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Moderate prenatal alcohol exposure and quantification of social behavior in adult rats

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Abstract:	Alterations in social behavior are among the major negative consequences observed in children with Fetal Alcohol Spectrum Disorders (FASDs). Several independent laboratories have demonstrated robust alterations in the social behavior of rodents exposed to alcohol during brain development across a wide range of exposure durations, timing, doses, and ages at the time of behavioral quantification. Prior work from our laboratory has identified reliable alterations in specific forms of social interaction following moderate prenatal alcohol exposure (PAE) in the rat that persist well into adulthood, including increased wrestling and decreased investigation. These behavioral alterations have been useful in identifying neural circuits altered by moderate PAE1, and may hold importance for progressing toward a more complete understanding of the neural bases of PAE-related alterations in social behavior. This paper describes procedures for performing moderate PAE in which rat dams voluntarily consume ethanol or saccharin (control) throughout gestation, and measurement of social behaviors in adult offspring.

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To the Editor:

This letter accompanies a revised manuscript 52407 entitled “Moderate prenatal alcohol exposure and quantification of social behavior in adult rats” submitted for your consideration as an original protocol article and video in *JoVE*. The changes to the manuscript were tracked (in MS Word) with the filename 52407_R1_082514_R2.docx. We thank the reviewers and veterinarian for the comments and suggestion on improving the manuscript and video. Rebuttal comments are contained in a separate document as requested.

Thank you for considering our manuscript for publication in *JoVE*.

Sincerely,

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TITLE:
Moderate prenatal alcohol exposure and quantification of social behavior in adult rats

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RUNNING HEAD:
Moderate prenatal alcohol exposure and social behavior

KEYWORDS:
Aggression; Alcohol Teratogenesis; Alcohol-related Neurodevelopmental Disorders; ARND; Fetal Alcohol Spectrum Disorders; FASD; Fetal Alcohol Syndrome; FAS; Social interaction

SHORT ABSTRACT:
The goal of the protocol presented here is to describe procedures to expose rats to moderate levels of alcohol during prenatal brain development and to quantify resulting

alterations in social behavior during adulthood.

LONG ABSTRACT:
Alterations in social behavior are among the major negative consequences observed in children with Fetal Alcohol Spectrum Disorders (FASDs). Several independent laboratories have demonstrated robust alterations in the social behavior of rodents exposed to alcohol during brain development across a wide range of exposure durations, timing, doses, and ages at the time of behavioral quantification. Prior work from this laboratory has identified reliable alterations in specific forms of social interaction following moderate prenatal alcohol exposure (PAE) in the rat that persist well into adulthood, including increased wrestling and decreased investigation. These behavioral alterations have been useful in identifying neural circuits altered by moderate PAE[1], and may hold importance for progressing toward a more complete understanding of the neural bases of PAE-related alterations in social behavior. This paper describes procedures for performing moderate PAE in which rat dams voluntarily consume ethanol or saccharin (control) throughout gestation, and measurement of social behaviors in adult offspring.

INTRODUCTION:
An estimated 1%-5% of children are diagnosed with Fetal Alcohol Spectrum Disorders (FASDs)[2], which include Fetal Alcohol Syndrome (FAS), partial FAS (pFAS), and Alcohol-Related Neurodevelopmental Disorders (ARNDs)[3]. Deficits in social behavior and cognition are among the most common adverse outcomes observed in children with FASDs[4-7]. Negative consequences are not limited to heavy prenatal alcohol exposure (PAE), as moderate PAE that does not lead to the conspicuous morphological, behavioral and cognitive deficits characteristic of FAS can cause comparatively subtle, but nonetheless persistent, deficits in humans with FASDs[8-10] and non-human animals exposed to ethanol during brain development[11]. The importance of understanding the behavioral and corresponding neurobiological consequences of moderate PAE is underscored by current estimates indicating that the large majority of FASD cases fall within the less severe range of the spectrum[12].

Several independent laboratories have reported alterations in rodent social behavior related to ethanol exposure during brain development, including decreased investigation and interaction[1,13-15], altered play[14,16,17], increased aggressive interactions[17,18], alterations in responsiveness to social stimuli[19-21], and deficits in socially acquired food preferences and social recognition memory[22]. Social behavior deficits have been observed following exposure to heavy (blood ethanol concentrations (BECs) ~300mg/dl)[22,23] or more moderate levels of ethanol (BECs ~80mg/dl)[1], and across a broad range of parameters for other significant factors including exposure timing, duration of exposure, and age at the time of behavioral measurement.

Previous research has demonstrated that alterations in specific aspects of social interaction in adulthood discriminate rats exposed to moderate levels of alcohol from control animals exposed to saccharin[1,18]. In particular, moderate PAE has consistently been associated with robust increases in wrestling, which suggests increases in

aggressive behavior, and lower levels of social investigation (e.g., sniffing of the partner) in adulthood. Because alterations in social behavior are reliable consequences of PAE, the quantification of social behavior following PAE may hold importance for progressing toward a more complete understanding of the neural bases of PAE-related alterations in social behavior and the development of interventional approaches. The goal of this paper and the associated video is to provide instruction on the moderate PAE protocol and methods for quantification of social behavior in adult offspring that have reliably distinguished prenatal alcohol-exposed from non-exposed rat offspring.

