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Prenatal alcohol exposure disrupts male adolescent social behavior and oxytocin receptor binding in rodents

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Abstract

Social behavior deficits resulting from prenatal alcohol exposure (PAE) emerge early in life and become more pronounced across development. Maturation changes associated with adolescence, including pubertal onset, can have significant consequences for social behavior development, making adolescence a unique period of increased vulnerability to social behavior dysfunction. Unfortunately, little is known about the underlying neurobiology supporting PAE-related social behavior impairments, particularly in the context of adolescence, when the transition to a more complex social environment may exacerbate existing deficits in social behavior function. Here we perform a comprehensive evaluation of social behavior development in PAE animals during two different periods in adolescence using three separate but related tests of social behavior in increasingly complex social contexts: the social interaction test, the social recognition memory test (i.e. habituation-dishabituation test), and the social discrimination test. Additionally, we investigated the underlying neurobiology of the oxytocin (OT) and vasopressin (AVP) systems following PAE, given their well-documented role in mediating social behavior. Our results demonstrate that compared to controls, early adolescent PAE animals showed impairments on the social recognition memory test and increased OT receptor binding in limbic networks, while late adolescent PAE animals exhibited impairments on the social discrimination test and increased OTR binding in forebrain reward systems. Taken together, these data indicate that PAE impairs adolescent social behavior – especially with increasing complexity of the social context – and that impairments are associated with altered development of the OT but not the AVP system.

Keywords

adolescence; oxytocin; prenatal alcohol exposure; social behavior; social recognition memory; vasopressin

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Conflict of Interest All authors declare no conflict of interest.

Introduction

Of the cognitive, physiological and behavioral impairments associated with prenatal alcohol exposure (PAE) documented in the clinical literature, lifelong social behavior deficits are particularly pervasive (Kelly et al., 2000; Thomas et al., 1998). PAE-related social behavior deficits emerge early in development and become more pronounced with age (Kully-Martens et al., 2012; Marquardt and Brigman, 2016). Animal models have demonstrated social behavior deficits parallel to those observed in humans with PAE. In neonatal rats, for example, PAE has been linked with disrupted attachment (Subramanian, 1992) and reduced ability to elicit retrieval by the mother (Ness and Franchina, 1990). Social deficits persist and often worsen during adolescence, with PAE rats showing disrupted play behavior and changes in social investigation (Lawrence et al., 2008; Mooney and Varlinskaya, 2011), which in themselves may have long-lasting effects on normal social development (Auger and Olesen, 2009). Indeed, social behavior deficits are present into adulthood, as PAE rats show altered social interactions and increased aggression with conspecifics (Hamilton et al., 2010; Hellemans et al., 2010; Royalty, 1990). Taken together, these data suggest that the transition to a more complex social environment, such as occurs during adolescence, which is characterized by expansions in social networks and increases in peer-directed social interaction, may exacerbate some of the social behavior impairments observed following PAE (Kully-Martens et al., 2012; Spear, 2000).

Adolescence is a critical developmental period in which significant neurobehavioral, cognitive and physiological changes occur, including the attainment of sexual maturity (i.e. puberty; McCormick and Mathews, 2010; Vetter-O'Hagen and Spear, 2012). Notably, many of the maturational changes that occur around pubertal onset can have significant consequences for social behavior development, such as steroid-dependent organization of neural circuits, making adolescence a unique period of increased vulnerability to social behavior dysfunction (Sisk and Zehr, 2005). In rats, play behavior peaks during adolescence (Spear, 2000), and most studies of adolescent social behavior following PAE have focused on observations of play, with relatively few assessing other aspects of social function such as social recognition memory or social discrimination (Marquardt and Brigman, 2016). Here, we expand this literature by performing a comprehensive evaluation of social behavior development in PAE animals during two different periods in adolescence, using a battery of social behavior tests.

Despite progress in characterizing social behavior deficits following PAE in humans and animal models, more research is needed to identify the underlying neurocircuitry. The neuropeptides oxytocin (OT) and vasopressin (AVP) have been implicated in the regulation of various aspects of social behavior (Bredewold and Veenema, 2018; Dore et al., 2013), including social motivation (Lim and Young, 2006), social recognition (Bielsky and Young, 2004; Engelmann et al., 1994; Ferguson et al., 2002; Veenema et al., 2012) and aggression (Ferris, 1992; Veenema et al., 2010). OT/AVP act by binding to their respective receptors, OTR or V1_aR. These receptors are widely distributed in the brain and correlate with sites of peptide release, particularly in brain areas implicated in social behavior function, including the amygdala, prefrontal cortex (PFC), bed nucleus of the stria terminalis (BNST), nucleus accumbens (NAcc), and lateral septum (LS; Dumais et al., 2013; Neumann and van den

Burg, 2013; Stoop et al., 2015). Despite their well-characterized role in regulating social behavior and other key functions, relatively few studies have assessed the OT/AVP systems within the context of PAE-related social behavior impairment. Kelly et al. demonstrated that PAE results in sexually dimorphic deficits in social recognition memory and reductions in OT-receptor binding in amygdala homogenates of adult rats (Kelly et al., 2009). In adult female mandarin voles, PAE resulted in reductions in OT fibers within the PVN and SON as measured by OT immunohistochemistry (He et al., 2012). Studies investigating the effects of PAE on the AVP system have generally done so within the context of stress regulation and have shown long-lasting alterations to the AVP system (Godino and Renard, 2018). Additionally, previous work from our laboratory indicates that PAE alters sensitivity of central AVP pathways to testosterone in adult males (Lan et al., 2009, 2006). These studies suggest an enduring effect of PAE on the OT and AVP systems; however, more work is needed to understand the role of the OT/AVP systems in mediating the deleterious effects of PAE on social behavior, particularly during the key developmental period of adolescence.

Here, we utilize a well-established rat model of PAE to investigate the neurobehavioral effects of PAE on adolescent social behavior development using three separate, but related tests of social behavior in increasingly complex social contexts: 1) the social interaction test, 2) the social recognition memory test (i.e. habituation-dishabituation test), and 3) the social discrimination test. To further address the specificity of the PAE insult to the social behavior domain and to confirm that deficits are not simply a downstream effect of impaired olfactory function, an additional set of animals was assessed using tests of olfaction and social odor discrimination (Wesson et al., 2010). Given the role of OT and AVP in regulating social behavior function, we assess the central OT/AVP systems, measuring hypothalamic mRNA expression of OT and AVP as well as receptor binding of the OTR and V1_aR in key brain regions. Finally, as the OT/AVP systems have significant cross talk with the hypothalamic-pituitary-adrenal and -gonadal systems (Dore et al., 2013; Neumann and van den Burg, 2013), we also assess plasma levels of corticosterone and testosterone.

