significant Social $\times$ Diet $\times$ Sex interaction for AID basilar spine density (panel B) as is illustrated in Fig. 4.

Figure 4.

Mean (+ SEM) basilar spine density in AID for male and female rats from each combination of Diet and Social experience conditions. These data correspond to the values shown in Fig. 3B. Values are expressed as percentages of combined SAC-Control mean for both sexes. * indicates a significant Diet effect at $p < .05$. # indicates a simple main effect of Social experience in male saccharin rats that approached significance ($p = .056$).

Figure 5.

Fluorescence images illustrating representative differences in *c-fos* (red) and *Arc* (green) expression for saccharin rats in the Caged Control (A–D) and Social interaction (E–H) conditions. Localization of *c-fos* and *Arc* to nuclei stained with DAPI (blue) is shown at 40X magnification (A,B,E,F) and 750X magnification (C,D,G,H).

Figure 6.

Mean (+SEM) *c-fos* and *Arc* expression for Diet and Social conditions of Experiment 2. Means are expressed as the percentage of SAC-Control means for each measure. A) AID *c-fos*, B) AID *Arc*, C) Cg3 *c-fos*, D) Cg3 *Arc*. Because there were no significant sex differences all values are collapsed across Sex. * indicates a significant effect of the social treatment within a particular Diet condition at $p < .05$.

Table 1

Mean (SEM) effects of daily four-hour ethanol consumption on female dams (weight gain, ethanol consumption, and serum ethanol concentration) and offspring (litter size and pup birthweight).

	Saccharin	Ethanol
Maternal weight gain during pregnancy	127g (3.0); n = 44	121g (4.0); n = 51
Daily 4-hr 5% ethanol consumption	N/A	2.81g/kg (0.13); n = 51
Maternal serum ethanol concentration	N/A	84.0 mg/dL (5.50); n = 24
Litter size	12.5 (0.13); n = 41	12.4 (0.30); n = 42
Pup birthweight	6.17g (0.13); n = 41	6.13g (0.11); n = 42

Maternal weight gain was measured over gestational days 1–21. Maternal serum ethanol concentration was measured 30 minutes following a 4-hour drinking period. Litter size reflects number of live births per litter.

Mean (SEM) time and frequency that for each behavior measured in Experiment 1. For each measure, means collapsed across sex are presented first (bold) followed by means for males and females (italics).

Table 2

	Saccharin		Ethanol	
	Control	Social	Control	Social
Rearing (Freq)_c	54.05 (2.41)	47.12 (4.87)	47.71 (4.23)	44.64 (4.11)
<i>Male</i>	<i>55.19 (3.20)</i>	<i>36.39 (7.90)*</i>	<i>40.71 (6.93)</i>	<i>38.83 (5.94)</i>
<i>Female</i>	<i>52.90 (3.81)</i>	<i>55.17 (4.70)</i>	<i>54.71 (3.61)</i>	<i>51.29 (4.89)</i>
Rearing (Time)_{a,c}	250.62 (15.27)	188.68 (22.34)*	247.80 (24.75)	172.72 (22.30)*
<i>Male_a</i>	<i>224.90 (15.60)</i>	<i>135.44 (35.05)*</i>	<i>209.39 (41.63)</i>	<i>137.77 (31.72)</i>
<i>Female_a</i>	<i>276.33 (23.39)</i>	<i>228.61 (20.79)</i>	<i>286.21 (20.72)</i>	<i>212.68 (25.42)*</i>
AG sniff (Freq)_{a,ac,bc}	4.05 (1.15)	6.14 (0.73)	2.74 (0.56)	6.29 (0.92)*
<i>Male_b</i>	<i>6.33 (1.96)</i>	<i>5.33 (1.39)</i>	<i>1.86 (0.46)</i>	<i>4.13 (1.13)</i>
<i>Female_a</i>	<i>1.76 (0.41)</i>	<i>6.75 (0.76)*</i>	<i>3.62 (0.94)</i>	<i>8.76 (0.79)*</i>
AG sniff (Time)_{a,ac,bc}	8.75 (3.07)	14.12 (1.78)	5.40 (1.38)	15.24 (2.81)*
<i>Male</i>	<i>14.24 (5.49)</i>	<i>12.70 (3.59)</i>	<i>3.71 (1.56)</i>	<i>10.53 (3.92)</i>
<i>Female_a</i>	<i>3.26 (0.88)</i>	<i>15.19 (1.73)*</i>	<i>7.09 (2.22)</i>	<i>20.63 (3.14)*</i>
Body sniff (Freq)_{a,ab,abc}	9.74 (2.12)	13.69 (1.53)	7.02 (1.01)	17.67 (1.71)*
<i>Male_{a,ab}</i>	<i>13.05 (3.88)</i>	<i>12.83 (3.31)</i>	<i>6.48 (1.52)</i>	<i>20.04 (2.38)*</i>
<i>Female_a</i>	<i>6.43 (0.82)</i>	<i>14.33 (1.27)*</i>	<i>7.57 (1.42)</i>	<i>14.95 (2.18)*</i>
Body sniff (Time)_{a,ab,abc}	17.20 (5.07)	23.94 (3.02)	10.99 (1.79)	36.22 (4.44)*
<i>Male_{a,ab}</i>	<i>23.89 (9.66)</i>	<i>23.73 (6.34)</i>	<i>10.37 (2.76)</i>	<i>43.33 (5.81)*</i>
<i>Female_a</i>	<i>10.51 (1.84)</i>	<i>24.09 (2.87)*</i>	<i>11.61 (2.49)</i>	<i>28.10 (5.75)*</i>
Wrestling (Freq)_a	0.79 (0.37)	1.88 (0.42)	0.64 (0.18)	2.80 (0.59)*
<i>Male</i>	<i>1.33 (0.70)</i>	<i>2.17 (0.86)</i>	<i>0.90 (0.26)</i>	<i>2.38 (0.64)†</i>
<i>Female_a</i>	<i>0.24 (0.10)</i>	<i>1.67 (0.41)*</i>	<i>0.38 (0.22)</i>	<i>3.29 (1.06)*</i>
Wrestling (Time)_a	2.23 (0.89)	6.90 (1.34)*	1.78 (0.83)	12.99 (3.39)*
<i>Male_a</i>	<i>3.68 (1.60)</i>	<i>8.20 (2.59)</i>	<i>2.86 (1.53)</i>	<i>10.53 (3.23)†</i>