PROTOCOL TEXT:

All procedures described here and in the accompanying video have been approved by the Institutional Animal Care and Use Committees of the Health Sciences Center and the main campus of the University of New Mexico.

1. Prenatal ethanol exposure

1.1) Obtain all required materials and chemicals : Saccharin sodium salt hydrate, 190 proof ethanol (95% alcohol by volume), beaded glass drinking tubes , natural rubber white #4 stopper one hole , 1" bend tubes-ball point, paper rulers printed from www.vendian.org/mncharity/dir3/paper_rulers/.

1.2) Obtain proven adult breeder rats from a vendor or alternative source. Use Long-Evans breeders for the methods and representative data presented in this protocol. Females weigh 125-150g and 6-7 weeks of age upon arrival such that they are approximately 9-10 weeks old at the time of breeding (step 1.5). Ensure that all prospective dams are first-time mothers. Make sure that Males are 12 weeks of age at arrival and 15 weeks old at the start of the breeding protocol.

1.3) House the animals individually in plastic cages at 22°C on a reverse 12h light/dark schedule (lights on from 2100 to 0900 hours). Provide access to food and tap water ad libitum throughout the study, including during all drinking sessions. Allow at least 1 week for acclimation to the facility before proceeding to step 1.4.

1.4) Obtain baseline body weights for each female rat.

1.5) Evaluate pre-pregnancy drinking levels in female rats for 2 weeks. Follow steps 1.5.1 – 1.5.6 in order.

1.5.1) On days 1 and 2 fill a drinking tube containing 0.066% saccharin solution (no ethanol) in tap water. To quantify consumption affix a paper ruler with mm precision to the drinking tube prior to filling it. Use this method as it reduces error in measurement associated with weighing tubes to quantify ethanol consumption. Fill the tube to the 20mm mark on the ruler. Begin four-hour drinking sessions (steps 1.5.1-1.5.6 and 1.7) 1 h after the onset of the dark phase (1000h) when activity and drinking levels are highest.

1.5.2) At the end of each drinking session quantify the volume of the saccharin solution that was consumed. To facilitate measurement, determine the volume of solution per mm on the ruler in advance and convert mm to volumes. For the tubes recommended here each mm corresponds to 0.366 ml.

1.5.3) On days 3 and 4 fill a tube with 2.5% ethanol (v/v) and 0.066% saccharin solution in tap water. At the end of each session quantify the amount of ethanol solution (weight per kg of body weight) that was consumed.

1.5.4) On day 5 and thereafter fill a tube with 5% ethanol (v/v) and 0.066% saccharin solution in tap water. At the end of each session quantify the amount of ethanol solution (weight per kg of body weight) that was consumed.

1.5.5) Upon completion of the pre-pregnancy drinking phase weigh the rats and calculate the mean ethanol consumption and standard deviation for the entire group. Remove rats for which mean consumption is greater than 1 standard deviation above or below the group mean from the study to reduce the variability in voluntary drinking during pregnancy.

1.5.6) Assign the remaining rats to either the saccharin control or PAE conditions such that the pre-pregnancy drinking levels are matched as closely as possible for the two groups.

1.6) Within 1-14 days pair each female rat with a proven male breeder. Female rats do not consume ethanol during the breeding phase. Confirm pregnancy by the presence of a vaginal plug, weigh the female rat, and house her individually. This is defined as gestational day 1. Optionally, leave the female rats with a male breeder for up to 5 days, after which remove the females from the study.

NOTE: Because ethanol consumption during pregnancy begins at 1000 hours on gestational day 1 inspection for the presence of a vaginal plug should be performed prior to this time.

1.7) Provide ethanol or saccharin solutions for 4 hours per day (1000 to 1400 hours) for the duration of pregnancy.

1.7.1) Beginning on gestational day 1 provide the female rat with either 0% or 5% ethanol and 0.066% saccharin solution in tap water based on the group assignment. Ensure that the volume of 0% ethanol solution provided to saccharin control rats is matched to the mean volume of 5% ethanol solution consumed by ethanol rats. At the end of each session quantify the amount of ethanol solution (weight per kg of body weight) or saccharin that was consumed.

1.7.2) Weigh rat dams weekly to assess maternal weight gain.

1.7.3) Provide food and tap water at all times including the drinking sessions.

1.7.4) Cease the ethanol exposure procedures when the offspring are born. Record the number of live pups and pup weights at birth. Designate the day of birth as postnatal day 0.

1.7.5) Cull the litter to 10 pups around postnatal day 2-3. Attempt to maintain an equal ratio of male to female animals in each litter. If this is not possible, cull to 10 pups with an unequal number of males and females.

1.7.6) Record pup weights 2-3 days after birth.

1.7.7) Wean the animals at approximately postnatal day 21-24 and house in same-sex pairs with an animal from the same prenatal treatment condition. Do not use more than 1-2 rats from each litter per experiment to limit potential litter effects.

2. Social behavior

2.1) Obtain and prepare all required materials and equipment.

2.1.1) Obtain an apparatus for social interaction. Use a chamber with an open top constructed of material that is easy to clean and sanitize. The front of the apparatus should be covered with rigid transparent plastic (~2mm thickness) for filming. Line the interior walls and floor with transparent plastic (~2mm thickness) to aid in cleaning and odor control. Place mirrors along the back interior wall to aid analysis.