Methods

Animals and Breeding

Male and female Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, Canada). Rats were pair-housed by sex and maintained at a constant temperature ($21 \pm 1^\circ\text{C}$) and on a 12 h light-dark cycle (lights on at 0700 h) with *ad libitum* access to water and standard lab chow (Harlan, Canada). After a 10-day acclimation period, male and female pairs were placed together for breeding. Vaginal smears were taken each morning, and the presence of sperm was used as an indicator of pregnancy (gestation day 1; G1). All experiments were performed in accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals, Canadian Council on Animal Care guidelines, and were approved by the University of British Columbia Animal Care Committee.

Prenatal Alcohol Exposure

On G1, females were single-housed and randomly assigned to one of three treatment groups: Prenatal Alcohol Exposure (PAE), Pair-Fed (PF), or *ad libitum*-fed Control. Dams in the PAE group (n = 41) were offered *ad libitum* liquid ethanol diet (6.37% v/v) with 36% ethanol-derived calories. The liquid ethanol diet was introduced gradually over the first 3 days with bottles containing: Day 1 – 66% control diet, 34% ethanol diet; day 2 – 34% control diet, 66% ethanol diet; day 3 – 100% ethanol diet. This diet is formulated to provide adequate nutrition to pregnant rats regardless of ethanol intake (Lan et al., 2006). To determinate blood alcohol levels (BALs) of alcohol consuming dams, tail blood samples from a subset of dams (n = 14) were taken on GD15 during various times across the light/dark cycle. Serum was collected and stored at –20 °C until the time of assay. BALs were measured using Pointe Scientific Inc. Alcohol Reagent Set (Lincoln Park, MI, USA); the minimum detectable concentration of alcohol is 2 mg/dl. Alcohol-consuming dams showed an mean of 135.3 ± 50.8 mg/dL (max BAL = 215.1 mg/dL; min BAL = 52.08 mg/dL). For reference, most jurisdictions set 80–100 mg/dL as the legal limit of intoxication. Pair-fed dams (n = 33) were offered a liquid control diet with maltosedextrin isocalorically substituted for ethanol, in an amount matched to the consumption of an alcohol-fed partner according to gestation day (g/Kg body weight/day of gestation). *Ad libitum*-fed Control dams (n = 39) were offered *ad libitum* access to a pelleted form of the liquid control diet. Diets were prepared by Dyets Inc., Bethlehem, PA (Weinberg-Keiver High Protein Experimental Diet # 710324; Control Diet #710109; Weinberg/Keiver High Protein Pelleted Control Diet #710109). All animals had *ad libitum* access to water, and were provided with fresh diet daily within 1h of lights off to prevent a shift in corticosterone circadian rhythms, which occurs in animals that are on a restricted feeding schedule, such as the pair-fed dams (Gallo and Weinberg, 1981).

Experimental diets were continued through G21; beginning on G22, all animals were offered *ad libitum* access to standard laboratory chow and water, which they received throughout lactation. Pregnant dams were left undisturbed except for cage changing and weighing (G1, G7, G14, and G21). On the day of birth (postnatal day 1 – PN1), litters were weighed and culled to 12 pups with an attempt to preserve an equal number of males and females per litter. Dams and pups were left undisturbed except for cage changing and weighing (PN1, PN8, PN15, P22). Subjects were male offspring housed in same-prenatal treatment, non-sibling pairs in standard rat cages (17”L × 10.5”W × 7.3”H, Allentown, Inc., Allentown, NJ) at P25. For behavioral testing, no more than 1 male per litter was at each age of testing. Behavioral testing occurred in early (P30–35) or late (P43–47) adolescence to account for potential effects of gonadal hormone changes across puberty. Unmanipulated juvenile male rats (P23–28), housed 2–4 per cage, were used as social stimuli for social motivation, recognition memory, and discrimination testing.

Experimental design

For all experiments, subjects were male offspring in either early (~P32) or late (~P45) adolescence. Behavioral testing was performed using three experimental cohorts to reduce repeated testing effects, as social experience has the potential to affect later function (Veenema, 2012). Cohort 1: Olfactory testing followed by social odor discrimination testing

4 days later (n=6–12 per prenatal treatment/age); **Cohort 2**: Social motivation testing (3-chamber) followed by social recognition memory testing on the following day (n=8–11 per prenatal treatment/age); **Cohort 3**: Social motivation testing (2-chamber) followed by social discrimination testing on the following day (n=8–10 per prenatal treatment/age). Brain analyses were performed only for cohort 3.

Buried Food Olfactory Testing

Olfactory testing was performed in early (~P30) or late (~P40) adolescence using a modified buried food olfactory test (Yang and Crawley, 2009). Odor familiarization to a food stimulus (Froot Loops® cereal; Kellogg's®, Mississauga, ON) occurred across 4 days preceding testing. On the testing day, subjects were briefly transferred to a clean holding cage, and the food stimulus was buried approximately 1 cm beneath the bedding surface, in a random corner of the home cage. Subjects were reintroduced to the home cage and the latency to find the food stimulus was recorded for each subject.

Social Odor Habituation/Discrimination Testing

Five days following buried food olfactory testing, early (~P35) and late (~P45) adolescent males were evaluated using a modified odor habituation/discrimination test using adult male and female urine odors (Wesson et al., 2010). Home cage bedding was replaced with fresh bedding 24 hr prior to testing. Previously collected urine samples were pooled into either male or female aliquots, which were applied to cotton applicator sticks enclosed in a piece of odorless plastic tubing to prevent contact of the liquid odor with the testing chamber or animal yet still allow volatile odor delivery. Odors were delivered for four successive trials, 20 sec each, separated by 10 sec intertrial intervals, by inserting the odor stick into a port on the side of the animal's home cage; odor testing order (male vs. female urine) was counterbalanced among groups. The duration of time spent investigating, defined as snout-oriented sniffing within 1 cm of the odor presentation port, was recorded across all trials by a single observer blind to prenatal treatments. To test for habituation, animals' first investigation of an odor was compared to its last investigation; discrimination was tested by comparing the last investigation of the first odor to the first investigation of the second odor presented. Only animals that investigated each odor a minimum of two times were included in the current analysis, and rates of exclusion were similar to those observed in periadolescent animals in previous studies of olfactory learning (Galef and Cem Kaner, 1980). Moreover, binomial logistic regression confirmed that exclusion of animals was not different among the different prenatal treatment groups at each age (P30: 34 animals excluded of 56 tested; P45: 26 animals excluded of 57 tested).

Social Motivation Testing

Male offspring were tested during early (~P32) or late (~P45) adolescence using a modified social motivation test. The test apparatus was constructed of transparent Plexiglas and divided into 2- or 3-chambers with openings large enough to allow animals to move between chambers (Page et al., 2009). The three-chambered apparatus consisted of a central "neutral" chamber and two outer chambers, and measured 10.5"L × 28.5"W × 16.25"H for early adolescent and 20"L × 32"W × 17"H for late adolescent animals. For early adolescent testing, the central chamber was 10.5"L × 6"W with 10.5"L × 11.5"W outer chambers; for

late adolescent testing, the central chamber was 20”L × 8”W with 20”L × 12”W outer chambers. The two-chambered apparatus was a Plexiglas box (21.5”L × 20”W × 21.5”H) divided in half to form two chambers (10.5” × 20”W). After 5 min of habituation to the testing apparatus, experimental rats were placed in the apparatus; one chamber was empty (Non-Social) and the other contained a social stimulus juvenile (~P25). Animals in the social chamber were retained behind a clear Plexiglas barrier (with holes to allow passage of odors) to prevent physical contact and thus isolate the specific motivation of the experimental animal to interact with the stimulus animal (location of social stimuli was counterbalanced among groups). Behavior was recorded and later scored using video tracking software (Noldus Ethovision, Netherlands) to quantify duration spent in each chamber.

