EFFECTS OF PRENATAL ALCOHOL EXPOSURE ON SOCIAL BEHAVIOR NEUROBIOLOGY IN RATS

by

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Abstract

Estimates indicate a 1-5% prevalence of Fetal Alcohol Spectrum Disorders (FASD) among American children, making prenatal alcohol exposure (PAE) a leading cause of developmental disability in North America. Among the cognitive, physiological and behavioral impairments associated with PAE in the clinical/pre-clinical literature, lifelong social behavior deficits serve as a unifying feature across the entire spectrum. Impaired social behavior in individuals with FASD has widespread implications for function in other domains and may contribute to difficulties within school, social rejection, trouble with the law, and later mental health problems. Importantly, early-life adversity (ELA) can also modulate social behavior development, and individuals with PAE are more likely to experience ELA. However, few studies have assessed the interactive effects of these two insults on social behavior development.

Maturational changes associated with adolescence have significant consequences for social behavior development, making adolescence a unique period of increased vulnerability to social behavior dysfunction. My dissertation research focuses on establishing a comprehensive neurobehavioral profile of adolescent social behavior in a rat model of PAE. Our assessments of play behavior, social preference and recognition memory, as well as central oxytocin and vasopressin systems, critical modulators of social behavior function, indicate PAE impairs adolescent social behavior – especially with increasing complexity of the social context – and behavioral impairments are associated with altered neural activity and development of the oxytocin/vasopressin systems. Specifically, we found that PAE disrupts sustained play bouts with unexposed playmates, impairs social recognition memory – particularly in males – and alters oxytocin receptor expression and neural activity (*c-fos* expression) in limbic and forebrain regions important for social behavior function. Moreover, ELA generally exacerbated and

extended the effects of PAE, highlighting the ability of the early environment to mediate outcomes of PAE and power of animal models to interrogate this relationship. The relevance of the current research stems from the need for establishing a more specific social neurobehavioral profile that could support the development of strategies for earlier diagnoses and more targeted interventions for FASD. Taken together, our results highlight that PAE directly impacts multiple aspects of social behavior and its underlying neurobiology and identifies potential targets for therapeutic intervention.

Lay Summary

Of the cognitive, physiological and behavioral impairments associated with prenatal alcohol exposure (PAE) documented in the clinical and pre-clinical literature, lifelong social behavior deficits serve as a unifying feature across the entire continuum of Fetal Alcohol Spectrum Disorders (FASD). Social behavior deficits emerge early in development and become more pronounced prior to and during adolescence, when the transition to a more complex social environment may exacerbate existing social behavior impairments. This proposal examines social behavior and its underlying neurobiology during the key developmental period of adolescence using a well-established animal model of PAE. Moreover, we also combine our model of PAE with a naturalistic model of early life adversity to understand how postnatal experience affects may further shape social behavior development in the context of PAE.

Preface

Portions of **Chapter 1** (introduction) have been modified from a previously published book chapter: Dobson, C. C., Holman, P. J., Comeau, W., Bodnar, T., Lam, V., Brien, J. F., Reynolds, J.N., & Weinberg, J. (2016). The Effects of Alcohol Exposure on Fetal Development. In N. Reissland & B. Kisilevsky (Eds.), Fetal Development (pp. 331–364). Cham: Springer International Publishing.

A version of the material presented in **Chapter 2** has been published as: Holman, P. J., Baglot, S. L., Morgan, E., & Weinberg, J. (2019). Effects of prenatal alcohol exposure on social competence: Asymmetry in play partner preference among heterogeneous triads of male and female rats. Developmental Psychobiology, 61(4), 513–524. J. Weinberg and I designed the experiments. S.L. Baglot and E. Morgan performed a portion of the behavioral analyses under my supervision. I executed the animal experiments with assistance from members of the Weinberg laboratory, performed behavioral and statistical analyses, and wrote the manuscript. All authors provided critical feedback and suggested edits prior to submission of the final manuscript.

A version of the material presented in **Chapter 3** has been published as: Holman, P. J., Ellis, L., Morgan, E., & Weinberg, J. (2018). Prenatal alcohol exposure disrupts male adolescent social behavior and oxytocin receptor binding in rodents. Hormones and Behavior, 105(June), 115–127. J. Weinberg and I designed the experiments. L. Ellis and I performed receptor binding assays. E. Morgan assisted with brain sectioning and image analysis for the hypothalamus under my supervision. I executed the animal experiments with assistance from members of the Weinberg laboratory, performed behavioral, image, and statistical analyses, conducted hormone

assays (with assistance from W. Yu), sectioned brains and wrote the manuscript. All authors provided critical feedback and suggested edits prior to submission of the final manuscript.

Chapter 4 is original and unpublished. J. Weinberg, C. Raineki and I designed the experiments. A. Chao, R. Grewal and S. Haghighat performed a portion of the behavioral analyses under my supervision. C. Fung, S. Sarkar and E. Morgan conducted a portion of the image analyses under my supervision. L. Ellis and I performed *in situ* hybridization assays. I executed the animal experiments with assistance from members of the Weinberg laboratory, performed behavioral, image and statistical analyses, sectioned brains and wrote the manuscript. J. Weinberg provided critical feedback on the content.

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The animal studies presented in this thesis were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of British Columbia Animal Care Committee (certificates: A06-0017, A10-0136, A10-0016, A10-0157, A14-0156, A14-0258, A15-0070).

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List of Abbreviations

aBNST	Anterior Bed Nucleus of the Stria Terminalis
ACC	Anterior Cingulate Cortex
aNAcc	Anterior Division of the Nucleus Accumbens
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorders
AVP	(Arginine) Vasopressin
BAL	Blood Alcohol Level
BL	Basal Nucleus of the Amygdala
BLA	Basolateral Complex of the Amygdala
BNST	Bed Nucleus of the Stria Terminalis
C	Control
CBCL	Child Behavior Checklist
CCE	Control-Control-PAE Play Triad
CCP	Control-Control-Pair Fed Play Triad
CeA	Central Nucleus of the Amygdala
CeL	Lateral Subdivision of the Central Amygdala
CeM	Medial Subdivision of the Central Amygdala
CoA	Cortical Nucleus of the Amygdala
CORT	Corticosterone
DTT	Dithiothreitol
EEC	PAE-PAE-Control Play Triad
ELA	Early Life Adversity
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorers

fMRI	Functional Magnetic Resonance Imaging
G	Gestational Day
HPA	Hypothalamic-Pituitary-Adrenal
HPG	Hypothalamic-Pituitary-Gonadal
IL	Infralimbic Cortex
i.p.	Intraperitoneal
IQ	Intelligent Quotient
ISH	*in situ* Hybridization
LA	Lateral Nucleus of the Amygdala
LLOD	Lower Limit of Detection
LS	Lateral Septum
magnoPVN	Magnocellular Division of the Paraventricular Nucleus of the Hypothalamus
MeA	Medial Nucleus of the Amygdala
mPFC	Medial Prefrontal Cortex
NAcc	Nucleus Accumbens
OB	Olfactory Bulb
OFC	Orbitofrontal Cortex
OR	Object Recognition
OT	Oxytocin
OTR	Oxytocin Receptor
P	Postnatal Day
PAE	Prenatal Alcohol Exposure
parvoPVN	Parvocellular Division of the Paraventricular Nucleus of the Hypothalamus
pBNST	Posterior Bed Nucleus of the Stria Terminalis

PBS	Phosphate Buffered Saline
PCX	Piriform Cortex
PF	Pair Fed / Pair Feeding
PFC	Prefrontal Cortex
pNAcc	Posterior Division of the Nucleus Accumbens
PPC	Pair Fed-Pair Fed-Control Play Triad
PrL	Prelimbic Cortex
PVN	Paraventricular Nucleus of the Hypothalamus
RCT	Randomized Control Trial
RIA	Radioimmunoassay
SD	Social Discrimination
SEM	Standard Error of the Mean
SON	Supraoptic Nucleus of the Hypothalamus
SSC	Saline-Sodium Citrate
SSRS	Social Skills Rating System
TEA	Triethanolamine-Hydrochloride
$V1_aR$	Vasopressin Receptor Subtype 1a
VABS	Vineland Adaptive Behavior Scales

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Dedication

This thesis is dedicated to Drs. Joanne Weinberg and Charlis Raineki.