NOTE : For the representative data reported here a custom chamber (95 cm long × 47 cm wide × 43 cm tall) with an open top and rigid transparent plastic (2mm thickness) covering the interior sides and floor of the apparatus was used. Mirrors were placed along the back wall. The specific dimensions and materials are not critical for measurement of social behavior, however, it is recommended that the dimensions be sufficiently large to ensure that genuine social interaction can be distinguished from other behaviors. That is, the apparatus should be large enough so that animals are not always in close proximity to one another.

2.1.2) Obtain video cameras capable of recording under low or no-light conditions as all filming is conducted with little or no ambient lighting within the visible spectrum. Ensure that the camera has a high resolution within the infrared spectrum, however, any camera capable of recording under dark conditions in its native night mode or with additional infrared illuminators should be sufficient.

2.1.3) Position infrared illuminators around the apparatus to improve lighting of the apparatus in the video recording.

2.1.4) Obtain laboratory grade wood chips (aspen chip).

2.1.5) Obtain a brush, dustpan, chlorine dioxide and isopropyl alcohol (70%) for cleaning the apparatus between sessions and odor control.

2.2) Apparatus acclimation and social behavior.
For this phase of experiment, make measurement in adult rats that are at least 90 days of age.

2.2.1) Prior to each session remove any woods chips, wipe the apparatus clean with isopropyl alcohol to control odors between sessions and provide fresh wood chips. Ensure that the wood chips entirely cover the bottom of the apparatus. Clean and sanitize the apparatus with an appropriate agent, such as chlorine dioxide, as necessary.

2.2.2) For three consecutive days place an animal and its cage-mate into the chamber for 30 minutes to acclimate the animals to the apparatus. During the acclimation sessions all room lights are turned off.

2.2.3) At the end of the third acclimation session house the animals individually in new cages with fresh bedding, food, and water for 24 hours to motivate social interaction.

2.2.4) Record social interaction on the following day 24 hours after the animals were separated.

2.2.4.1) Remove wood chips from the apparatus, clean and sanitize with chlorine dioxide, wipe with isopropyl alcohol to control odors, and replace the wood chips prior to the session.

2.2.4.2) Position one or more cameras to record the interaction. Position at least one camera in front of the apparatus so that the mirrors on the back wall of the apparatus can provide an additional perspective for analysis.

2.2.4.3) Retrieve animals one at a time and hold the animal in front of the camera so that the unique features of the fur pattern can be noted. These identifying features of the fur can be used to distinguish rats during analysis rather than artificially marking the animals.

NOTE: Because many aspects of rodent social interaction involve olfactory signals and smelling the partner, wherever possible introducing foreign odors should be avoided. Long Evans rats typically have some feature of the fur pattern that can be utilized to distinguish any given pair of animals. For other strains (e.g., Sprague-Dawley rats) alternative approaches such as marking the tail with an unscented dye could be used. It is important to recognize that many behaviors of interest are directed toward specific targets (e.g., anogenital sniffing directed near the base of the tail, playful attacks directed at the nape of the neck, or aggressive attacks directed toward the flanks or belly). Marking the animals closer to the tip of the tail far from these targets of interest is recommended.

2.2.4.4) Record video of the social interaction for at least 12 minutes.

2.2.4.5) Monitor animals for fighting throughout the session. If possible, watch the animals via a monitor or window so that the experimenter is not in the room during the session.

NOTE: Fighting has been only rarely observed in studies with adult rats, however, animals should be monitored throughout the session. The session should be ceased if there is excessive fighting or there are signs of harm or injury to an animal.

2.3) Behavioral coding and analysis.

2.3.1) Identify the following behaviors of interest as per previous work with PAE in [1,18]. Quantify the duration, frequency and latency to first occurrence of the following behaviors; wrestling (including pinning), boxing, crawling (crossing) over/under the partner, anogenital sniffing, other sniffing of the partner's body (body sniffing), allogrooming (grooming of the partner), rearing, and sniffing/digging in the wood chips. Examples of each behavior are illustrated in the video component of this article.

2.3.2) Quantify the social behaviors of interest from the video. Obtain the frequency, total duration and latency to first instance for each behavior of interest.

NOTE: Obtaining these measures can be achieved manually, however, quantification of these measures using computerized analyses of digitized video is recommended. A Matlab (www.mathworks.com) script for playback of the video and quantification of behaviors is provided as a supplement to this article. Software packages capable of registering codes for observed events/behaviors and maintaining the relative timing and duration of these events would be adequate for the types of quantification described here.

2.3.3) After coding is completed for all rats the resulting duration, frequency and latency data are analyzed with a statistical package.

REPRESENTATIVE RESULTS:

Over the course of many breeding rounds female rats in the ethanol condition consistently drink an average of about 2.1g/kg of ethanol per four-hour drinking session. Rat dams consume approximately one-half of the four-hour total during the first 15 to 30 minutes after the introduction of the drinking tubes, resulting in a peak maternal serum ethanol concentration of about 60 mg/dl, measured at the 45 minute time point. Over the remaining 3.5 hours of the drinking period, they continue to consume 5% ethanol at a lower, but relatively stable rate of 0.4 g/kg body weight/hour. This level and pattern of voluntary ethanol consumption has no significant effects on maternal weight gain, offspring birth weight, litter size, maternal care, placental wet weight, offspring weight at behavioral testing, or whole brain, hippocampal or cerebellar wet weights.