Social Recognition Memory Testing

Immediately following social motivation testing, animals were singly housed for 24 hr to increase salience of the social stimulus animals (Niesink and Van Ree, 1989). On the following day, animals underwent social recognition memory testing within the home cage (Dore et al., 2013; Todeschin et al., 2009). The habituation phase of the test involves four 2-min trials (18-min intertrial interval) during which the same juvenile social stimulus (~P25) is introduced into the home cage. The recognition phase occurs on a fifth 2-min session, when a novel social stimulus is introduced. Testing was filmed from the front of the clear home cage and scored later by a trained observer blind to prenatal treatment using a computer-assisted data acquisition system (Noldus Observer, Netherlands). In addition to assessing olfactory investigation as an indicator of social recognition, the latency to initiate play was scored as a potentially more sensitive and age-relevant measure of social recognition memory, particularly in early adolescence. Specifically, the duration of social stimulus investigation (body sniff, anogenital sniff, allogroom) as well as the latency to initiate play (wrestle, pounce, boxing, pin) were used as measures of both habituation (trial 1 vs. trial 4) and recognition (trial 4 vs. trial 5).

Social Discrimination Testing

On the day following social motivation testing, animals were singly housed for 4 hr prior to social discrimination testing to increase salience of the social stimulus animals. Testing occurred in a Plexiglas box (16.5”L × 16.5”W × 15”H) filled with clean bedding, and consisted of a 5-min familiarization phase with a same-sex social stimulus animal (~P25) and a 5-min discrimination phase (novel vs. familiar social stimulus) separated by a 15-min retention period (Engelmann et al., 2011). Testing was filmed and scored later by a trained observer blind to prenatal treatment and social stimulus identity using a computer-assisted data acquisition system (Noldus Observer, Netherlands). The duration and frequency of non-social behaviors (rearing, environmental investigation, self-groom), social stimulus investigation (body sniff, anogenital sniff, allogroom) and play (wrestle, pounce, boxing, pin) with each social stimulus was recorded. Cohort 3 animals were decapitated 30-min after the end of social discrimination testing for collection of trunk blood and brains.

Hormone assays for corticosterone and testosterone

Trunk blood was collected in glass tubes containing EDTA, and then centrifuged at 3500 *g* for 10 min at 4 °C, and serum stored at –80 °C until assay. Plasma corticosterone and testosterone levels were assessed using ImmunChem™ Double Antibody Corticosterone and Testosterone ¹²⁵I RIA kits (MP Biomedicals, Orangeburg, NY). Plasma testosterone was measured with an adapted protocol such that all reagent volumes were halved. The minimum detectable concentration was 7.7 ng/mL for corticosterone and 0.1 ng/mL for testosterone, with intra- and interassay coefficients of variation of less than 10% for both assays.

Tissue collection

30-min after the end of social discrimination testing, cohort 3 animals were decapitated and brains were collected, quickly frozen on dry ice and stored at –80 °C. Brains were sectioned coronally (20 µm) using a cryostat (–16 °C) and stored at –80 °C until receptor binding or OT/AVP *in situ* hybridization assays. Regions of interest for receptor binding assays included the amygdala [basolateral complex (BLA), medial (CeM) and lateral (CeL) divisions of the central amygdala (CeA), cortical (CoA), and medial (MeA) subnuclei], anterior bed nucleus of the stria terminalis (aBNST), posterior BNST (pBNST), lateral septum (LS), medial PFC (mPFC) [anterior cingulate (ACC), prelimbic (PrL), and infralimbic (IL) cortices], anterior and posterior divisions of nucleus accumbens (aNAcc, pNAcc), and olfactory bulb (OB). Only the paraventricular (PVN) and supraoptic nuclei (SON) were assayed using *in situ* hybridization. Brain tissue early and late adolescent animals were run simultaneously by brain region to be able to compare groups.

Receptor binding assays

OTR autoradiography procedure was performed using [125I]- Ornithine Vasotocin Analog d(CH₂)₅[Tyr(Me)₂,Thr₄,Orn₈,[125I]Tyr₉-NH₂]-OVT (Perkin Elmer, USA) as tracer; V_{1a}R autoradiography procedure was performed using V_{1a}R antagonist [125I]-d(CH₂)₅Tyr(Me)AVP (Perkin Elmer, USA) as tracer. Briefly, slides were thawed and dried at room temperature. Sections were outlined with hydrophobic pen and slides were then fixed in 0.25% buffered formalin and washed two times in Tris buffer (50mM; pH 7.4). The slides were then exposed to tracer buffer (Tris + 10 mM MgCl₂, 0.1% BSA, and tracer) for 60 min, and then washed four times in Tris + MgCl₂. The slides were then dipped in distilled water, dried, and exposed to film (Kodak) for 2–10 days depending on brain region. The optical density of OTR and V_{1a}R binding was measured using ImageJ (NIH, <http://rsb.info.nih.gov/ij/>). Each measurement was subtracted by tissue background (corpus callosum), and receptor densities were calculated by taking the mean of 2-4 (depending on the region being analyzed) bilateral brain section measurements per region of interest per rat. Receptor binding assays for early and late adolescent animals were run simultaneously by brain region allow for comparisons by age.

In situ hybridization for OT and AVP in hypothalamus

Oligonucleotide probes were used to measure OT and AVP mRNA. Probes were synthesized at the Oligonucleotide Synthesis Laboratory, University of British Columbia as follows: antisense OT (5'-CTC GGA GAA GGG AGA CTC AGG GTC GCA GGC GGG GTC GGT

GCG GCA GCC-3') (Calcagnoli et al., 2014); antisense AVP (5'-GTA GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG-3') (Ivell and Richter, 1984; Young et al., 1986). Sense oligos for OT and AVP mRNA were used as negative controls. Probes were 3' tail labeled with 35S-dATP (Amersham Biosciences, Piscataway, NJ, USA) using terminal deoxytransferase (New England Biolabs Inc., Pickering, ON, Canada) as per supplier protocol. Probes were purified using Roche DNA G-25 Sephadex Columns (Roche Scientific, Indianapolis, IN., USA). 1 M dithiothreitol (DTT) was added to prevent oxidation.