Joanne, thank you for taking a chance on a high school teacher who wanted to make the leap back into research. To borrow a word you taught me – not just by defining, but by truly embodying – you are a real "mensch."

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Chapter 1: Introduction

1.1 General overview and hypotheses

Fetal Alcohol Spectrum Disorder (FASD) refers to the wide range of neurobiological, neurobehavioral and physiological impairments resulting from prenatal alcohol exposure (PAE; Manning & Hoyme, 2007; J. F. Williams, Smith, & the COMITTEE ON SUBSTANCE ABUSE, 2015). Among the associated impairments across the entire spectrum, social behavior deficits are pervasive (Kelly, Day, & Streissguth, 2000; Kully-Martens, Denys, Treit, Tamana, & Rasmussen, 2012; Mooney & Varlinskaya, 2011); indeed, impaired social function is the second most commonly reported behavioral deficit after attention among individuals with FASD (Steinhausen & Spohr, 1998). Notably, individuals with FASD are at an increased risk of experiencing early life adversity (ELA; O'Connor & Paley, 2006; Streissguth & O'Malley, 2000), which alone can produce long-term social behavior deficits (Conaway & Hansen, 1989; Raineki, Cortés, Belnoue, & Sullivan, 2012), making it difficult to separate effects of PAE from those of ELA on outcomes. PAE-related social behavior deficits emerge early in development and become more pronounced around adolescence (Duquette, Stodel, Fullarton, & Hagglund, 2006; Streissguth, Barr, Kogan, & Bookstein, 1996; S. E. Thomas, Kelly, Mattson, & Riley, 1998). Importantly, adolescence is a critical period of development encompassing significant maturational changes that can have dramatic consequences for social behavior, making this a unique period of increased vulnerability (Blakemore, 2008; Blakemore, Burnett, & Dahl, 2010; Sisk & Zehr, 2005).

The overarching goal of my dissertation is to investigate how PAE can impact adolescent social behavior and elucidate possible mechanisms underlying these impairments, concentrating particularly on alterations of oxytocin (OT) and vasopressin (AVP) – key neuropeptides involved

in regulating social behavior – and focusing on key neural structures that comprise the "social behavior neural network" (Benarroch, 2013; Newman, 1999). Moreover, given the critical role of postnatal experience in shaping social behavior development (Kelly, Goodlett, & Hannigan, 2009; Pryce & Feldon, 2003), embedded in our comprehensive assessment of adolescent social behavior are progressive increases to the complexity of the social environment and testing parameters. Similarly, we also combine our well-established model of PAE with a naturalistic model of ELA to begin to understand the unique and potentially interactive effects of these two perinatal insults. Specifically, we will test the hypothesis that PAE results in social behavior impairments that stem, at least in part, from altered development of the OT/AVP systems in the brain. The significance of my dissertation is its potential contribution to establishing a more distinctive neurobehavioral profile that could support the development of specific strategies for earlier diagnoses and more targeted interventions for FASD.

1.2 PAE and FASD

Fetal Alcohol Syndrome (FAS), which represents the most severe form of FASD, was first described contemporaneously by physicians who noticed a pattern of characteristic facial dysmorphology, central nervous system abnormalities and growth retardation among infants with a history of PAE (Jones & Smith, 1973; Lemoine, Harouseau, Borteryu, & Menuet, 1968). Because facial dysmorphology can serve as a reliable physical marker of PAE, FAS can be diagnosed even in the absence of maternal confirmation of alcohol consumption. However, it was noted soon after these first reports that doses and patterns of maternal alcohol consumption that do not result in full FAS can still lead to a range of significant cognitive and neurobehavioral impairments (Petrelli, Weinberg, & Hicks, 2018). From a clinical standpoint, diagnosis of an FASD in the absence of facial dysmorphology can be challenging, in part because these

diagnoses require confirmation of alcohol exposure during gestation (Mattson, Crocker, & Nguyen, 2011; Oberlander et al., 2010). Obtaining confirmation of maternal alcohol consumption can be difficult for many reasons, including underreporting by birth mothers due to stigmatization associated with acknowledging alcohol use during pregnancy or a lack of maternal history, such as occurs among children living in foster care (Bertrand, Floyd, & Weber, 2005). Notably, a recent metanalysis suggests an overrepresentation of FASD among children in care, estimating a 32 times higher prevalence than is observed in the general population (Popova, Lange, Shield, Burd, & Rehm, 2019). Without a reliable biomarker to identify evidence of PAE, diagnosis of an FASD for children without facial dysmorphology is further complicated by the heterogenous and complex neurocognitive and behavioral presentations among individuals with PAE (Mattson, Bernes, & Doyle, 2019). Indeed, the difficulty in identifying individuals affected by PAE has likely led to an underestimation of its already high prevalence (2-5%) in the general population (Chasnoff, Wells, & King, 2015; May & Gossage, 2001; May et al., 2009; Warren & Foudin, 2001). Moreover, individuals with FAS constitute a relatively small proportion of FASD among epidemiological prevalence estimates (May et al., 2018). Taken together, these findings highlight the critical need for more distinctive neurobehavioral profiles that could support diagnostic, as well as more targeted interventions for FASD.

To aid in the description and identification of the spectrum of adverse effects associated with PAE, multiple diagnostic schema have been developed. Beside assessment for facial dysmorphology and growth restriction, diagnosis of an FASD requires documentation of neurocognitive and behavioral impairments, which can vary across different domains including learning and memory, self-regulation and adaptive function (Warren, Hewitt, & Thomas, 2011). Deficits in neurocognitive/behavioral function result from PAE-related damage to brain

development, and manifest as developmental delays, low IQ, attention difficulties, impaired executive function, impulsivity, learning disabilities, as well as poor adaptive functioning (Cook et al., 2016; Hoyme et al., 2016). Moreover, the level of impairment typically – but not always – aligns to diagnosis along the FASD spectrum (i.e., individuals with FAS are most severely affected). Deficits in these domains appear early in development and persist into adulthood, as individuals with FASD frequently lag behind age-matched, unexposed peers throughout development. Of the cognitive, physiological and behavioral impairments associated with PAE, lifelong social behavior deficits serve as a unifying feature across the entire continuum of FASD (Kully-Martens et al., 2012). Though the complex pattern of neurobehavioral deficits likely mediate and/or contribute to impaired social function, social behavior deficits are not merely a secondary effect of cognitive deficits or intellectual function (Mattson & Riley, 2000); indeed, several studies have shown that PAE-related impairments in social behavior function persist even when experimental or statistical controls for cognitive function are employed (Doyle et al., 2019; S. E. Thomas et al., 1998). In this way, PAE appears to directly impact on social neurobehavioral function, however, the underlying mechanisms that lead to impaired social behavior in individuals with FASD requires further study. Moreover, because of considerable overlap in characteristics of FASD and other childhood disorders with social behavior impairments – including autism spectrum disorders (ASD) – reaching a definitive diagnosis presents a challenge (Benz, Rasmussen, & Andrew, 2009; Coles et al., 1997). Likewise, many clinical studies of FASD collapse subjects across gender or are underpowered to investigate these differences, making the study of potential sex differences critically important (Beery & Zucker, 2011; Kully-Martens et al., 2012).