Representative means and SEMs from male saccharin- and ethanol-exposed rats for each behavioral measure are shown in Table 1. These data were pooled from prior

experiments and include 16 males for each prenatal treatment condition. All animals were paired with partners from the same prenatal treatment condition. Robust alcohol-related alterations in the social behavior of female animals have not been observed in our studies[1], however, alcohol-related differences in female social behavior have been documented using other procedures[15,23]. Separate univariate analyses of variance (ANOVAs) performed in SPSS ver. 21 for Macintosh revealed that male ethanol-exposed rats had significantly higher duration [$F(1, 30) = 19.12$] and frequency [$F(1, 30) = 6.80$] of wrestling and decreased latency to the first instance of wrestling [$F(1, 30) = 9.41$]. Ethanol-exposed rats also spent less time engaged in anogenital sniffing [$F(1, 30) = 5.17$].

[PLACE TABLE 1 HERE]

In addition to performing ANOVAs, performing a linear discriminant analysis to evaluate which variables best discriminate ethanol-exposed from saccharin-exposed animals is recommended[18]. For the present sample, the Box M's test to test for equal variances could not be calculated because the number of independent variables was greater than the number of cases (a 5:1 ratio is typically recommended). The discriminant function revealed a significant association between groups and predictors, accounting for 74% of between group variability. An analysis of the structure matrix revealed that duration of wrestling (.470), and latency to first occurrence of wrestling (-.330) were significant predictors. Counts (frequency) of wrestling (.280) and duration of anogenital sniffing (-.244) were slightly weaker predictors. The cross-validated classification showed that overall, 71.9% of cases were correctly classified.

TABLE LEGENDS:

Table 1. Mean (±SEM) frequency (1a), duration (1b) and latency to first occurrence (1c) for each behavior quantified during the social interaction session for saccharin- (SAC) and prenatal alcohol-exposed (PAE) rats (n=16 per prenatal treatment group). [* $p < 0.05$, ** $p < 0.005$]

DISCUSSION:

The prenatal alcohol exposure paradigm described here involves voluntary consumption of ethanol (5% v/v) by rat dams during pregnancy. There are a number of protocols for exposing non-human animals to ethanol during brain development represented in the literature, which differ with respect to the timing, dose, duration and route of ethanol administration as well as the species under investigation. Although a thorough treatment of the advantages of various exposure protocols is not provided here, several advantages of the voluntary drinking method for PAE described in this protocol are highlighted. Previously we utilized a liquid diet protocol, an approach commonly employed in this field of research, in which rat dams consume 5% ethanol as part of the primary food source[24]. Control conditions for this approach include a pair-fed group in which the caloric intake was yoked to that of ethanol-consuming dams and a group that has *ad libitum* access to chow. In the voluntary drinking paradigm described here rat dams in both groups (ethanol and saccharin) consume the same rat chow diet which reduces between group variability in nutrition and caloric intake, and minimizes the potential confound of stress associated with forced consumption of an unfamiliar food source[25]. This feature of the voluntary drinking paradigm also eliminates the need for a pair-fed control as with the liquid diet approach, which provides some practical and

ethical benefits, including reductions in the number of experimental groups (from three to two), the number of animals used in the research, and the associated costs to perform the research. The four-hour intermittent exposure pattern of voluntary ethanol consumption yields less variable drinking levels than observed with the 5% ethanol liquid diet protocol, which might reasonably be expected to similarly diminish variability in outcome measures observed in PAE offspring. Because blood ethanol concentrations achieved with any protocol are important to quantify and communicate, measurements of maternal serum ethanol concentrations should be conducted periodically (e.g., annually) to ensure that comparable BACs are being achieved across breeding rounds. Due to the potential for interactions between prenatal stress and ethanol exposure these measures should be performed in a separate cohort of females for which the offspring are not used in subsequent studies (see ref. [26]). It is important to recognize that peak serum ethanol concentrations will occur approximately 45-60 minutes after the drinking tube is introduced. In lieu of BACs from the dams producing experimental offspring, the most useful measure to correlate with outcome measures is a rat dam's daily ethanol consumption during pregnancy. However, restricting the range of drinking in rat dams to one standard deviation of the mean for the group would likely constrain any meaningful correlation between ethanol consumed and a given outcome measure. Finally, evaluation of pre-pregnancy drinking is utilized to identify rats that drink at desired levels for subsequent phases of the drinking protocol. This aspect of the ethanol exposure paradigm also ensures that all female rats have experience drinking prior to pregnancy, which more accurately models human behavior in that drinking is unlikely to begin during pregnancy.

Several additional methodological issues and caveats related to the alcohol exposure paradigm should be considered. The voluntary drinking PAE paradigm described here occurs throughout gestational development, which in the rat corresponds roughly to the first two trimesters of gestational development in humans. Exposure during early postnatal development is utilized by many laboratories to model third trimester human equivalence (see e.g., ref. [27]). Further, we note that the procedures presented here represent a chronic exposure protocol (rat dams drink every day). Importantly, precisely timed exposure to higher levels of alcohol (~287mg/dl) limited to gestational day 15 has also been shown to alter social behavior[15]. The paradigm presented here involves voluntary alcohol consumption by rat dams, therefore, there is a limited range of blood ethanol concentrations that can be achieved with this approach. Achieving higher blood ethanol concentrations requires other methods of exposure (e.g., gavage, injection, vapor exposure). An additional point of importance concerns potential for alterations in maternal care that could complicate interpretation of behavioral effects associated with prenatal ethanol exposure. To address this, assessments of maternal care during pregnancy should be evaluated periodically. No effects of moderate drinking during pregnancy on maternal care have been detected using the procedures described here in the Long-Evans rat[28]. Evaluation of whether alterations in behavioral indicators of maternal care (see ref. [29]), however, should be evaluated initially and periodically thereafter, particularly if deviations from the methods described here, including species and strain of animal, or higher concentrations of ethanol are utilized.