Sections were thawed (20 min) and went through prehybridization as follows: formalin (30 min), 1 × PBS (10 min) twice, 0.1M triethanolamine-hydrochloride - 0.9 % NaCl + 0.25 % acetic anhydride (10 min), 2 × SSC (5 min), dehydrated through a graded series of ethanol concentrations, chloroform (5 min) followed by 100 % ethanol, and then air-dried. Hybridization buffer (50 % formamide, 3 × SSC, 1 × Denhardt's solution, 100 µg/ml yeast tRNA, 25 mM sodium phosphate buffer (pH 7.4), 10 % dextran sulphate, 55 mM DTT, 30 % deionized water) was applied; sections were covered with hybrislips (Sigma-Aldrich Canada Ltd., St Louis, ON, Canada), and incubated overnight at 40 °C in 50 % formamide humidified containers. Hybrislips were removed and slides were washed in 2 × SSC (20 min) twice, 2 × SSC/0.01 M DTT (45 °C, 20 min), 1 × SSC (45 °C, 15 min), 1 × SSC/50 % formamide (45 °C, 30 min), 1 × SSC (10 min), 0.5 × SSC (10 min). Sections were dipped briefly in water five times then plunged into 70 % ethanol (5 min), then air dried overnight. Sections were first exposed to Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY, USA). All slides were dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) diluted 1:1 (Deionized H₂O) and exposed for 3 days for OT and 24 hours for AVP in desiccated, light tight boxes at 4 °C. Slides were developed with Kodak D-19 developer at 14 °C and fixed with Kodak fixer at 14 °C, then counterstained with Cresyl Violet. Coverslips were mounted with Permount (Fisher Scientific Ltd., Nepean, ON, Canada).

In situ signals were visualized with a Q-imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope. Images were captured using Northern Elite 6.0v (Empix Imaging Inc., Mississauga, ON, Canada) and semiquantitative densitometric analyses were performed using Image J 1.50i software (National Institutes of Health, Bethesda, MD). The mean optical density (OD) of hybridization signal, corrected by background subtraction, was taken under dark-field illumination. Background signal was measured over the optic tract immediately adjacent to each side of the area of interest. The corrected grey levels from both sides of two sections of each region were averaged to obtain a mean corrected grey level of the 4 measurements for each animal.

Statistical Analyses

All data are expressed as mean ± SEM. Dam and offspring weights were analyzed using a repeated measures ANOVA [prenatal treatment (between-subject factor) × day (within-subject factor)]. Behavioral and brain data were first analyzed using two-way ANOVAs (age × prenatal treatment) with or without repeated measures depending on the specific dataset. Results indicated that for the majority of measures, a significant main effect of age was detected. Accordingly, subsequent one-way ANOVA analyses were performed

independently by age. For social motivation testing (2- or 3-chamber), the duration of time (sec) animals spent in each chamber (social vs. non-social) was analyzed using repeated measures ANOVA [prenatal treatment $\times$ duration in social/non-social chamber (within-subject factor)]. For social recognition memory testing, social investigation of and latency to initiate play with the social stimulus were analyzed using repeated measures ANOVA [prenatal treatment $\times$ trial (within-subject factor)]. For social discrimination testing, total social investigation time (investigation of familiar social stimulus + investigation of novel social stimulus) among prenatal treatments was analyzed using one-way ANOVA. Social discrimination was measured by testing whether the time subjects spent investigating the novel versus familiar rat differed using paired t-tests. Differences in the percent time subjects spent investigating the novel rat were analyzed using a one-way ANOVA for the effect of prenatal treatment.

Normality assumptions were investigated by visual inspection of the data and explicitly tested using Shapiro-Wilk test. Homogeneity of variance assumptions were tested using Levene's tests for ANOVA analyses; only P30 IL OTR binding violated this assumption, and was therefore transformed using Box-Cox transformation). Mauchley's sphericity tests were run for repeated measures ANOVAs, and if significant, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (play latency data). Where appropriate, Newman-Keuls post hoc tests were used to test for differences among groups. For all tests, the software packages Statistica 13 (Statsoft, USA) and Graphpad Prism 7.0 (USA) were used. Significance was set at $p = 0.05$.

Results

Prenatal treatment reduced dam weights but not offspring weights

As expected, dam weights increased across gestation for all treatment groups, with PF and PAE dams showing attenuated weight gain relative to controls [Table 1; group $\times$ day interaction ($F = 47.53$, $p < 0.0001$, $\eta_p^2 = 0.6327$, $p = .47$)]. Specifically, dam weights were not different on GD1, but PF and PAE dam weighed less than controls from GD7–21. We did not identify any differences pregnancy viability among dams from any of the three diet groups (control, PF, PAE), and this is further reflected by the uniformity of mean litter size (Table 1) across prenatal treatments. No differences in offspring weights among the prenatal treatments were observed, and as expected, offspring weights increased across the pre-weaning period [Table 1; within-factor comparison of weigh date ($F_{3,330} = 7430$, $p < 0.0001$, $\eta_p^2 = .99$)].

General olfaction and social odor habituation/discrimination not impaired by PAE

Prenatal treatment groups did not differ in latency to find the hidden food reward in early or late adolescence. (Figure 1a,b). Likewise, investigation patterns in the social odor habituation/discrimination were indistinguishable among prenatal treatment groups in both early and late adolescence [Figure 1c,d; within-factor comparison of olfactory investigation of odors across trials (early adolescent: $F_{1,57} = 76.67$, $p < 0.0001$, $\eta_p^2 = .80$; late adolescent: $F_{3,84} = 74.82$, $p < 0.0001$, $\eta_p^2 = .73$)]. Specifically, all animals showed a reduction in investigation across similar odor presentations (first investigation vs. last investigation of

odor A) and an increase in investigation between different odors (last investigation of odor A vs. first investigation of odor B).

Social motivation is not affected by PAE

Results from the social interaction test showed that all animals, regardless of prenatal treatment or apparatus (three- vs. two-chambered), spent significantly more time in the social chamber versus the non-social chamber (Figure 2; [within-factor comparison of time in each chamber (early adolescent: $F_{1,20}=148.9$, $p<0.0001$, $\eta_p^2=.88$; late adolescent: $F_{1,26}=54.19$, $p<0.0001$, $\eta_p^2=.68$]). A similar pattern was observed in the 2-chamber apparatus [within-factor comparison of time in each chamber (early adolescent: $F_{1,26}=141.4$, $p<0.0001$, $\eta_p^2=.84$; late adolescent: $F_{1,26}=203.3$, $p<0.0001$, $\eta_p^2=.89$)].

PAE impairs social recognition memory in early adolescence

In early adolescence, we observed markedly different olfactory investigation patterns among animals in the three prenatal treatments across social recognition testing trials [Figure 3a,b; interaction of prenatal treatment $\times$ trial ($F_{4,42}=6.697$, $p<0.001$, $\eta_p^2=.39$)]. Specifically, PAE and PF animals showed the expected decrease in investigation from trials 1–4 (i.e. habituation), but PAE animals failed to show increased investigation of the novel social stimulus between trials 4 and 5 (i.e. recognition) observed in PF animals. Early adolescent controls did not show any significant changes in olfactory investigation across testing trials. Given that play behaviors peak during early adolescence (Meaney and Stewart, 1981), we used the latency to initiate play as an age-relevant measure of social recognition memory (Figure 3c,d). During the habituation phase (trial 1 vs. 4), early adolescent PAE and control males exhibited a reduction in the latency to initiate play with the social stimulus animal [interaction of prenatal treatment $\times$ trial ($F_{3,46,31.53}=3.462$, $p<0.03$, $\eta_p^2=.25$); calculated using Greenhouse-Geisser correction ($\epsilon=.75$)]. However, PAE males failed to show an increase in latency to initiate play with the novel social stimulus during the recognition phase.