1.3 Social behavior

Social behavior encompasses the reciprocal interactions among two or more individuals of a group, and is generally split into two broad categories: affiliative (e.g., behaviors that brings individuals together) and agonistic (e.g., behaviors that keep individuals apart) behaviors (Caldwell, 2017). When considering different neurocognitive processes, social behavior is often treated as its own domain. However, social behavior can be considered as the mobilization of interconnected neurocognitive functions – such as sensory processing, learning and memory, motivation, emotional regulation, and executive function – in the service of social interaction (Adolphs, 2003). Human social behavior can be considered under the umbrella of adaptive function, defined as the ability to handle everyday demands of life, live independently, and utilize "social skills." Social skills involve the range of social behaviors required to navigate our intensely social world, including the ability to initiate and manage social interactions, establish and maintain short- and long-term relationships, express and interpret emotions, and successfully use interpersonal/communication skills (Little, Swangler, & Akin-Little, 2017). Moreover, social behavior does not simply involve behaviors that are expressed, but also requires the ability to inhibit inappropriate/negative behaviors. In this way, social behavior can be considered a dynamic collaboration, involving not only the accurate perception of social stimuli and subsequent execution of appropriate social responses, but also the capacity to elicit appropriate behavioral reciprocity to sustain social interactions.

Development of social behavior is an ongoing process that involves the complex interaction of behavioral, cognitive, and emotional factors (Milligan, Sibalis, Morgan, & Phillips, 2017; Nelson, 2017). Given the reciprocal nature of social interactions, development of social behavior is also shaped by experience, such that the changing social demands experienced across

development further guide and refine the social behavioral repertoire (Kendrick, Haupt, Hinton, Broad, & Skinner, 2001; Parent & Meaney, 2008; Sevelinges et al., 2007). Two critical neurobehavioral processes underlying social behaviors are social motivation (Caldwell & Albers, 2016) and social recognition memory (Camats Perna & Engelmann, 2017). Social motivation refers to the drive to engage or not in social interactions and depends on the perceived positive or negative valence of social stimuli. Social recognition memory – or the ability to learn and distinguish between familiar and novel conspecifics – builds on social motivation and allows organisms to use experience to guide future behaviors (Bielsky & Young, 2004). Reflecting the experience-dependent nature of social behavior development, key social behaviors emerge and expand across development, beginning with attachment to the caregiver during the early postnatal period, play behavior during the juvenile/early adolescent period, and morphing into more complex behaviors such as aggression, pair bonding, and parental behavior into adulthood (Insel, 2000; Meaney & Stewart, 1981).

1.4 Adolescence and social behavior development

Adolescence refers to the unique transitional period from childhood to adulthood, and encompasses a variety of sexually dimorphic developmental changes in behavioral, cognitive, physiological and neurological parameters, including the attainment of sexual maturity (i.e. puberty; Brenhouse & Andersen, 2011; McCormick & Mathews, 2010; Vetter-O'Hagen & Spear, 2012). Adolescence also involves significant alterations in social demands and behavioral repertoire, as this period involves expansions in social networks and increases in peer-directed social interaction (Spear, 2000). For example, male and female rats show a dramatic age-related change in social behavior expression during early adolescence that includes peak levels of play

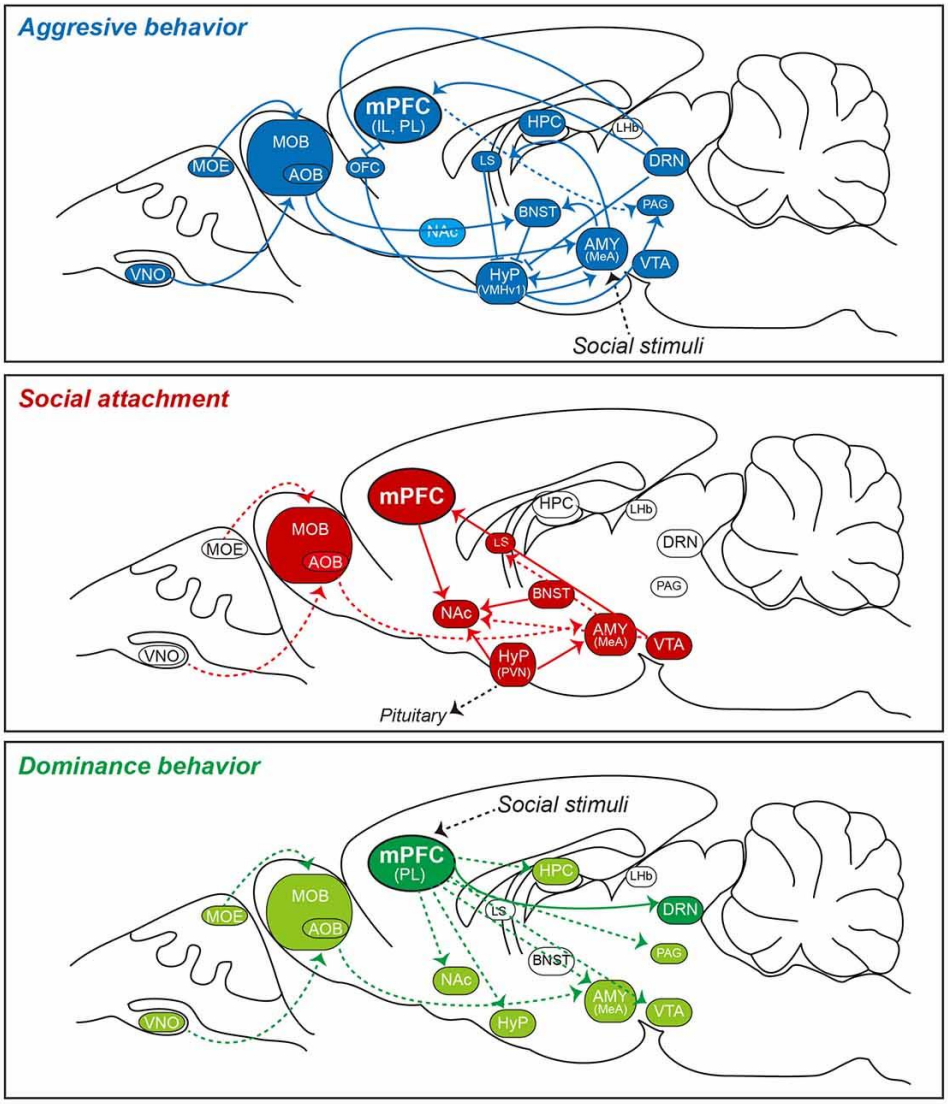
behavior occurring at postnatal day (P) 35 (Meaney & Stewart, 1981; Panksepp, Siviy, & Normansell, 1984; Thor & Holloway, 1982).