The behavioral procedures described here have yielded reliable alterations in specific aspects of social behavior (wrestling and investigation) in adult male rats exposed to moderate levels of alcohol during prenatal brain development[1,18,30]. The behaviors quantified here were selected based on a large body of extant literature[31] to target partner-directed behaviors (e.g., wrestling, investigation) and other behaviors directed toward the environment (e.g., rearing, digging) that can be easily measured by way of video analysis. Discriminant analyses revealed that increases in wrestling provide the best discrimination between alcohol-exposed and saccharin-exposed rats among a broad range of social and non-social behavioral variables[18]. It is important to point out that effects of PAE on social behavior in female rats have not been observed using the methods described here[1], although main effects of sex have been reported for several dependent measures including anogenital sniffing (female > male), body sniffing (male > female), wrestling (male > female), and boxing (male > female).

Although wrestling has been shown to be the primary aspect of social behavior altered by moderate PAE, the inclusion of other behaviors is important for establishing the selectivity of the behavioral effects and ruling out generalized behavioral deficits. The set of behaviors quantified here is by no means exhaustive. Behaviors of interest should be selected during preliminary work to capture the overall pattern of effects observed in a given set of data. This is particularly important if different alcohol exposure paradigms, or parameters, are utilized as variations in procedures could reasonably be expected to yield different behavioral outcomes. In addition to the behaviors described here, initially evaluating a broader set of behaviors including biting, scratching (self), full grooming sequences, truncated grooming sequences (indicative of anxiety), “lateral” display[32,33], body shakes, chasing, and play behavior[34-37] is recommended. Observation in 6-12 pairs of animals should be sufficient to identify behaviors that distinguish alcohol-exposed from non-exposed animals.

It is also important to consider that wrestling, depending upon the age at the time of measurement, could reflect genuine aggressive behavior or play behavior. Early lesions of the ventrolateral frontal cortex in rats, which has been linked to moderate PAE effects on social behavior[1,18,30], result in increased play behavior[38]. In the rat, play behavior peaks during post-weaning development around postnatal days 30-40 [33,34] and declines as animals approach adulthood. Alcohol exposure during brain development alters play behavior when measured prior to adulthood[6,16,23] and could affect the rate at which play behavior decreases with age. Because the topographies of play and aggressive behaviors are similar a clear distinction can be difficult to achieve. In previous studies, conspicuous behavioral indicators of aggression, such as fighting or biting, have not been observed in moderate PAE rats. However, additional behavioral indicators can provide clues regarding the classification of these behaviors[18]. For example, attacks directed at the nape of the neck, the primary target of playful attacks, rarely occur in adult PAE rats[18]. In contrast, attacks directed toward the rump, a target of non-playful attacks[39] were observed more frequently, suggesting that PAE-related increases in wrestling reflect aggression rather than play. Play behavior should be included in the analysis if social behavior is measured prior to adulthood or play behavior is

conspicuous in the behavior of adult animals. Detailed methods for the analysis of play behavior are described by Himmler et al[35].

Finally, it is important to note that social behavior using the methods described here can be influenced by the selection of the partner animal. In the representative results presented here animals were paired with familiar animals (cage-mates) from the same prenatal treatment condition[1,18]. The rationale for this selection was based largely on data demonstrating that housing control animals with ethanol-exposed rats alters social behavior in control rats[14], as well as similar and reliable unpublished observations from our laboratory. These effects can potentially complicate identification of and interpretation of PAE-related alterations in social behavior. The methods described here for quantification of social behavior could, however, be applied to any variation of the source of the social partner, of which there are several possibilities including using a non-treated partner that comes from neither treatment condition[1], using a partner animal from the same treatment condition (as described here), or a partner animal from a different treatment condition. Further, the familiarity of the partner animal can be manipulated to affect social interaction[1]. The selection of the partner condition and other variables related to social housing, sex, and exposure paradigm can be tailored to best meet the scientific goals of the particular laboratory while still utilizing the basic procedures for quantification of social behavior described here.

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The authors have no conflicts of interest to disclose.

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Table 1. Mean (±SEM) frequency (1a), duration (1b) and latency to first occurrence (1c) for each behavior quantified during the social interaction session for saccharin- (SAC) and prenatal alcohol-exposed (PAE) rats (n=16 per prenatal treatment group). [* $p < 0.05$, ** $p < 0.005$]

1a. Frequency	***SAC***	***PAE***
Wrestling *	2.00 (0.58)	8.00 (2.23)
Boxing	1.81 (0.80)	3.56 (1.47)
Cross over/under	1.06 (0.48)	1.00 (0.29)
Anogenital sniffing	6.25 (1.20)	3.75 (0.73)
Body Sniffing	19.75 (1.64)	18.56 (2.06)
Allogrooming	2.31 (0.93)	0.75 (0.27)
Rearing	56.50 (5.39)	56.06 (5.40)
Dig/Sniff Bedding	32.06 (6.03)	30.06 (5.27)