In late adolescence, all animals regardless of prenatal treatment showed typical social recognition memory, with a reduction in investigation during the habituation phase followed by an increase in investigation during the recognition phase [within-factor comparison of trial ($F_{2,52}=20.404$, $p<0.0001$, $\eta_p^2=.44$)]. This same pattern was observed for play latency, such that all animals regardless of prenatal treatment showed a reduction in play latency during the habituation phase followed by an increase play latency during the recognition phase [within-factor comparison of trial ($F_{1,47,38.14}=12.933$, $p<0.001$, $\eta_p^2=.33$); calculated using Greenhouse-Geisser correction ($\epsilon=.73$)].

Late adolescent social discrimination is impaired by PAE

Familiarization phase.—There were no significant differences in total social investigation of the social stimulus animal among prenatal treatments in either early or late adolescence (Table 2). PAE resulted in significantly higher play initiation (i.e. pounce) frequency in early adolescence as compared to control and PF animals (Figure 4a; $F_{2,26}=4.10$, $p<0.05$, $\eta_p^2=.24$).

Discrimination phase.—There were no significant differences in total social investigation (investigation of familiar social stimulus + novel social stimulus) among prenatal treatments in either early or late adolescence (Table 2). Early adolescent animals did not show differential investigation of the novel versus familiar social stimulus, regardless of prenatal treatment group (control: $t_8=2.06$, $p=0.07$; PF: $t_9=0.85$, $p=0.41$; PAE: $t_9=0.79$, $p=0.45$). In late adolescence, however, different from control ($t_8=2.43$, $p<0.05$, $d=0.81$) and PF ($t_9=2.28$, $p<0.01$, $d=0.72$) animals, PAE rats failed to discriminate between the novel and familiar social stimuli ($t_9=0.42$, $p=0.69$).

Corticosterone and testosterone levels were not affected by PAE

Corticosterone levels were not different among prenatal treatment groups in either early or late adolescence (Table 3). In early adolescence, testosterone levels (66%) were generally below the lower limit of detection (LLOD) so no statistical comparisons were made. Additionally, binomial logistic regression analysis indicated no difference among prenatal treatment groups for the number of samples below the LLOD. In late adolescence, all but one sample were above the LLOD and testosterone levels were not different among prenatal treatment groups (Table 3).

Oxytocin receptor binding is altered in age-dependent manner following PAE

Early adolescence.—PAE animals exhibited increased OTR binding in the IL subdivision of the mPFC as compared to control and PF animals [(Figure 5a; $F_{2,26}=3.503$, $p<0.05$, $\eta_p^2=.21$); statistics run on Box-Cox transformed data]. Additionally, PAE and PF animals showed higher OTR binding than controls in the CeL relative to control animals (Figure 5d; $F_{2,26}=5.36$, $p<0.05$, $\eta_p^2=.29$). There were no differences in OTR binding among prenatal treatment groups for any of the other brain regions analyzed.

Late adolescence.—PAE exhibited increased OTR binding in the lateral septum as compared to control but not PF animals (Figure 5h; $F_{2,24}=3.76$, $p<0.05$, $\eta_p^2=.24$). OTR receptor binding in the posterior NAcc (pNAcc) was also increased in PAE and PF animals relative to controls (Figure 5k; $F_{2,23}=4.79$, $p<0.05$, $\eta_p^2=.29$). No other differences in OTR binding among prenatal treatment groups were observed.

Adolescent vasopressin 1_a receptor binding is not affected by PAE

There were no differences among prenatal treatment groups in V_{1a}R binding across any of the brain regions analyzed for either early or late adolescent animals.

OT and AVP mRNA expression is not altered by PAE

There were no differences among prenatal treatment groups in OT or AVP mRNA expression in the PVN or SON for either early and late adolescent animals.

Discussion

Here we used a battery of social behavior tests with increasing social complexity to evaluate distinct aspects of social behavior and to capture a broad neurobehavioral profile for identification of age-specific social behavior deficits following PAE. Overall, our results

indicate that PAE delays adolescent social behavior development and impairs social recognition memory, particularly in a complex social context (i.e. social discrimination test). Importantly, the social behavior deficits observed appear to stem from specific insult to the social behavior domain and are not simply a result of more general PAE-related olfactory impairment, as PAE rats did not differ from controls on their general olfaction or ability to discriminate between two different social odors. Assessment of the neurobiological systems implicated in the regulation of social behavior indicate that PAE alters the oxytocinergic system, while the AVP system appears to be spared. Specifically, PAE animals showed increased OTR binding in the CeL and IL in early adolescence, and increased OTR binding in the LS and NAcc in late adolescence, whereas V1_aR binding was not altered in PAE relative to controls in any of the regions examined. Together, these results suggest a marked effect of PAE on the adolescent development of social behavior and of the oxytocinergic system.

PAE-related social behavior deficits uncovered with increasing test complexity

Our first assessment of adolescent social behavior was accomplished using the social interaction test, which provided a measure of social motivation (Moy et al., 2004). Our social interaction testing was completed using a standard three-chambered apparatus (non-social, neutral start, and social chambers); additionally, a separate cohort of animals was tested using a two-chambered apparatus in an attempt to increase test sensitivity (de Chaumont et al., 2012). Results from the social interaction test showed that all animals, regardless of prenatal treatment, age, or apparatus, spent significantly more time in the social chamber, suggesting that social motivation is not impaired by PAE. This is in contrast to previous studies in similarly aged PAE rats, which found reduced social motivation in a modified social interaction test (Ignacio et al., 2014; Mooney and Varlinskaya, 2011). Importantly, in these previous studies, experimental animals were able to interact freely with age-matched social stimulus animals (Ignacio et al., 2014; Mooney and Varlinskaya, 2011), whereas in the present study, social stimulus animals were retained behind a clear Plexiglas barrier (with holes to allow passage of odors) to prevent physical contact and thus isolate the specific motivation of the experimental animal to interact with the stimulus animal. Moreover, these divergent results may also stem from the different alcohol exposure paradigm utilized. In contrast to our chronic alcohol exposure across gestation, these earlier studies utilized an acute, binge-like method of PAE targeting a single day during gestation. Our results parallel findings from the human literature, which demonstrate that, rather than a lack of social motivation, individuals with PAE show typical or enhanced levels of social motivation, even being described as being inappropriately friendly (Kully-Martens et al., 2012; Nanson, 1992). Indeed, reductions in social investigation in the social interaction test – which was originally designed as an assay to test social deficits in animal models of autism spectrum disorder (ASD) – might be more reflective of neurodevelopmental disorders in general, where deficits in social motivation are thought to be a core feature (Bishop et al., 2007; Chevallier et al., 2012).