		Saccharin		Ethanol	
		Control	Social	Control	Social
Boxing (Freq)_{ac}	<i>Female_a</i>	0.78 (0.38)	5.93 (1.38)*	0.69 (0.48)	15.81 (6.42)*
		1.36 (0.72)	2.69 (1.00)	0.26 (0.15)	2.36 (0.72)*
	<i>Male_a</i>	2.71 (1.28)	5.33 (1.85)	0.48 (0.29)	3.83 (1.07)*
Boxing (Time)_{ac,ac}	<i>Female_a</i>	0.00 (0.00)	0.71 (0.36)	0.05 (0.05)	0.67 (0.36)
		2.68 (1.44)	6.88 (3.08)	0.82 (0.55)	5.04 (1.70)*
	<i>Male_a</i>	5.35 (2.57)	14.73 (5.95)	1.61 (1.06)	8.39 (2.65)*
Grooming (Freq)_{abc}	<i>Female_a</i>	0.00 (0.00)	1.00 (0.56)	0.02 (0.02)	1.22 (0.76)
		3.71 (0.78)	2.76 (0.41)	2.48 (0.49)	3.49 (0.60)
	<i>Male_{ab}</i>	3.95 (1.40)	2.28 (0.55)	1.76 (0.58)	4.71 (0.90)*
Grooming (Time)	<i>Female</i>	3.48 (0.81)	3.13 (0.58)	3.19 (0.72)	2.10 (0.38)
		19.87 (3.58)	16.44 (2.94)	15.69 (4.24)	20.65 (4.03)
	<i>Male</i>	18.07 (5.62)	15.68 (3.70)	11.63 (5.91)	25.22 (6.16)
Allogroom (Freq)_a	<i>Female</i>	21.68 (4.79)	17.00 (4.54)	19.75 (6.12)	15.43 (4.68)
		0.55 (0.14)	0.90 (0.26)	0.62 (0.17)	1.44 (0.37)
	<i>Male</i>	0.48 (0.20)	0.89 (0.44)	0.76 (0.31)	1.25 (0.22)
Allogroom (Time)	<i>Female</i>	0.62 (0.21)	0.92 (0.33)	0.48 (0.16)	1.67 (0.78)
		1.99 (0.78)	2.51 (0.75)	2.35 (0.90)	4.90 (1.81)
	<i>Male</i>	1.51 (0.75)	2.18 (0.97)	3.64 (1.65)	4.45 (2.57)
Crawl OU (Freq)_{ac}	<i>Female</i>	2.48 (1.42)	2.76 (1.14)	1.06 (0.44)	5.42 (2.73)
		1.05 (0.77)	0.74 (0.25)	0.48 (0.23)	0.58 (0.31)
	<i>Male</i>	1.86 (1.54)	0.06 (0.06)	0.48 (0.38)	0.21 (0.11)
Crawl OU (Time)	<i>Female_a</i>	0.24 (0.10)	1.25 (0.33)*	0.48 (0.28)	1.00 (0.64)
		1.42 (1.15)	0.82 (0.29)	0.47 (0.22)	0.59 (0.33)
	<i>Male</i>	2.58 (2.29)	0.08 (0.08)	0.47 (0.36)	0.21 (0.12)
Bedding (Freq)_{ac}	<i>Female</i>	0.27 (0.11)	1.38 (0.40)*	0.48 (0.28)	1.02 (0.68)
		35.10 (1.68)	30.00 (2.94)	33.36 (3.37)	30.40 (1.82)
	<i>Male_a</i>	35.95 (3.03)	24.17 (5.94)	34.19 (6.85)	26.79 (2.47)
Bedding (Time)	<i>Female</i>	34.24 (1.66)	34.38 (1.67)	32.52 (1.48)	34.52 (1.77)
		105.45 (9.63)	84.30 (10.24)	106.44 (16.28)	83.44 (6.32)