1b. Duration (sec)	***SAC***	***PAE***
Wrestling **	9.14 (2.31)	39.81 (6.62)
Boxing	2.55 (1.43)	3.81 (1.62)
Cross over/under	0.83 (0.39)	1.03 (0.27)
Anogenital sniffing*	11.21 (2.10)	5.69 (1.22)
Body Sniffing	27.21 (2.33)	27.09 (3.73)
Allogrooming	13.50 (5.68)	3.82 (1.79)
Rearing	120.31 (13.32)	121.48 (12.13)
Dig/Sniff Bedding	119.59 (24.45)	109.15 (21.41)

1c. Latency (sec)	***SAC***	***PAE***
Wrestling **	430.75 (50.51)	209.98 (51.25)
Boxing	569.52 (48.14)	525.63 (74.75)
Cross over/under	544.4 (65.21)	429.01 (75.78)
Anogenital sniffing	107.68 (39.35)	164.31 (44.09)
Body Sniffing	22.77 (6.14)	16.80 (3.21)
Allogrooming	471.44 (70.82)	588.52 (48.47)
Rearing	20.92 (7.65)	11.94 (1.20)
Dig/Sniff Bedding	76.78 (25.78)	117.66 (44.64)

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Saccharin sodium salt hydrate	Sigma	S1002	
190 proof ethanol	Sigma	493538	
Beaded glass drinking tubes	Fisher	14-955K	
Natural rubber white #4 stopper one hole	Plasticoid	LSG4M181	
1" bend tubes-ball point	Ancare	TD-199-3"	
Paper rulers	N/A	N/A	www.vendian.org/mncharity/dir3/paper_rulers
Apparatus for social interaction	Custom built	N/A	95 cm X 47 cm X 43 cm
Video cameras	N/A	N/A	Capable of recording low/no light conditions
Infrared illuminators	Vitek	VT-IR1-12	
Teklad laboratory grade sani-chips	Harlan	7090A	
Brush and dustpan	N/A	N/A	
Isopropyl alcohol	Sigma	W292907	
Chlorine Dioxide (1.5 mg Tablets)	Quiplabs	N/A	Prepare per manufacturer's recommendation

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Vet Review Comments:

1. The video shows the custom made box which was fabricated without adherence to the building material standard of the Guide. Wood is not an acceptable building material according to the 8th edition of the Guide.

The custom box depicted in the revised video is constructed of materials that adhere to the 8th edition of the guide (see Video 4:58-5:21).

2. The video demonstrates the insufficient cleaning of the custom box with an inappropriate cleaning agent. A 70% solution of alcohol is not an acceptable cleaning solution to assure disinfection of the box containing rats and aspen chip bedding. Documenting the cleaning procedure in this manner will lower the acceptable standard of sanitation. I do not believe that JOVe would like to provide the citation for lowering the sanitation standard.

The function of wiping the apparatus with 70% isopropanol is to control odors between sessions. Reference to “cleaning” the apparatus with isopropanol have been removed from the video and text and replaced with references to the odor control function of this step. Because the frequency and agent used for sanitization of the apparatus could vary based on a number of factors that are not related to the methods described in this protocol (e.g., heterogeneity in the microbiological status of the animals under study) we have directed the reader to clean and sanitize the apparatus with an appropriate agent as needed.

Editorial Comment: Regarding the following comments from this reviewer pertaining to data, please note that only representative results are required for publication in JoVE. However please ensure that any claims presented are fully substantiated in the video by data or references to published works. While we do not require a hypothesis statement for JoVE videos, please take this opportunity to ensure that the goal of the protocol is properly established in the video.

1. A hypothesis statement is not given in this research paper.
The goal of the protocol is to describe prenatal ethanol exposure methods and methods for quantification of social behaviors. These goals can be achieved without an explicit hypothesis statement.

2. Data is not demonstrated in in this video.
Data were presented in the original video. Representative results for the critical groups are shown from 9:47 to 10:20.

3. At 7:12 in this video there is an unsubstantiated claim that two groups could be distinguished using behavior testing, but data is not given.

This claim is not unsubstantiated. In addition to the representative results

depicted in the video at 9:47 to 10:20, we reference published articles using these methods that support this claim in the manuscript (see e.g., ref 1).

Reviewers' comments:

Reviewer #1:

1) Lines 174-175. Should this read "Follow steps 1.5.1-1.5.6…"?
This error has been fixed.

2) Line 209: How long does the breeding phase typically last? Is there a cut-off if a female does not become pregnant within 4-5 days (1 cycle)? What is the interval between the pre-pregnancy and pregnancy phases? It appears that the pregnancy phase begins immediately after the pre-pregnancy phase, but please note this specifically.

A female rat is placed with a proven male breeder for up to five days. She is removed on the morning that a vaginal plug is observed in the bedding below the hanging cage. If no plug is observed after five days, the female is removed from the study.

The period during which breeding is conducted is dependent on the number of male breeders on hand and the number of female rats to breed. We typically maintain about one male for every three females to be bred. The entire process normally takes up to two weeks to complete. Thus, the interval between the end of pre-pregnancy drinking and the start of pregnancy drinking can range from one day up to two weeks. During this time, females are not consuming 5% ethanol.

These aspects of the method have been included in section 1.6.