We next used a more complex social behavior test, the social recognition memory test (i.e. habituation-dishabituation test; Gheusi et al., 1994). Compared to the social interaction test, experimental animals in the social recognition memory test could freely interact with social

stimuli, thus allowing for assessment of social interaction with physical contact. The social recognition memory test takes advantage of the rodent's natural tendency to investigate a novel social stimulus more than a familiar social stimulus (Gabor et al., 2012). Though this test is most frequently used to assess adult social recognition memory, previous research has demonstrated that early adolescent animals (~P32) are capable of exhibiting typical social recognition memory, especially with short intertrial intervals (Marco et al., 2011; Thor and Holloway, 1982). In addition to assessing social investigation as an indicator of social recognition, play behaviors were scored as potentially sensitive and age-relevant measures of social recognition memory (Cirulli et al., 1996). Results from the habituation phase demonstrated that all animals, regardless of age or prenatal treatment, habituated to repeated presentations of a familiar social stimulus animal as indicated by a reduction in the latency to initiate play with the social stimulus animal. During the recognition phase, however, only early adolescent PAE males exhibited impaired social recognition memory as indicated by a failure to show an increase in the latency to initiate play with the novel social stimulus. These data indicate that measures of play behavior can provide important information for assessing social recognition memory in adolescence, when the frequency of play behavior is at its highest expression. In late adolescence, though, all animals performed the task similarly, regardless of prenatal treatment. Taken together, these results suggest that PAE results in delays to social recognition memory development. Our findings of delayed social behavior development parallel findings from the clinical literature, which demonstrate that adolescents with PAE consistently lag behind peers in social behavior function (Streissguth et al., 1991). Additional indirect evidence of impaired social behavior comes from reports showing that adults with PAE are more likely to have had disrupted school experiences, trouble with the law, and histories of inappropriate sexual behavior (Streissguth et al., 1996). PAE-related delays in social behavior development during adolescence have important implications not just for later life social function, but can also have downstream effects on many other aspects of neurobehavioral development (Brenhouse and Andersen, 2011).

Given the relative simplicity of the social recognition memory test (i.e. only one social stimulus presented per trial), we next used the social discrimination test in order to investigate further the effects of PAE on social recognition memory in a more complex social context. Unlike the social recognition memory test, the social discrimination test assesses whether experimental animals are able to discriminate between a familiar and a novel social stimulus during simultaneous presentation (Engelmann et al., 1995), and thus offers a direct measure of social recognition memory in a complex social context (Engelmann et al., 2011). In early adolescence, no animals showed social discrimination as assessed by time spent in olfactory investigation of the familiar versus novel social stimulus, regardless of prenatal treatment. These results were somewhat surprising, as previous research has demonstrated social discrimination abilities in early adolescent rats following a 1-hr retention interval between the familiarization and discrimination phases (Veenema et al., 2012). An important difference between these earlier studies and the present study is that we tested animals in an unfamiliar apparatus rather than a home cage, which may represent a more stressful environment for early adolescent animals (Camats Perna and Engelmann, 2016). Nevertheless, in late adolescent animals, only PAE animals were unable to show social discrimination such that, in contrast to control and PF animals, PAE rats did not

differentially investigate the familiar and novel social stimuli. These results suggest that deficits in social recognition memory are still present in late adolescence; however, they may only be uncovered with increased complexity of the social context.

One possible limitation of our findings is that only male animals were tested in our experiments. The animal literature has identified many sex differences in social behavior function in general as well as in the context of PAE (Dhakar et al., 2013). For example, several groups have described extended social recognition memory in females versus males (Engelmann et al., 2011). Sex differences in social behavior function have also been observed in the PAE literature. For example, sensory encoding for and memory duration of social information in adult rats has been shown to be sexually dimorphic, such that females were less able to encode social information whereas males were deficient at retaining social memories over longer periods of time (Kelly et al., 2009). Our pilot studies investigating adolescent social behavior function following PAE failed to detect robust deficits of social recognition memory/discrimination in female subjects. The goal of the current experiments was to determine whether PAE-related changes in behavior were associated with changes in the central OT/AVP systems. In the absence of any apparent social recognition memory/discrimination deficits in females, we chose to investigate male animals.

Overall, results from our social behavior profile across adolescent development demonstrate the dynamic nature of social behavior deficits following PAE, and underscore the importance of tracking development across adolescence when investigating the effects of early life insults.

PAE results in age-specific changes in OTR but not V1_aR binding

The underlying neurobiology of social behavior involves the complex interplay of many neural structures and neuroendocrine systems. In particular, the OT/AVP neuropeptide systems are critical for regulating various aspects of social behavior via actions in key limbic and reward brain regions implicated in social behavior function (Bielsky and Young, 2004; Ferguson et al., 2002; Ross and Young, 2009; Veenema and Neumann, 2008). PAE resulted in several age-specific alterations in OTR but not V1_aR binding, such that PAE animals showed increased OTR binding in the IL and CeL in early adolescence and increased binding in the LS and pNAcc in late adolescence.

The amygdala is a key structure within the social behavior network (Adolphs, 2010), as amygdala lesions result in severe social behavior impairments, such as decreased play behavior in infant/adolescent rats (Daenen et al., 2002; Meaney et al., 1981), reductions in affiliative behavior in voles (Kirkpatrick et al., 1994) and altered social communication in primates (Rasia-Filho et al., 2000). The preclinical literature has demonstrated that in adulthood, PAE produces structural and functional alterations in the amygdala, which may underlie social behavior deficits observed following PAE (Cullen et al., 2013; Kelly and Dillingham, 1994; Zhou et al., 2010). Importantly, in the CeA, OT signaling appears to regulate social interest, as OTR antagonism attenuates social investigation of juvenile conspecifics (Dumais et al., 2016). Additionally, a positive correlation between aggression and OTR binding in CeA has also been described (Calcagnoli et al., 2014). Our present results demonstrate PAE-related increases in OTR binding in the CeL subdivision of the

CeA during early adolescence, an age when PAE rats show deficits in social recognition memory, suggesting a potential role for altered amygdala OTR signaling in the manifestation of social behavior deficits following PAE.

The PFC – a brain region generally associated with higher cognitive tasks and executive function (Arnsten, 1998; Diamond, 2011; Floresco et al., 1997) – is also a critical regulator of social behavior and social cognition (Bicks et al., 2015; Minami et al., 2017). Similar to the amygdala, the pre-clinical literature has also demonstrated that PAE produces structural and functional alterations in the and PFC (Hamilton et al., 2010; Lawrence et al., 2012; Mihalick et al., 2001). Our present results add to this literature by showing that PAE increased mPFC OTR binding in early adolescence.