Many of the diverse maturational changes occurring during adolescence depend on increased secretion of gonadal hormones that occurs at puberty, making pubertal onset an important landmark of adolescent development (Brenhouse & Andersen, 2011). For example, steroid-dependent organization of neural circuits can have significant consequences for social behavior development, making adolescence a unique period of increased vulnerability to social behavior dysfunction (Schulz, Molenda-Figueira, & Sisk, 2009; Sisk & Zehr, 2005). Timing of pubertal onset in rats is also sexually dimorphic, and can be estimated by changes in physical markers such as vaginal opening (~P32) and preputial separation (~P39; McCormick & Mathews, 2007). Separating puberty-dependent from puberty-independent maturation processes is complex, especially given earlier pubertal onset in females. To examine sex differences during adolescence there are two approaches: pubertal-dependent effects can be assessed by testing males and females at similar pubertal stages, which has the confound of testing at different ages; or pubertal-independent effects can be assessed by testing males and females at the same age, which has the confound of testing at different pubertal stages.

1.5 Social behavior neurocircuitry

The neurobiology of social behavior involves the complex interplay of many neural structures and neuroendocrine systems collectively referred to as the "social behavior neural network" (Figure 1.1; Bielsky & Young, 2004; Ferguson, Young, & Insel, 2002; Newman, 1999; Ross & Young, 2009; Veenema & Neumann, 2008). Included in this interconnected neural network are the olfactory bulb (OB) and associated olfactory cortices such as the piriform cortex (PCX; Richter, Wolf, & Engelmann, 2005; Young, 2002), lateral septum (LS; Albers, 2012),

Figure 1.1 Social behavior neural network



Simplified schematic of proposed social neurocircuits comprising the social behavior neural network. Abbreviations: AMY, amygdala; AOB, accessory olfactory bulb; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; DRN, dorsal raphe nucleus; HPC, hippocampus; Hyp, hypothalamus; IL, infralimbic division of the mPFC; LHb, lateral habenula; LS, lateral septum; MeA, medial amygdala; mPFC; medial prefrontal cortex; MOB, main olfactory bulb; MOE, main olfactory epithelium; MPOA, medial preoptic area; NAc, nucleus accumbens; OFC, orbitofrontal cortex; PAG, periaqueductal gray; PL, prelimbic division of the mPFC; VMHv1, ventrolateral subdivision of the ventromedial hypothalamus; VNO, vomeronasal organ; and VTA, ventral tegmental area. From (Ko 2017).

amygdala (Amaral, 2003; Katayama et al., 2009), bed nucleus of the stria terminalis (BNST; DiBenedictis, Nussbaum, Cheung, & Veenema, 2017), prefrontal cortex (PFC; Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009; Diamond, 2011) and hypothalamus (Ross & Young, 2009). Each of these brain regions have been implicated in modulating several aspects of social behavior function in multiple species (Amaral, 2003; Emery et al., 2001; K. M. Thomas et al., 2001).

Conspecific odor cues serve as salient social sensory stimuli – particularly in rodents – and have been shown to guide a variety of social behaviors, including attachment, social recognition memory, and sexual behavior (Camats Perna & Engelmann, 2017). Accordingly, the OB and PCX detect and provide the initial processing of these socially relevant olfactory stimuli (Richter et al., 2005; Young, 2002). These olfactory areas project to limbic and cortical areas – such as the amygdala, LS, and PFC – which provide higher-order processing and integration that support social recognition memory and subsequent behavioral responses (Dias, Golino, Oliveira, Moraes, & Pereira, 2016). The amygdala is a critical hub of the social behavior neural network and is thought to be involved in socioemotional processing by assigning salience to socially relevant sensory stimuli and connecting this information with brain regions mediating cognitive processes like motivation, emotion and executive function (Adolphs, 2001, 2010; Camats Perna & Engelmann, 2017; Garrido Zinn et al., 2016; Tanimizu et al., 2017). The amygdala can be subdivided into several interconnected subnuclei – including the lateral (LA), basal (BA), central (CeA), cortical (CoA), and medial (MeA) subnuclei – which each differentially contribute to socioemotional processing (McDonald, 1998). Despite a more prominent role for the MeA, all of the amygdala subnuclei participate in multiple aspects of social behavior (Insel & Shapiro, 1992; Katayama et al., 2009; Maaswinkel, Baars, Gispen, & Spruijt, 1996). Indeed, amygdala lesions result in severe social behavior impairments, such as decreased play behavior in rats (Daenen,

Wolterink, Gerrits, & Van Ree, 2002; Meaney, Dodge, & Beatty, 1981), reductions in affiliative behavior in voles (Kirkpatrick, Carter, Newman, & Insel, 1994) and altered social communication in primates (Rasia-filho, Londero, & Achaval, 2000). Additionally, expression of the immediate early gene *c-fos*, a marker of neural activity (Guzowski, Setlow, Wagner, & McGaugh, 2001), increases in the medial amygdala following maternal behavior in rats (Fleming, Suh, Korsmit, & Rusak, 1994) and after nonsexual social encounters in voles (Kirkpatrick et al., 1994).

The amygdala communicates bidirectionally with the LS, a forebrain region associated with social motivation and essential for intact social recognition memory formation, particularly in males; indeed, silencing of the LS can block social recognition memory (Engelmann, Wotjak, & Landgraf, 1995; Lukas, Toth, Veenema, & Neumann, 2013; Mesic et al., 2015; Popik & van Ree, 1999; Sheehan & Numan, 2000; Veenema, Bredewold, & De Vries, 2012). Also located in the basal forebrain, the nucleus accumbens (NAcc) functions classically as part of the reward circuitry, and in the present context, supports aspects of social motivation and social reward (Caldwell & Albers, 2016; Dölen & Malenka, 2014).

The amygdala also has reciprocal connections with the PFC, and this amygdala-PFC circuit within the social behavior neural network is important for social approach and coordinating socially appropriate responses as well as the encoding of social memories (Bicks, Koike, Akbarian, & Morishita, 2015; Gee et al., 2013; Tan et al., 2019). The PFC is generally associated with higher cognitive tasks including executive function and behavioral flexibility (Arnsten, 1998; Diamond, 2011; Floresco, Seamans, & Phillips, 1997), and thus it is not surprising that this region is also essential for the cognitive demands of social behavior function (Adolphs, 2001; Anderson, Damasio, Tranel, & Damasio, 2010). Consistent with this