3) Lines 222-223: There is no explicit mention that the volume of alcohol/saccharin that the dams are drinking during the pregnancy phase should be recorded. This information is included in the video and is specifically mentioned in the pre-pregnancy drinking phase section, but should be added here for clarity.

We thank reviewer 1 for pointing out this omission. We have added this to section 1.7.1.

4) Line 232: While culling to an even sex ratio (5 males:5 females) is implied from the diagrams in the video, it is not mentioned explicitly in the manuscript. It would be helpful to indicate whether or not the male:female sex ratio is considered as part of the experimental methodology.

We sex the rat pups when we cull a litter to ten and we attempt to have five pups of each sex. However, on occasion when there is a disproportionate number of one sex, we will keep all of the sex with the smaller numbers represented, but still cull the litter to ten with the other sex.

We have modified section 1.7.5 in response to this suggestion.

5) Line 243. Instructions are to use a custom built chamber exactly as that used by these investigators. Should there be a comment that a similar chamber would be acceptable as long as it meets the basic requirements (type of floor and walls, open front, etc) or must it be exactly this chamber?

It is not necessary for the materials or dimensions of the custom chamber to match. We have simply described what we use in our laboratory. We do recommend that the dimensions provide sufficient space to identify genuine social interaction. That is, it should be large enough that the animals are not always in close proximity to one another. We have included a "NOTE" to this effect in section 2.1.1.

6) Line 271. A more general issue:
In this paradigm, experimental animals are interacting with same-condition cage-mates. It would probably be useful to have a brief discussion about this choice. Many social behavior studies use a non-treated social stimulus animal as the partner for testing. Having a cage-mate (same prenatal condition) vs a novel social stimulus animal (typically a non-treated control but could be from the same prenatal condition) as the partner likely makes a significant difference for the interactions that occur and therefore in the behaviors observed in the experimental animal. Some discussion here would be very helpful for an investigator just getting into the social behavior area and trying to make decisions about testing conditions, and in fitting the data from this paradigm into the broader social behavior literature.

This is an excellent point and we have included several sentences addressing this issue in the revised Discussion section (lines 536 – 552).

Finally, it is important to note that social behavior using the methods described here can be influenced by the selection of the partner animal. In the representative results presented here animals were paired with familiar animals (cage-mates) from the same prenatal treatment condition[1,18]. The rationale for this selection was based largely on data demonstrating that housing control animals with ethanol-exposed rats alters social behavior in control rats[14], as well as similarly reliable unpublished observations from our laboratory. These effects can potentially complicate identification of and interpretation of PAE-related

alterations in social behavior. The methods described here for quantification of social behavior could, however, be applied to any variation on the source of the social partner, of which there are several possibilities including using a non-treated partner that comes from neither diet condition[1], using a partner animal from the same diet condition (as described here), or a partner animal from a different diet condition. Further, the familiarity of the partner animal can be manipulated to affect social interaction[1]. The selection of the partner condition and other variables related to social housing, sex, and exposure paradigm can be tailored to best meet the scientific goals of the particular laboratory while still utilizing the basic procedures for quantification of social behavior described here.

We have also added the following sentence in the representative results section on lines 396-397 to make it clear which partner condition is represented in the representative results.

All animals were paired with partners from the same prenatal treatment condition.

7) Line 303: How long is the social interaction session? Based on the data presented in Table 1, it appears that the session lasts for 5 minutes. This information is important for interpreting your representative results. Moreover, it would be useful to know the rationale for session length (i.e. is session length a critical factor for observing group differences?).

Approximately 12 minutes is recommended as a session duration. This is included as section 2.2.4.4. A 12 minute session was utilized for the representative results.

8) Line 324. Would it be useful to mention other possible programs to use for playback of the video and quantification of behaviors, and possible pros and cons of Matlab vs other programs (eg., Noldus, Anymaze, etc)?

Although other approaches to analysis can be used, including alternative software packages, it was our understanding that specific products and their associated trade names could not be mentioned in the article or video. Thus, we are hopeful that our general “NOTE” added in section 2.3.2 will help alert researchers to the possibility of alternative approaches and the important functions to consider when identifying a software package for analysis.

The added note reads as follows :
NOTE : Software packages capable of registering codes for observed events/behaviors and maintaining the relative timing and duration of these events would be adequate for the types of quantification described here.

10) Line 350: Are all of these results in male animals? Based on the previous sentence ("Robust alcohol-related alterations in the social behavior of female animals have not been observed .. by us…"), it would seem so. However, it should be explicitly stated here.

Yes, the representative results reported here are from male animals. We have stated this explicitly in the representative results of the revised manuscript.
(See also our response to reviewer 2's point 6).

Video Comments:
11) ~3:30: It might be nice to have an animation showing rat dams being assigned to either the EtOH or saccharin groups; this will make it clear that all dams have had EtOH experience prior to pregnancy.

We have modified the video to include a segment depicting assignment to drinking conditions. It begins at ~3:31.

Minor:
12) Lines 137-138: Consider revising "compared to" to "from"
We have made this modification

13) Line 182: It might be helpful to remind the reader that "1 h after the onset of the dark phase" corresponds to "(1000 hours)"
We have included this reminder.

14) Lines 222-223: Sentence is missing the word "is" before the word "matched"
This has been corrected.

15) Line 348: Delete the word "in" after the word "observed"
This error has been corrected.

Reviewer #2:

1. While the authors state that pre-pregnancy drinking should be at least two weeks, they need to provide a reasonable range (say, 2-4 weeks?).