Notably, the amygdala has specific and reciprocal connections with the IL, PL, ACC and orbitofrontal cortices comprising the PFC (Kita and Kitai, 1990; McDonald, 1998; Swanson, 2003); moreover, the mPFC and amygdala also show functional connectivity as demonstrated by correlated expression of the neural activity marker *c-fos* during social play behavior in adolescence (van Kerkhof et al., 2014). Importantly, OT activity within the mPFC-amygdala circuit is thought to be an important modulator of social behavior (Adolphs, 2010; Bredewold and Veenema, 2018), as OT infusions in the IL have been shown to mediate synaptic plasticity thought to allow for the top-down regulation of subcortical structures such as the amygdala (Ninan, 2011). Indeed, alterations to the OT system within the mPFC-amygdala network when the mPFC is undergoing significant maturational changes relative to the earlier developing amygdala (Anderson et al., 2000; Diamond, 2002; Steinberg, 2005) may underlie the failure of PAE animals to respond appropriately to a novel social stimulus (i.e. increased latency to initiate play to a novel social stimulus).

The PAE-related alterations in OTR binding in limbic areas observed in early adolescence were transient, as we did not detect increased OTR binding in those areas in late adolescence. However, PAE-related changes in OTR binding in late adolescence were observed in the LS and NAcc, forebrain regions critical for both social recognition memory and reward. The LS is a key hub in the social recognition neurocircuitry and shares important connections with other brain structures critical for social behavior function, including the amygdala, BNST, and hypothalamus (Grinevich et al., 2016; Wacker and Ludwig, 2012). LS-mediated social recognition memory requires the coordinated activity of the OT/AVP systems, as receptor antagonism of V1_aR (Veenema et al., 2012) or OTR (Lukas et al., 2013), as well as genetic knockout of OTR (Mesic et al., 2015) all result in social recognition deficits. Moreover, septal infusion of AVP (Engelmann and Landgraf, 1994) or OT (Popik and van Ree, 1999) enhance social recognition. Given the PAE-related deficits in social discrimination in late adolescence, our finding of a corresponding increase in septal OTR binding is noteworthy, particularly given the significant role of this brain region in facilitating social discrimination.

Also located in the basal forebrain, the NAcc functions classically as part of the reward circuitry, and in the present context, supports aspects of social motivation and social reward (Caldwell and Albers, 2016; Dölen and Malenka, 2014). For example, OTR antagonism within the NAcc attenuates social novelty seeking (Smith et al., 2017) as well as social

reward associated with social interaction (Dölen et al., 2013). Interestingly, PAE has previously been shown to alter play behavior and reduce *c-fos* expression in the NAcc in adolescent male rats (Lawrence et al., 2008), but combined prenatal alcohol and nicotine exposure also result in increased OTR binding in the NAcc of adult male offspring (Williams et al., 2009). Our finding of increased OTR binding in the NAcc of PAE animals replicates these previous data suggesting that PAE effects on OTR in the NAcc is long-lasting. Moreover, PAE-related changes to the ventral striatal OT system underscore the importance of considering motivation in interpreting our behavioral data. Indeed, deficits in social discrimination following PAE may not necessarily stem from deficits in social recognition memory *per se*, but may instead reflect alterations in processing social cues and dysregulation of social motivation. For example, deficits in social discrimination observed here may be due to increased saliency of the familiar social stimulus, decreased saliency of the novel social stimulus, or some combination of both. Indeed, our finding of increased play behavior in PAE animals during the habituation phase appears to support this notion.

Importantly, the LS and NAcc are both responsive to changes in gonadal hormones such as occur during puberty (Blakemore et al., 2010; Grinevich et al., 2015). Given that we do not detect changes in OTR binding in the LS and NAcc until late adolescence, pubertal-related increases in circulating levels of testosterone may uncover PAE-related changes in these brain regions, which may contribute to the deficits observed in the social discrimination task – particularly given its more complex social context. Developmentally, both the LS and NAcc appear to show peak OTR expression/binding early in adolescent development (Grinevich et al., 2015), suggesting that, much like the delays observed in social behavior development following PAE, development of the OT system may be similarly delayed.

Analysis of brains revealed no differences among prenatal treatments in OT or AVP expression (mRNA) within the paraventricular nucleus (PVN) or supraoptic nucleus (SON) of the hypothalamus, which are the primary production sites for OT/AVP. These results are in contrast with findings of reduced OT immunoreactive fibers following PAE in voles (He et al., 2012), though because that study only investigated adult PAE females, it is unclear whether this is a sex-, age-, exposure paradigm- and/or species-specific finding. Interestingly, VI_aR binding was not altered in PAE relative to control animals in any of the regions examined. Our results demonstrating increased OTR but not VI_aR binding suggest that the OT system is more vulnerable to PAE insult. Nevertheless, the OT/AVP systems have significant crosstalk (Song and Albers, 2017), and future studies should continue to explore the interaction of these systems in the context of PAE. Even so, given the increased interest in utilizing exogenous OT as a therapeutic agent for social behavior disorders, our data have important implications for the investigation of OT treatment within the context of PAE. An important caveat here though is that the increased OTR binding density might represent the potential for higher OTR activation in PAE animals. Indeed, increased OTR binding density following PAE may result from either a higher affinity or a greater expression of OTR, perhaps to compensate for reduced local OT release and/or availability (Hodges et al., 2017; Zoicas et al., 2014).

Patterns of brain OTR changes observed in the present study have also been observed following a variety of experimental manipulations in pre- and postnatal life (Bales and

Perkeybile, 2012; Veenema, 2012). For example, prenatal stress results in increased CeA OTR binding in adult offspring (Lee et al., 2007), and acute prenatal exposure to valproic acid potentiates OTR mRNA expression in the mPFC and CeA in adulthood that is associated with altered social behavior (Štefánik et al., 2015; but see Bertelsen et al., 2017). Increased OTR binding is also observed in the LS and NAcc following adolescent social instability stress (Hodges et al., 2017), in LS and CeA following adult social fear conditioning (Zoicas et al., 2014), and in the LS, CeA, and BNST following treatment with the synthetic glucocorticoid dexamethasone (Patchev et al., 1993). Taken together, these data highlight the ability of experience to shape development of the OT system. Accordingly, in addition to viewing the primary insult of PAE to central OT systems, it is important also to consider how the resulting mismatch between the increasing demands of the social environment across development and the ability for the delayed individual to meet those challenges may exacerbate the original insult.

Conclusions and Implications

Overall, our results suggest that PAE disrupts social behavior development during adolescence, and that these behavioral impairments are associated with age-related alterations in the oxytocinergic system. Interestingly, late adolescent PAE animals performed as well as controls on the social recognition memory test, but showed impairments when evaluated in the social discrimination test, indicating that increasing complexity of the social context may unmask PAE-induced impairments in social behavior function. These results suggest that PAE animals may be less capable of navigating the complex and dynamic social transition that occurs during adolescence, as this period is characterized by alterations in the normal behavioral repertoire, expansions in social networks and increases in peer-directed social interactions (Spear, 2000). In addition to inducing age-related social behavior deficits, PAE also resulted in age-related alterations in OT receptor binding, suggesting a role for altered OT system development in the ontogeny of PAE-related social behavior impairments.

