Video Article

Moderate Prenatal Alcohol Exposure and Quantification of Social Behavior in Adult Rats

Derek A. Hamilton[1,2], Christy M. Magcalas[1], Daniel Barto[1], Clark W. Bird[1], Carlos I. Rodriguez[1], Brandi C. Fink[3], Sergio M. Pellis[4], Suzy Davies[2], Daniel D. Savage[1,2]

[1]Department of Psychology, University of New Mexico

[2]Department of Neurosciences, University of New Mexico

[3]Department of Psychiatry and Behavioral Sciences, University of New Mexico

[4]Canadian Centre for Behavioural Neuroscience, University of Lethbridge

Correspondence to: Derek A. Hamilton at dahamilt@unm.edu

URL: https://www.jove.com/video/52407
DOI: doi:10.3791/52407

Keywords: Neuroscience, Issue 94, Aggression, Alcohol Teratogenesis, Alcohol-related Neurodevelopmental Disorders, ARND, Fetal Alcohol Spectrum Disorders, FASD, Fetal Alcohol Syndrome, FAS, Social interaction

Date Published: 12/14/2014

Citation: Hamilton, D.A., Magcalas, C.M., Barto, D., Bird, C.W., Rodriguez, C.I., Fink, B.C., Pellis, S.M., Davies, S., Savage, D.D. Moderate Prenatal Alcohol Exposure and Quantification of Social Behavior in Adult Rats. *J. Vis. Exp.* (94), e52407, doi:10.3791/52407 (2014).

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.

Video Link

The video component of this article can be found at https://www.jove.com/video/52407/

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

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. 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/.
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 125-150 g 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.
3. House the animals individually in plastic cages at 22 °C on a reverse 12 hr light/dark schedule (lights on from 21:00 to 09:00 hr). 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.
4. Obtain baseline body weights for each female rat.
5. Evaluate pre-pregnancy drinking levels in female rats for 2 weeks. Follow steps 1.5.1–1.5.6 in order.
    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 20 mm mark on the ruler. Begin four-hour drinking sessions (steps 1.5.1-1.5.6 and 1.7) 1 hr after the onset of the dark phase (10:00 hr) when activity and drinking levels are highest.
    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.
    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.
    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.
    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.
    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.
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 10:00 hr on gestational day 1 inspection for the presence of a vaginal plug should be performed prior to this time.
7. Provide ethanol or saccharin solutions for 4 hr per day (1,000 to 1,400 hr) for the duration of pregnancy.
    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.
    2. Weigh rat dams weekly to assess maternal weight gain.
    3. Provide food and tap water at all times including the drinking sessions.
    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.
    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.
    6. Record pup weights 2-3 days after birth.
    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

1. Obtain and prepare all required materials and equipment.
    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 (~2 mm thickness) for filming. Line the interior walls and floor with transparent plastic (~2 mm 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 (2 mm 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. 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.
3. Position infrared illuminators around the apparatus to improve lighting of the apparatus in the video recording.
4. Obtain laboratory grade wood chips (aspen chip).
5. Obtain a brush, dustpan, chlorine dioxide and isopropyl alcohol (70%) for cleaning the apparatus between sessions and odor control.

2. Apparatus acclimation and social behavior
    1. For this phase of the experiment, make measurements in adult rats that are at least 90 days of age.
    2. 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.
    3. 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.
    4. At the end of the third acclimation session house the animals individually in new cages with fresh bedding, food, and water for 24 hr to motivate social interaction.
    5. Record social interaction on the following day 24 hr after the animals were separated.
        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. 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.
        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.
        4. Record video of the social interaction for at least 12 min.
        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.

3. Behavioral coding and analysis.
    1. Identify the following behaviors of interest as per previous work with PAE[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. 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.
    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 2.1 g/kg of ethanol per 4 hr drinking session. Rat dams consume approximately one-half of the four hr total during the first 15 to 30 min after the introduction of the drinking tubes, resulting in a peak maternal serum ethanol concentration of about 60 mg/dl, measured at the 45 min time point. Over the remaining 3.5 hr 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 using these procedures[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 [$F(1, 30) = 5.17$].

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)

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 =1 6 per prenatal treatment group). [* p <0.05, ** p <0.005]

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 (0.470), and latency to first occurrence of wrestling (-0.330) were significant predictors. Counts (frequency) of wrestling (0.280) and duration of anogenital sniffing (-0.244) were slightly weaker predictors. The cross-validated classification showed that overall, 71.9% of cases were correctly classified.

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 4 hr 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 min 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 within 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 (~287 mg/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.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgements

Support provided by grant AA019462 to DAH and AA019884 to DDS.