The two-week interval of pre-pregnancy drinking is sufficient to obtain an accurate measure of voluntary drinking. We have modified the text in section 1.5, removing "at least" to specify that 2 weeks of pre-pregnancy drinking is needed to measure drinking levels.

2. Should the dams be first time mothers?

Yes, dams should be first time mothers. This is now stated in section 1.2 of the revised manuscript. Although it would seem that reusing rat dams as breeders would be both a practical and cost-effective approach, our experience with this paradigm over the past eight years indicates that voluntary drinking behavior from "first time moms" is the most consistent.

3. Are the male and female breeders kept together for multiple days in a row? This method does not seem as accurate for knowing GD0 as placing them together for one night only.

Females are maintained with proven male breeders for up to five days and evidence of a vaginal plug used to confirm pregnancy and Gestational Day 1.

4. Why are weaned pups housed with animals from the same condition and not counter-balanced?

It is important to note that housing with same condition partners the approach we favor, but we have included a discussion of alternatives in the revised Discussion (final paragraph lines 536-552) to emphasize that the particular housing preparation can be selected by the investigator. Based on our experience and other published data, it is important to recognize that the particular housing configurations may negatively affect behavior in control animals (e.g., if paired with PAE rats), which may complicate interpretation. See also our response to reviewer 1's point 6.

5. Do the mirrored tiles in the cage increase anxious behaviors in the rats since it might look like there are more stranger rats than there really are?

All video recording is performed in the dark, so it is unlikely that the rats are able to see reflected light from the mirrors. Although we have not systematically compared behavior with and without the mirrors in the back of the apparatus, our early work was conducted with no mirrors and in our experience the presence of the mirrors does not appear to affect behavior.

6. Male rats were used in the experiments presented in the paper. Obviously, sex could be an important factor in the behavioral experiments. Are there any sex-specific behaviors or prevalence of certain behaviors between males and females?

There are effects that discriminate male and female animals, however, we have not observed effects of moderate PAE on social behaviors in female rats. We have added the following sentence to the Discussion section (lines 496 – 500).

It is important to point out that effects of PAE on social behavior in female rats have not been observed using the methods described here[1], although main

effects of sex have been reported for several dependent measures including anogenital sniffing (female > male), body sniffing (male > female), wrestling (male > female), and boxing (male > female).

7. What is the rationale for isolating the rats for 24 hours before the social interaction? Is it to increase social interaction?

Yes, the function of the isolation period is to motivate social interaction. This is stated in section 2.2.3 of the revised manuscript.

8. Monitoring BACs after alcohol consumption is know to be useful in interpreting variation of the behavioral (as well as neuroanatomical) outcomes. BACs were not measured in these experiments, and the only mentioning about BACs is in the Discussion (lines 394-397): "Because blood ethanol concentrations achieved with any protocol are important to quantify and communicate, measurements of maternal serum ethanol concentrations should be conducted periodically (e.g.,annually???)". The authors should clarify how they recommend to monitor BACs.

Collecting blood from rat dams is stressful and, therefore, a confound in prenatal ethanol exposure paradigms. For this reason, periodic measures of maternal serum ethanol concentrations must be conducted in a separate set of rat dams. We typically run a separate set of rat dams about once a year to confirm maternal serum ethanol concentrations. In the revised manuscript we indicate that assessment of BACs should be conducted at least annually in a separate set of rat dams for which the offspring are not used. It is also important to note that the peak ethanol concentration will come about 45 minutes to an hour after the introduction of the drinking tube.

In lieu of BACs from the dams producing experimental offspring, the most useful measure to correlate with outcome measures is a rat dam's daily ethanol consumption during pregnancy. However, limiting the range of rat dams used in a study to those whose voluntary drinking is within one standard deviation of the mean for the group will usually constrain any meaningful correlation between ethanol consumed and a given outcome measure.
We have included the following sentences on this issue in the revised Discussion on lines 446 – 458.

Because blood ethanol concentrations achieved with any protocol are important to quantify and communicate, measurements of maternal serum ethanol concentrations should be conducted periodically (e.g., annually) to ensure that comparable BACs are being achieved across breeding rounds. Due to the potential for interactions between prenatal stress and ethanol exposure these measures should be performed in a separate cohort of

females for which the offspring are not used in subsequent studies (see ref. [26]). It is important to recognize that peak serum ethanol concentrations will occur approximately 45-60 minutes after the drinking tube is introduced. In lieu of BACs from the dams producing experimental offspring, the most useful measure to correlate with outcome measures is a rat dam's daily ethanol consumption during pregnancy. However, restricting the range of drinking in rat dams to one standard deviation of the mean for the group would likely constrain any meaningful correlation between ethanol consumed and a given outcome measure.

9. Pups' weight should be taken on PD2-3. Should the number of pups/litter be recorded as well?

The number of live pups is recorded along with the litter birth weight to provide a mean pup weight for the litter. This is stated in section 1.7.4 of the revised manuscript.

Supplemental : Instructions for Matlab code

Click here to download Supplemental File (as requested by JoVE): Social Coding AnalysisREADME.docx

Supplemental code file (if applicable)

Click here to download Supplemental code file (if applicable): SocialCodingAnalysis.m

Supplemental code file (if applicable)

Click here to download Supplemental code file (if applicable): SocialCodingAnalysis.fig