interpretation, the PFC and amygdala show functional connectivity as demonstrated by correlated neural activity (*c-fos*) during social play behavior among adolescent male rats (van Kerkhof et al., 2014). However, the PFC is not a single structure, but consists of multiple subregions including the medial PFC (mPFC) – which can be further subdivided into the infralimbic (IL), prelimbic (PrL), and anterior cingulate (ACC) cortices – as well as the orbitofrontal cortex (OFC; Felix-Ortiz, Burgos-Robles, Bhagat, Leppla, & Tye, 2016; Kita & Kitai, 1990; Ko, 2017; Mcdonald, 1998; Swanson, 2003). The mPFC is thought to mediate social behavior function via top-down control of subcortical regions, including the BL and LA (Bicks et al., 2015). Moreover, *c-fos* expression increases in the mPFC following social recognition, while inhibiting protein synthesis in this region blocks consolidation of social recognition memory (Minami, Shimizu, & Mitani, 2017; Tan et al., 2019; Tanimizu et al., 2017). Subregions of the mPFC appear to serve slightly different functions in regulating social behavior function. For example, the IL and PrL seem to be especially important for encoding information about environmental social contexts, as these regions show robust increases in neural activity among mice establishing dominance hierarchies following removal of an alpha male (Williamson, Klein, Lee, & Curley, 2019). In contrast, the ACC has also been hypothesized to process the motivational states of social conspecifics during social interactions (Apps, Rushworth, & Chang, 2016), and lesion studies have demonstrated an important role for the ACC in social recognition memory in rodents and non-human primates (Rudebeck, Buckley, Walton, & Rushworth, 2006; Rudebeck et al., 2007). Specifically, the ACC has been shown to influence responsiveness to social stimuli, such that male rats with ACC lesions fail to habituate to repeated presentations of a social stimulus. In contrast to the mPFC, the OFC appears to encode information about stimulus reward value to guide social and non-social behavioral responses (Bell et al., 2009;

Brenhouse & Andersen, 2011; Rolls, 2000). Indeed, previous research has shown that the OFC shapes behavioral responses to a play partner based on the partner's identity within a dominance hierarchy (e.g., dominant or subordinate; Himmler et al., 2018; Pellis et al., 2006).

1.6 Oxytocin and vasopressin: Neuropeptide modulators of social behavior

Oxytocin (OT) and vasopressin (AVP) are hypothalamic neuropeptides that have been implicated in the regulation of multiple aspects of social behavior across the lifespan (Dore, Phan, Clipperton-Allen, Kavaliers, & Choleris, 2013), including social motivation (Lim & Young, 2006), social recognition memory (Bielsky & Young, 2004; Engelmann, Ludwig, & Landgraf, 1994; Ferguson et al., 2002), parental behavior (Insel, 2000; Rilling & Mascaro, 2017), as well as aggression (Ferris, 1992; Veenema, 2012). OT/AVP are produced in magnocellular and parvocellular neurons located in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus; AVP is also synthesized in extrahypothalmic sites such as the amygdala and BNST (Neumann & van den Burg, 2013; Veenema & Neumann, 2008). Magnocellular PVN/SON neurons primarily project to the posterior pituitary from which OT/AVP are released into the peripheral circulation to regulate several homeostatic processes, including osmoregulation (Benarroch, 2013; Veenema & Neumann, 2008; Wotjak et al., 1998). Magnocellular collaterals also project to limbic and forebrain regions associated with social behavior function (Althammer & Grinevich, 2018). Parvocellular OT/AVP neurons project to many areas of the brain and contribute to socio-emotional processing and social behavior expression (Landgraf & Neumann, 2004). Microdialysis studies in rodents indicate coordinated release of central/peripheral OT but not AVP following certain stressful or social stimuli (Babygirija, Bülbül, Yoshimoto, Ludwig, & Takahashi, 2012; Landgraf & Neumann, 2004;

Wotjak et al., 1998), suggesting plasma OT may serve as a biomarker of central OT activity, though there are conflicting reports (Kagerbauer et al., 2013).

Receptors for OT (OTR) and AVP ($V1_aR$) are widely distributed in the brain and correlate with sites of peptide release, particularly within the social behavior neural network (Neumann & van den Burg, 2013). Specifically, OTRs are localized in the OB, the central, medial and basolateral nuclei of the amygdala, (Rosenfeld, Lieberman, & Jarskog, 2011), the LS, BNST, NAcc, ventromedial hypothalamus and to a lesser extent in PFC (Ostrowski, 1998; Yoshida et al., 2009), while $V1_aRs$ are expressed in the central nucleus of amygdala, LS and BNST (Ostrowski, Lolait, & Young, 1994). Interestingly, in brain areas where both receptors are coexpressed, OTR and $V1_aRs$ are segregated into distinct subregions (Stoop, 2012). For example, within the CeA, OTRs reside in the lateral subdivision (CeL) while $V1_aRs$ occupy the medial subdivision (CeM), and these complimentary distribution patterns are thought to mediate some of the opposing actions OT/AVP may produce within the same brain region (Stoop, Hegoburu, & van den Burg, 2015). Adding to the complexity of OT/AVP systems regulation of social behavior is the observation that these neuropeptides have significant crosstalk between receptors (Song & Albers, 2017).

Given the high expression of OTR/$V1_aRs$ within the social behavior neural network, it is not surprising that OT/AVP activity within these circuits modulate multiple aspects of social behavior function. OT/AVP agonist and antagonist infusion studies have provided important information about the roles of these neuropeptide systems (van der Kooij & Sandi, 2012). In the CeA, for example, OT signaling appears to regulate social interest, as OTR antagonism attenuates social investigation of juvenile conspecifics (Dumais, Alonso, Bredewold, & Veenema, 2016). Additionally, a positive correlation between aggression and OTR binding in

CeA has also been described (Calcagnoli et al., 2014). Importantly, OT activity within the mPFC-amygdala circuit is thought to be an important modulator of social behavior (Adolphs, 2010; Bredewold & 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 may mediate, at least in part, developmental changes in social behavior expression (Anderson et al., 2010; Diamond, 2002; Steinberg, 2005). AVP, and to a lesser extent OT, activity in the LS appears to be a major regulator of social recognition memory. Indeed, 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) each results in social recognition deficits. Moreover, septal infusion of AVP (Engelmann & Landgraf, 1994) or OT (Popik & van Ree, 1999) enhances social recognition. Within the NAcc, OTR antagonism attenuates social novelty seeking (Smith et al., 2017) as well as social reward associated with social interaction (Dölen, Darvishzadeh, Huang, & Malenka, 2013).

1.7 Hypothalamic-pituitary-gonadal axis: Interaction with OT/AVP systems

Sex hormone increases occurring at puberty strongly influence OTR/$V1_aR$ localization and, therefore, their sexually dimorphic function (Cushing, 2013; Dore et al., 2013). Indeed, OTR/$V1_aR$ localization is correlated with specific behavioral strategies of males and females (Insel, Young, Witt, & Crews, 1993; Neumann & van den Burg, 2013). In general, OT/AVP regulate many of the same social behaviors, though the literature suggests a more prominent role for OT in females and AVP in males (Insel & Young, 2001; Veenema et al., 2012). One of the

best examples of sexually dimorphic OT/AVP function comes from pair bonding studies in monogamous prairie voles, where partner preference could be blocked in females with OT antagonist infusions into the NAcc or PFC, while in males, partner preference could be blocked with $V1_aR$ antagonism within the ventral pallidum (Young & Wang, 2004). These differences are due partly to the organizational/activational effects of gonadal hormones on OT/AVP systems (Dhakar, Stevenson, & Caldwell, 2013). Evidence for hypothalamic-pituitary-gonadal and OT interactions comes from observations of correlated peaks in estradiol and hypothalamic OT at pubertal onset. Additionally, alterations in OT expression are synchronous with the estrous cycle; likewise, ovariectomy reduces OT mRNA expression that is restored by estradiol replacement (Miller, Ozimek, Milner, & Bloom, 1989). Conversely, gonadectomized male and intact female rats show reduced AVP mRNA expression, and testosterone treatment in both groups increases AVP mRNA expression in the medial amygdala and BNST (De Vries, Wang, Bullock, & Numan, 1994; Z. Wang & De Vries, 1993). Besides hypothalamic-pituitary-gonadal (HPG) effects on OT/AVP transcription, sex steroids can have direct effects on neuropeptide production, release and neurotransmission (Dhakar et al., 2013).