	Saccharin		Ethanol	
	Control	Social	Control	Social
Male	115.90 (16.97)	75.91 (21.37)	117.97 (31.56)	72.39 (7.76)
Female	95.00 (8.79)	90.60 (9.09)	94.90 (10.40)	96.07 (8.34)

Male/female numbers for each Diet-Social condition were: Saccharin-Control (7/7), Saccharin-Social (6/8), Ethanol-Control (7/7), and Ethanol-Social (8/7). AG Sniff = anogenital sniffing; Crawl OU = Crawling over/under.

† indicates Social effects that approached significance at $p = .06$. Subscripts indicate significant main effects and interaction for social housing condition (a), prenatal diet condition (b), and sex (c). The direction of the effects reported here are summarized in Table 3.

* indicates a significant Social effect within Diet conditions at $p < .05$.

Table 3

Summarization of the effects of social experience and prenatal diet condition on social behavior in male and female rats in Experiment 1. Effects of prenatal diet condition on social behavior in the control housing condition are not summarized here because only the diet effect on anogenital sniffing in males was significant.

	Female		Male	
	Saccharin	Ethanol	Saccharin	Ethanol
Rearing		–	–	
AG sniff	+	+		+
Body sniff	+	+		+
Wrestling	+	+		+ [†]
Boxing				+
Groom				+
Allogroom				
Crawl OU	+			
Bedding				

+ indicates a significant increase related to social experience for the time and/or frequency measures for each behavior. - indicates a decrease.

[†] indicates that effects approached significance $p \leq .06$. Group numbers and abbreviations are provided in Table 2.

Table 4

Mean (SEM) time and frequency that male and female rats from each prenatal Diet condition engaged in each behavior measured during the 10 minute social interaction session of Experiment 2.

	Female		Male	
	Saccharin	Ethanol	Saccharin	Ethanol
Rearing (Freq)	40.40 (2.83)	34.67 (3.76)	32.10 (2.72)	32.00 (4.54)
Rearing (Time)	73.94 (6.67)	62.39 (7.29)	61.34 (10.06)	55.47 (8.49)
AG sniff (Freq) _a	3.80 (0.59)	4.89 (0.95)	3.20 (0.80)	2.40 (0.56)
AG sniff (Time) _a	7.90 (1.64)	10.21 (2.82)	5.95 (1.52)	3.43 (0.80)
Body sniff (Freq) _a	8.80 (1.14)	10.00 (1.55)	11.90 (1.04)	12.10 (1.26)
Body sniff (Time) _a	9.81 (1.35)	9.93 (1.58)	13.63 (1.71)	13.77 (1.53)
Wrestling (Freq) _{a,b,ab}	0.10 (0.10)	0.00 (0.00)	0.60 (0.43)	*4.70 (1.51)
Wrestling (Time) _{a,b,ab}	0.09 (0.09)	0.00 (0.00)	0.77 (0.59)	*14.25 (3.94)
Boxing (Freq) _a	0.10 (0.10)	0.00 (0.00)	0.70 (0.47)	2.70 (0.96)
Boxing (Time) _a	0.05 (0.05)	0.00 (0.00)	0.69 (0.47)	2.98 (1.09)
Groom (Freq)	0.50 (0.31)	1.33 (0.41)	0.40 (0.22)	1.10 (0.59)
Groom (Time)	1.58 (0.98)	5.24 (3.01)	1.95 (1.09)	3.57 (2.26)
Allogroom (Freq)	0.30 (0.15)	0.44 (0.24)	0.20 (0.20)	0.10 (0.10)
Allogroom (Time)	0.40 (0.21)	0.76 (0.47)	0.39 (0.39)	0.09 (0.09)
Crawl OU (Freq)	0.90 (0.41)	0.56 (0.38)	0.60 (0.27)	0.90 (0.23)
Crawl OU (Time)	0.71 (0.34)	0.52 (0.34)	0.44 (0.23)	0.87 (0.24)
Bedding (Freq)	33.30 (2.19)	33.78 (1.65)	29.10 (1.79)	26.50 (1.69)
Bedding (Time)	96.35 (6.01)	90.64 (4.68)	105.35 (10.44)	82.62 (9.95)

Subscripts indicate main effects and interactions for Sex (*a*) and Diet (*b*) at $p < .05$.

* indicates a significant Diet effect within Sex at $p < .05$.