References

1. Hamilton, D. A. *et al.* 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. *Behav. Brain Res.* **207** (2), 290-304, doi:10.1016/j.bbr.2009.10.012 (2010).
2. May, P. A. *et al.* Approaching the prevalence of the full spectrum of Fetal Alcohol Spectrum Disorders in a South African population-based study. *Alcohol. Clin. Exp. Res.* **37** (5), 818-830, doi:10.1111/acer.12033 (2013).
3. Chasnoff, I. J., Wells, A. M., Telford, E., Schmidt, C., & Messer, G. Neurodevelopmental functioning in children with FAS, pFAS, and ARND. *Journal of Developmental and Behavioral Pediatrics.* **31** (3), 192-201, doi:10.1097/DBP.0b013e3181d5a4e2 (2010).
4. Disney, E. R., Iacono, W., McGue, M., Tully, E., & Legrand, L. Strengthening the case: Prenatal alcohol exposure is associated with increased risk for conduct disorder. *Pediatrics.* **122** (6), E1225-E1230, doi:10.1542/peds.2008-1380 (2008).
5. Kelly, S. J., Goodlett, C. R., & Hannigan, J. H. Animal models of fetal alcohol spectrum disorders: Impact of the social environment. *Dev Disabil Res Rev.* **15** (3), 200-208, doi:10.1002/ddrr.69 (2009).
6. Kelly, S. J., Day, N., & Streissguth, A. P. Effects of prenatal alcohol exposure on social behavior in humans and other species. *Neurotoxicol. Teratol.* **22** (2), 143-149 (2000).
7. Thomas, S. E., Kelly, S. J., Mattson, S. N., & Riley, E. P. Comparison of social abilities of children with fetal alcohol syndrome to those of children with similar IQ scores and normal controls. *Alcoholism: Clinical and Experimental Research* . **22** (2), 528-533 (1998).
8. Conry, J. Neuropsychological deficits in Fetal Alcohol Syndrome and fetal alcohol effects. *Alcohol. Clin. Exp. Res.* . **14** (5), 650-655 (1990).
9. Streissguth, A. P. *et al.* Fetal Alcohol Syndrome in adolescents and adults. *JAMA-Journal of the American Medical Association.* **265** (15), 1961-1967 (1991).
10. Streissguth, A. P., Barr, H. M., & Sampson, P. D. Moderate prenatal alcohol exposure: effects on child IQ and learning problems at age 7 1/2 years. *Alcoholism: Clinical and Experimental Research* . **14** (5), 662-669 (1990).
11. Valenzuela, C. F., Morton, R. A., Diaz, M. R., & Topper, L. Does moderate drinking harm the fetal brain? Insights from animal models. *Trends Neurosci.* **35** (5), 284-292, doi:10.1016/j.tins.2012.01.006 (2012).
12. May, P. A. *et al.* Prevalence of children with severe Fetal Alcohol Spectrum Disorders in communities near Rome, Italy: New estimated rates are higher than previous estimates. *Int. J. Env. Res. Public Health.* **8** (6), 2331-2351, doi:10.3390/ijerph8062331 (2011).
13. Tunc-Ozcan, E., Ullmann, T. M., Shukla, P. K., & Redei, E. E. Low-dose thyroxine attenuates autism-associated adverse affects of fetal alcohol in male offspring's social behavior and hippocampal gene expression. *Alcohol. Clin. Exp. Res.* **37** (11), 1986-1995, doi:10.1111/acer.12183 (2013).
14. Middleton, F. A., Varlinskaya, E. I., & Mooney, S. M. Molecular substrates of social avoidance seen following prenatal ethanol exposure and its reversal by social enrichment. *Dev. Neurosci.* **34** (2-3), 115-128, doi:10.1159/000337858 (2012).
15. Varlinskaya, E. I., & Mooney, S. M. Acute exposure to ethanol on gestational day 15 affects social motivation of female offspring. *Behav. Brain Res.* **261** 106-109 (2014).
16. Meyer, L. S., & Riley, E. P. Social play in juvenile rats prenatally exposed to alcohol. *Teratology* . **34** (1), 1-7 (1986).
17. Royalty, J. Effects of prenatal ethanol exposure on juvenile play-fighting and postpubertal aggression in rats. *Psychol Rep* . **66** (2), 551-560 (1990).
18. Hamilton, D. A. *et al.* Effects of moderate prenatal ethanol exposure and age on social behavior, spatial response perseveration errors and motor behavior. *Behav. Brain Res.* **269** 44-54 (2014).
19. Kelly, S. J., & Dillingham, R. R. Sexually dimorphic effects of perinatal alcohol exposure on social interactions and amygdala DNA and DOPAC concentrations. *Neurotoxicol. Teratol.* **16** (4), 377-384 (1994).
20. Lugo, J. N., Marino, M. D., Cronise, K., & Kelly, S. J. Effects of alcohol exposure during development on social behavior in rats. *Physiology and Behavior.* **78** (2), 185-194 (2003).
21. Lugo, J. N., Marino, M. D., Gass, J. T., Wilson, M. A., & Kelly, S. J. Ethanol exposure during development reduces resident aggression and testosterone in rats. *Physiology and Behavior.* **87** (2), 330-337 (2006).
22. Kelly, S. J., & Tran, T. D. Alcohol exposure during development alters social recognition and social communication in rats. *Neurotoxicol. Teratol.* **19** (5), 383-389 (1997).
23. Mooney, S. M., & Varlinskaya, E. I. Acute prenatal exposure to ethanol and social behavior: Effects of age, sex, and timing of exposure. *Behav. Brain Res.* **216** (1), 358-364, doi:10.1016/j.bbr.2010.08.014 (2011).
24. Savage, D. D., Becher, M., de la Torre, A. J., & Sutherland, R. J. Dose-dependent effects of prenatal ethanol exposure on synaptic plasticity and learning in mature offspring. *Alcohol. Clin. Exp. Res.* **26** (11), 1752-1758, doi:10.1097/01.alc.0000038265.52107.20 (2002).
25. Rasmussen, D. D. *et al.* Chronic daily ethanol and withdrawal: 1. Long-term changes in the hypothalamo-pituitary-adrenal axis. *Alcohol. Clin. Exp. Res.* **24** (12), 1836-1849, doi:10.1111/j.1530-0277.2000.tb01988.x (2000).
26. Savage, D. D. *et al.* Effects of a Novel Cognition-Enhancing Agent on Fetal Ethanol-Induced Learning Deficits. *Alcohol. Clin. Exp. Res.* **34** (10), 1793-1802, doi:10.1111/j.1530-0277.2010.01266.x (2010).
27. Goodlett, C. R., & Johnson, T. B. Neonatal binge ethanol exposure using intubation: Timing and dose effects on place learning. *Neurotoxicol. Teratol.* **19** (6), 435-446, doi:10.1016/s0892-0362(97)00062-7 (1997).
28. Staples, M. C., Rosenberg, M. J., Allen, N. A., Porch, M. W., & Savage, D. D. Impact of Combined Prenatal Ethanol and Prenatal Stress Exposure on Anxiety and Hippocampal-Sensitive Learning in Adult Offspring. *Alcohol. Clin. Exp. Res.* **37** (12), 2039-2047, doi:10.1111/acer.12190 (2013).

29. Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* **79** (3), 359-371, doi:10.1016/s0031-9384(03)00149-5 (2003).
30. Hamilton, D. A. *et al.* Patterns of social-experience-related c-fos and Arc expression in the frontal cortices of rats exposed to saccharin or moderate levels of ethanol during prenatal brain development. *Behav. Brain Res.* **214** (1), 66-74, doi:10.1016/j.bbr.2010.05.048 (2010).
31. Barnett, S. A. *A study in behaviour: Principles of ethology and behavioural physiology displayed mainly in the rat.* (1963).
32. Pellis, S. M., & Pellis, V. C. Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat *Rattus-Norvegicus*. *Aggressive Behav.* **13** (4), 227-242 (1987).
33. Meaney, M. J., & Stewart, J. A descriptive study of social-development in the rat (*Rattus-Norvegicus)*. *Anim. Behav.* **29** (FEB), 34-45, doi:10.1016/s0003-3472(81)80149-2 (1981).
34. Pellis, S. M., & Pellis, V. C. The prejuvenile onset of play fighting in laboratory rats (Rattus norvegicus). *Dev. Psychobiol.* **31** (3), 193-205 (1997).
35. Himmler, B. T., Pellis, V. C., & Pellis, S. M. Peering into the dynamics of social interactions: measuring play fighting in rats. *Journal of visualized experiments : JoVE*. (71), e4288-e4288, doi:10.3791/4288 (2013).
36. Panksepp, J., Siviy, S., & Normansell, L. The psychobiology of play : Theoretical and methodological perspectives. *Neurosci. Biobehav. Rev.* **8** (4), 465-492, doi:10.1016/0149-7634(84)90005-8 (1984).
37. Siviy, S. M., & Panksepp, J. Sensory modulation of juvenile play in rats. *Dev. Psychobiol.* **20** (1), 39-55, doi:10.1002/dev.420200108 (1987).
38. Pellis, S. M. *et al.* The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. *Behav. Neurosci.* **120** (1), 72-84, doi:10.1037/0735-7044.120.1.72 (2006).
39. Blanchard, R. J., Blanchard, D. C., Takahashi, T., & Kelley, M. J. Attack and defensive behavior in albino-rat. *Anim. Behav.* **25** (AUG), 622-634, doi:10.1016/0003-3472(77)90113-0 (1977).