Notably, previous research has documented alterations in HPG development and AVP function in both males and females following PAE. PAE can delay pubertal onset in both males (Lan, Hellemans, Ellis, Viau, & Weinberg, 2009; Mcgivern, Raum, Handa, & Sokol, 1992) and females (Lan, Yamashita, et al., 2009; McGivern & Yellon, 1992). Moreover, testosterone has reduced effects on central CRH pathways but greater effects on AVP pathways in PAE compared to control males (Lan, Hellemans, et al., 2009). PAE females have higher basal and stress E_2 levels in proestrus compared to other phases of the cycle (Lan, Yamashita, et al., 2009).

Furthermore, AVP mRNA levels are increased overall in PAE compared to control females (Lan et al., 2006).

1.8 Social behavior deficits in FASD

Social behavior deficits following PAE have been characterized across the lifespan, and are the second most commonly reported behavioral impairment after attention among individuals with FASD (Steinhausen & Spohr, 1998). Early in development, alcohol-exposed infants show disorganized attachment to caregivers as assessed using the Ainsworth strange situation procedure (O'Connor, Sigman, & Kasari, 1992) and show fewer "social monitoring" behaviors (e.g., attentive head orienting and eye contact with the caregiver) following a still-face paradigm (Jirikowic, Chen, Nash, Gendler, & Carmichael Olson, 2016). In addition to these more direct measures of PAE-related social impairments, alcohol-exposed infants also exhibit increased irritability as well as disrupted sleep/feeding cycles (Coles & Platzman, 1993; Platzman, Coles, Lynch, Bard, & Brown, 2001). Social deficits persist and worsen as the child reaches adolescence, as demonstrated by poor performance on various behavioral assessments such as the Vineland Adaptive Behavior Scales (VABS), Child Behavior Checklist (CBCL) as well as the Social Skills Rating System (SSRS; Carmichael Olson, Feldman, Streissguth, Sampson, & Bookstein, 1998; Rasmussen, Becker, McLennan, Urichuk, & Andrew, 2011; S. E. Thomas et al., 1998; Whaley, O'Connor, & Gunderson, 2001). Specifically, adolescents with FASD appear unresponsive to social cues, exhibit low peer cooperation, and show difficulties in establishing reciprocal friendships (Kully-Martens et al., 2012). Importantly, social behavior deficits are not merely a secondary effect of cognitive deficits or intellectual function (Doyle et al., 2019; Mattson & Riley, 2000); indeed, children with FAS scored significantly lower on general social skills when compared to unexposed children with similar verbal IQs (S. E. Thomas et al., 1998).

FASD-related social impairments persist into adulthood, as affected individuals continue to exhibit deficits in social responsiveness and interpersonal relationships (Kelly et al., 2000; Kully-Martens et al., 2012), and consistently lag behind peers in social behavior function (Streissguth et al., 1991). Additional indirect evidence of deficient social behavior comes from reports showing that adults with FASD are more likely to have "secondary disabilities," including disrupted school experiences, trouble with the law, and inappropriate sexual behavior (Kelly et al., 2000; Streissguth et al., 1996).

1.9 Animal models of PAE-related social behavior deficits

Animal models of PAE have shown social neurobehavioral deficits parallel to those observed in individuals with FASD (Hamilton et al., 2010; Hellemans, Verma, et al., 2010; Kelly et al., 2000; Kelly, Goodlett, et al., 2009). PAE disrupts mother-pup attachment, as PAE pups show increased latency to nipple attach and a reduction in overall time spent nipple attached (Subramanian, 1992). Moreover, PAE pups also show reduced vocalizations as well as aberrant isolation-induced vocalizations (Barron, Segar, Yahr, Baseheart, & Willford, 2000; Kehoe & Shoemaker, 1991; Marino, Cronise, Lugo, & Kelly, 2002; Raineki et al., 2017). Additionally, PAE rat pups show a diminished ability to elicit maternal retrieval, even by a foster dam that never consumed alcohol (Ness & Franchina, 1990). Social deficits persist into adolescence, with disruptions in play behavior commonly reported (Charles Lawrence, Cale Bonner, Newsom, & Kelly, 2008; Lugo, Marino, Cronise, & Kelly, 2003; Meyer & Riley, 1986; Mooney & Varlinskaya, 2011; Royalty, 1990; Waddell, Yang, Ho, Wellmann, & Mooney, 2016). In addition to impaired play behavior, preclinical research has identified deficits in adolescent social interaction (Mooney & Varlinskaya, 2011; Varlinskaya & Mooney, 2014) and social motivation (Diaz, Mooney, & Varlinskaya, 2016). Social behavior deficits in adolescence can

have long-lasting effects on social development (Auger & Olesen, 2009), as PAE effects are still present in adulthood, including increased aggression (Royalty, 1990) and atypical social interactions (Hamilton et al., 2010). For example, adult PAE rats show sexually dimorphic alterations of social interactions, such that males but not females show a reduction in affiliative behaviors (Hellemans, Verma, et al., 2010); however more work is needed to elucidate underlying mechanisms supporting social behavior deficits during adolescence.

PAE produces sexually dimorphic structural and functional alterations in structures within the social behavior neural network. For example, in the amygdala, PAE is associated with increased dendritic length and synaptic spine number (Cullen, Burne, Lavidis, & Moritz, 2013), increased glutamatergic activity and diminished GABAergic inhibition in the amygdala (Baculis, Diaz, & Fernando Valenzuela, 2015; Zhou, Wang, & Zhu, 2010), disrupted cytokine balance (Raineki et al., 2017) and reduced DNA concentration – a surrogate measure of cell number (Kelly & Dillingham, 1994). The PFC also shows PAE-related changes, including atypical dendritic length and neural activity (Hamilton et al., 2010), reduced spine density and altered distribution patterns (Charles Lawrence, Otero, & Kelly, 2012), diminished overall cell numbers (Mihalick, Crandall, Langlois, Krienke, & Dube, 2001), as well as irregular expression of activity-related immediate early genes (Heroux, Robinson-Drummer, Kawan, Rosen, & Stanton, 2019). Unfortunately, few studies have assessed the effects of PAE on the amygdala and PFC during adolescence, when the PFC is undergoing significant maturational changes (Diamond, 2002; Steinberg, 2005). Importantly, the PFC shows relatively protracted development through adolescence (Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000), suggesting ongoing functional consequences for PFC-amygdala interactions on social behavior development – particularly in the context of ELA.