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term that refers to the spectrum of neurobiological, neurobehavioral and physiological impairments resulting from PAE. Despite progress in characterizing neurobehavioral deficits following PAE in humans and animal models, reaching a definitive diagnosis can still present a challenge for the clinical community (Benz et al., 2009). Indeed, it has been suggested that the difficulty in identifying individuals affected by PAE has led to an underestimation of its already high prevalence (2–5%) in the general population (May et al., 2018). Impaired social behavior in individuals with FASD has widespread implications for other domains and may contribute to difficulties in school, social rejection, trouble with the law, and later mental health problems. To this end, understanding the mechanisms that support impaired social behavior function observed following PAE is critical for developing specific strategies for earlier diagnoses and more targeted interventions for individuals living with FASD.

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Highlights

- Social motivation was not altered by prenatal alcohol exposure (PAE)
- PAE impaired social recognition memory in early adolescence and social discrimination in late adolescence
- PAE resulted in age-dependent increases in oxytocin but not vasopressin 1a receptor binding

Figure 1.

Tests of olfaction using the buried food olfactory (a-b) and social odor habituation/discrimination (c-d) tests in early (a, c) and late (b, d) adolescence. Data are expressed as mean $\pm$ SEM of latency to find the buried food or duration of the first and last investigation each social odor. * indicates a significant difference between the last investigation and first investigation for each odor; § indicates significant difference between the first investigation of Odor B vs the last investigation of Odor A. (n = 6–10 for all groups)

Figure 2.

Social motivation testing in 3- (a-b) or 2-chamber (c-d) apparatuses in early (a, c) and late (b, d) adolescence. Data are expressed as mean $\pm$ SEM of the time spent in the non-social or social chamber. * indicates a main effect of chamber location, such that all animals spent significantly more time in the social versus the non-social chamber. (n = 8–11 for all groups)

Figure 3. Social investigation (a-b) and play latency (c-d) during the social recognition memory test (habituation–dishabituation paradigm) in early (a, c) and late (b, d) adolescence. Data are expressed as mean $\pm$ SEM of investigation/play latency across four repeated 2-min exposures to the same juvenile (trials 1–4) followed by one final 2-min exposure to a different juvenile (trial 5). # indicates a main effect of trial, such that trial 4 is significantly different from trial 1 for all groups; \$ indicates a main effect of trial, such that trial 5 is significantly different from trial 4 for all groups; & indicates a main effect of trial, such that trial 5 is significantly different from trial 1 for all groups; + indicates trial 4 is significantly different from trial 1 for controls; × indicates trial 4 is significantly different from trial 1 for PFs; ^ indicates trial 5 is significantly different from trial 4 for controls; \$ indicates trial 5 is significantly different from trial 4 for PFs; † indicates trial 5 is significantly different from trial 1 for PAEs; * indicates that controls are significantly different from both PF and PAE animals; & indicates that PAE animals are significantly different from both control and PF animals. (n = 8–11 for all groups)

Figure 4.

Pounce behavior during the familiarization phase (a-b) and social investigation during the discrimination phase (c-d) of the social discrimination test in early (a, c) and late (b, d) adolescence. Data are expressed as mean $\pm$ SEM of the time spent investigating a familiar versus a non-familiar social stimulus juvenile. # indicates a significant main effect of prenatal treatment, where PAE are different from control and PF animals. * indicates a significant difference of investigation time, such that novel social stimuli were investigated significantly more than non-social stimuli. (n = 9–10 for all groups)

Figure 5. Effects of PAE on oxytocin receptor binding in early (a, d, g, j) and late (b, e, h, k) adolescence. Atlas images (images are used with permission from Paxinos and Watson, 2005) and corresponding representative OTR autoradiograms. Data are expressed as mean $\pm$ SEM (mean gray value) of OTR binding in infralimbic cortex (a-b), lateral division of the central amygdala (d-e), lateral septum (g-h), and posterior nucleus accumbens (j-k). * indicates a significant main effect of prenatal treatment, where PAE and/or PF are different from control animals. (n = 8–10 for groups)

Table 1.

Litter size and gestational and developmental weights.

	Prenatal treatment group		
	Control	PF	PAE
Dam N	39	33	41
Litter size at birth	15.8 ± 0.4	15.1 ± 0.5	15.1 ± 0.4
Dam weight (g)			
GD1	281.9 ± 3.1	287.1 ± 3.3	290.4 ± 3.0
GD7	319.1 ± 3.2	302.1 ± 3.4 *	305.6 ± 3.1 *
GD14	376.3 ± 3.5	347.5 ± 3.8 *	348.2 ± 3.4 *
GD21	485.5 ± 4.7	443.4 ± 5.0 *	432.0 ± 4.5 *
Pup weight (g)			
PN1	6.9 ± 0.1	7.1 ± 0.1	6.7 ± 0.1
PN8	15.8 ± 0.4	16.8 ± 0.5	15.8 ± 0.4
PN15	32.2 ± 0.6	34.2 ± 0.7	33.5 ± 0.6
PN22	54.4 ± 1.0	56.7 ± 1.0	55.6 ± 0.9

Data are expressed as mean ± SEM.

* indicates a significant main effect of prenatal treatment, where PAE and/or PF are different from control animals.

Table 2.

Total investigation during familiarization and discrimination phases of the social discrimination test.

Social Investigation Data			
Familiarization Phase	Prenatal treatment group		
	Control	PF	PAE
Early Adolescent	149.6 ± 7.5	159.8 ± 7.2	152.4 ± 7.2
Late Adolescent	144.3 ± 8.8	164.6 ± 8.8	160.2 ± 8.4
Discrimination Phase (Familiar + Novel)			
Early Adolescent	178.9 ± 8.5	190.7 ± 8.1	172.7 ± 8.1
Late Adolescent	184.5 ± 8.5	182.8 ± 8.1	189.2 ± 8.1

Data are expressed as mean ± SEM.

Table 3.

Plasma hormone levels collected following behavioral testing.

Hormone Data			
	Prenatal treatment group		
Corticosterone ($\mu\text{g/dL}$)	Control	PF	PAE
Social Recognition			
Early Adolescent	26.3 ± 3.0	27.0 ± 5.3	26.0 ± 3.3
Late Adolescent	17.4 ± 2.6	17.0 ± 3.8	13.8 ± 2.2
Social Discrimination			
Early Adolescent	28.4 ± 6.6	27.1 ± 4.5	30.2 ± 4.7
Late Adolescent	33.52 ± 4.99	24.7 ± 3.6	24.1 ± 3.7
Testosterone (ng/mL)			
Early Adolescent	n.d.	n.d.	n.d.
Late Adolescent	1.8 ± 0.5	1.3 ± 0.2	1.7 ± 0.3

Data are expressed as mean $\pm$ SEM.