Despite their well-characterized role in regulating social behavior and other key functions, more research is needed to assess the OT/AVP systems with respect to PAE-related social behavior impairments. Kelly et al. demonstrated that PAE results in social recognition memory deficits and reductions in OTR binding in amygdala of female adult rats (Kelly, Leggett, & Cronise, 2009). PAE has previously been shown to reduce hypothalamic OT in adult voles (Feng et al., 2019; F. Q. He, Zhang, & Guo, 2012) but not in adult rats with both PAE and nicotine exposure (S. K. Williams et al., 2009). Moreover, PAE alters sensitivity of central AVP pathways to testosterone in adult males, as gonadectomized PAE rats show enhanced AVP expression in the BNST and MeA following high physiological testosterone replacement (Lan, Hellemans, et al., 2009; Lan et al., 2006). Taken together, these findings suggest an enduring effect of PAE on OT/AVP systems; however, the role of OT/AVP in mediating the deleterious effects of PAE on social behavior needs further study, particularly in the context of adolescent development.

1.10 Early-life adversity and social behavior

The clinical and preclinical literature have consistently provided data demonstrating that early-life adversity (ELA) – including trauma, physical/sexual abuse, and/or neglect – can have dramatic and long-lasting negative consequences on development, including impaired social behavior function and increased risk for developing mental health problems (Alink, Cicchetti, Kim, & Rogosch, 2012; Andersen & Teicher, 2008; Conaway & Hansen, 1989; McEwen, 2003; Shonkoff et al., 2012). This has important ramifications for individuals with FASD, as children prenatally exposed to alcohol are at increased risk of experiencing ELA (Price, Cook, Norgate, & Mukherjee, 2017; Streissguth & O'Malley, 2000). Indeed, it is not uncommon for children with FASD to live in unstable and/or stressful environments, which may include multiple foster care

placements or living with an alcoholic parent (Streissguth et al., 2004). Additionally, children with FASD are at a higher risk for sexual and/or physical abuse (O'Connor & Paley, 2006). Dissociating the contribution of each insult is extremely difficult, if not impossible, in clinical studies, due not only to the fact that they often co-occur but also to an inability to control for the many PAE and ELA exposure parameters. Relatively few clinical studies have systematically investigated social behavior function following combined PAE and ELA exposure during adolescence. However there is some evidence that, perhaps not unexpectedly, alcohol-exposed children who also experience ELA have poorer social function than children with ELA alone (Henry, Sloane, & Black-Pond, 2007).

Animal models have been successful in reproducing various aspects of social behavior deficits following ELA, including alterations in infant attachment and adult social interaction (Raineki et al., 2012; Raineki, Moriceau, & Sullivan, 2010; Rincón-Cortés & Sullivan, 2016). Specifically, rat dams are provided with limited bedding during the early postnatal period, which increases abusive-like maternal behaviors such as rough handling of and stepping on pups, reduces arched-backed nursing and results in inconsistent patterns/sequences of maternal care (Molet, Maras, Avishai-Eliner, & Baram, 2014; Raineki et al., 2017; Walker et al., 2017). Importantly, rat pups reared using this paradigm show increased basal levels of the stress hormone corticosterone; of note, this occurs during the stress hyporesponsive period (~P4-14), a period of development characterized by low levels of corticosterone and a diminished ability to respond to stress (Levine, 2006).

ELA-related changes in mPFC-amygdala circuitry/development are well-documented, including data obtained from the clinical literature (Fan et al., 2014; Gee et al., 2013; Tottenham et al., 2010) as well as preclinical models using low bedding (Fan et al., 2014; Rincón-Cortés &

Sullivan, 2016) and maternal separation (Brenhouse, Lukkes, & Andersen, 2013; Chocyk et al., 2013; Holland, Ganguly, Potter, Chartoff, & Brenhouse, 2014; Muhammad & Kolb, 2011; Sandi & Haller, 2015), which generally show precocial functional coupling of this circuit. Interestingly, ELA has also been shown to alter the OT/AVP systems in humans and animal models (Seltzer, Ziegler, Connolly, Prososki, & Pollak, 2014; Veenema, 2012). For example, childhood abuse has been associated with decreased OT in urine of children (Fries, Ziegler, Kurian, Jacoris, & Pollak, 2005) and in cerebrospinal fluid of adult women (Heim et al., 2009). Animal models of ELA – most of which have employed maternal separation – have also shown reductions of hypothalamic OT and increases in AVP (Veenema, 2012). In view of these data, it is likely that ELA may exacerbate any PAE-related changes in social behavior neurobiology, however, it remains unclear as to how ELA and PAE may interact to alter the OT/AVP systems.

1.11 Thesis overview

The experimental data in this thesis will be presented in three chapters, addressing the specific hypotheses listed in section 1.1 above. Chapter 2 – entitled "Effects of prenatal alcohol exposure on social competence: Asymmetry in play partner preference among heterogeneous triads of male and female rats" – evaluates play partner preference within same-sex triads comprised of animals from mixed prenatal treatments to understand how the social environment may shape PAE-related social behavior deficits, particularly in more complex social contexts. Chapter 3 – entitled "Prenatal alcohol exposure disrupts male adolescent social behavior and oxytocin receptor binding in rodents" – presents 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 followed by assessment of OTR/$V1_aR$ binding in the social behavior neural network. Chapter 4 – entitled

"Prenatal alcohol exposure and early-life adversity: Unique and interactive effects on the neurobiology of adolescent social recognition memory" – builds on the neurobehavioral profile from Chapter 3, combining animal models of PAE and ELA to investigate their unique and/or interactive effects on social recognition memory followed by assessment of neural activity (*c-fos*) within key regions of the social behavior neural network as well as hypothalamic OT/AVP expression. Finally, the concluding chapter will integrate the major findings from the data chapters and discuss the potential limitations as well as future directions for research.

Chapter 2: Effects of prenatal alcohol exposure on social competence: Asymmetry in play partner preference among heterogeneous triads of male and female rats

2.1 Introduction

Fetal Alcohol Spectrum Disorder (FASD) refers to the spectrum of physical, neurobehavioral and physiological impairments resulting from prenatal alcohol exposure (PAE; Williams, Smith, & the COMMITTEE ON SUBSTANCE ABUSE, 2015). Among the associated impairments across the entire spectrum, social behavior deficits are pervasive and have widespread implications for other domains such as executive function and emotional processing (Kully-Martens et al., 2012; Streissguth et al., 1996). PAE-related social behavior deficits emerge early in development and become more pronounced as the individual approaches adolescence, when the transition to a more complex social environment may exacerbate existing social behavior impairments (Duquette et al., 2006; Streissguth et al., 1996; S. E. Thomas et al., 1998). Social behavior deficits in individuals with FASD are frequently described in terms of impaired social competence, which can broadly be defined as the effectiveness in social interaction or the ability to employ social skills successfully within various interpersonal contexts (Dodge, 1986; Rose-Krasnor, 1997).

Development of social competence is an ongoing process that begins in infancy through interactions with the primary caregiver and involves the complex interaction of behavioral, cognitive, and emotional factors (Milligan et al., 2017; Parent & Meaney, 2008; Raineki et al., 2015). PAE has been shown to impair the development of social competence in the early postnatal period, as alcohol-exposed infants may show disorganized attachment to caregivers

(O'Connor et al., 1992; Platzman et al., 2001). As children with FASD transition into adolescence, impairments in social competence persist and generally worsen, as demonstrated by poor performance on behavioral assessments such as the Vineland Adaptive Behavior Scales, a parental rating of adaptive functioning in communication, daily living, and socialization domains (Whaley et al., 2001). More specifically, children with FASD often struggle with reading social cues, engaging in socially appropriate interactions, and establishing reciprocal peer relationships (Bishop, Gahagan, & Lord, 2007; Stevens, Clairman, Nash, & Rovet, 2017).

Data from animal models of PAE have shown neurobehavioral deficits parallel to those observed in individuals with FASD (Driscoll, Streissguth, & Riley, 1990; Jänicke & Coper, 1993; Marquardt & Brigman, 2016; Sulik & Johnston, 1983; Weinberg, 1989; Weinberg & Bezio, 1987), including social behavior impairments (Hamilton et al., 2010; Hellemans, et al., 2010; Kelly, Day, & Streissguth, 2000; Kelly, Goodlett, & Hannigan, 2009). In particular, disruptions in play behavior are a commonly reported deficit and provide an important behavioral context for investigating how PAE impacts social behavior development during adolescence (Charles Lawrence, et al., 2008; Lugo et al., 2003; Meyer & Riley, 1986; Mooney & Varlinskaya, 2011; Royalty, 1990).

Play behavior is a characteristic form of social interaction observed in many mammalian species, particularly during the transition into adolescence when the expression of play behavior is highest (Meaney & Stewart, 1981; Spear, 2000; Trezza, Baarendse, & Vanderschuren, 2010). As one of the first non-maternal social interactions in which young animals participate, play behavior is critical for developing social competence, as well as for motor, cognitive, and emotional development (Graham & Burghardt, 2010; Pellis, Burke, Kisko, & Euston, 2018). Evidence for the critical role of play behavior comes from studies employing social isolation,

which demonstrate long-lasting neurobehavioral deficits into adulthood, including reduced social approach and/or interaction, increased aggression, and increased anxiety-like behavior (Cooke & Shukla, 2011; Siviy, Deron, & Kasten, 2011; Vanderschuren & Trezza, 2014). As in other forms of social behavior, play behavior is a dynamic collaboration, requiring each playmate to process salient cues and respond accordingly, so that the interaction can proceed in a coordinated fashion (Adolphs, 2003; Vanderschuren & Trezza, 2014). In this way, play is essentially dyadic, with playmates frequently switching between solicitor and responder across a social interaction such that each reciprocally influences the other. Moreover, play behavior involves not only the accurate perception of social stimuli and subsequent execution of an appropriate social response, but also the capacity to elicit appropriate behavioral reciprocity with a play partner in order to sustain play interactions (Thor & Holloway, 1984). Accordingly, investigations of play behavior offer important insight into the development of social competence.

In the dyadic protocols typically used in studies of play behavior, an experimental animal interacts either with a partner matched by treatment or with a naïve control animal (Himmler, Pellis, & Pellis, 2013; Thor & Holloway, 1984; Vanderschuren & Trezza, 2014). These paradigms offer useful information about how play may be altered by different experimental treatments or conditions, and provide important comparisons to increase understanding of social behavior (Himmler, Himmler, Pellis, & Pellis, 2016). For example, it was shown that the playfulness of one partner is influenced by the playfulness of the other partner, as high-playing rats exhibited decreased play behavior when paired with low playing playmates (Pellis & McKenna, 1992). Likewise, increasing play by systematically varying amount of social isolation results in increased play behavior by non-isolated playmates (Varlinskaya, Spear, & Spear, 1999).

While experiments utilizing dyadic protocols have significantly advanced the field, they do not allow for assessment of play in more complex social situations or for phenomena such as play partner preference. Our novel approach of utilizing triads of mixed-treatment composition allows us to assess not only play behavior, but also play partner preference. Specifically, we directly assessed play partner preference in adolescent male and female rats using same-sex triads of mixed-treatment composition to determine not only the impacts of PAE on social competence but also the impacts of the social environment on play behavior development.

2.2 Methods

2.2.1 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 ± 1ºC) and on a 12 hr light-dark cycle (lights on at 0700 hr) with *ad libitum* access to water and standard laboratory 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) Guide for the Care and Use of Laboratory Animals (2011), Canadian Council on Animal Care guidelines, and were approved by the University of British Columbia Animal Care Committee.

2.2.2 Prenatal alcohol exposure

On G1, females were single-housed and randomly assigned to one of three treatment groups: Prenatal Alcohol Exposure (PAE; n = 24), Pair-Fed (PF; n = 17), or *ad libitum*-fed Control (C; n = 25). Dams in the PAE group were offered *ad libitum* liquid ethanol diet (6.37% v/v) with 36% ethanol-derived calories (Weinberg-Keiver High Protein Experimental Diet

#710324, Dyets Inc., Bethlehem, PA). Pair-fed dams were offered a liquid control diet (Weinberg-Keiver High Protein Control Diet #710109) with maltose-dextrin 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). Control dams were offered *ad libitum* access to a pelleted form of the liquid control diet. 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. Diets are formulated to provide adequate nutrition to pregnant rats regardless of ethanol intake (Lan et al., 2006). To determine blood alcohol levels (BALs) of alcohol consuming dams, tail blood samples from a subset of dams (n = 14) were taken on G15 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); the minimum detectable concentration of alcohol is 2 mg/dl. Alcohol-consuming dams showed an average of 95.85 ± 10.79 mg/dL (max BAL = 165.68 mg/dL; min BAL = 38.95 mg/dL). All animals had *ad libitum* access to water, and were provided with fresh diet daily within 1 hr 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 & 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, which occurred on G1, G7, G14, and G21. On the day of birth (postnatal day 1 – P1), litters were weighed and culled to 12 pups with equal numbers of males and females per litter when possible. Dams and pups were weighed

on P1, P8, P15, P22, and after play behavior testing (P30 ± 1 for females; P37 ± 1 for males). No more than 2 males or females per litter were used for behavioral testing.

2.2.3 Play behavior triad testing

To examine play behavior during adolescence there are two approaches: pubertal-dependent effects can be assessed by testing males and females at similar pubertal stages, which has the confound of testing at different ages; or pubertal-independent effects can be assessed by testing males and females at the same age, which has the confound of testing at different pubertal stages. In this study we chose to assess pubertal-dependent effects and therefore tested males and females at different ages (P30 ± 1 for females and P37 ± 1 for males). Habituation to the testing apparatus occurred over two consecutive days immediately prior to testing. Specifically, animals in same-sex littermate groups were habituated to the play arena (21.5" × 20" × 21.5"), which consisted of 3 mirrored side panels and a front panel made of clear Plexiglas and contained clean corn cob bedding. At the end of the second habituation period, pups were singly housed overnight in order to promote social play at testing (Himmler et al., 2013). The next day, animals were tested for 10 min in mixed-prenatal treatment triads, with four possible triad combinations: (1) two controls and a PAE (CCE), (2) two controls and a PF (CCP), (3) two PAEs and a control (EEC), (4) two PFs and a control (PPC). All triads consisted of same-sex, non-littermate animals that had never interacted.

Play behavior was filmed from the front of the clear enclosure and behavior scored later by trained observers blind to triad composition using a computer-assisted data acquisition system (Observer 5.0, Noldus, Netherlands). In each triad, the behavior of each of the two rats from the same treatment group was analyzed to determine the duration and frequency with which the experimental animals interacted with either the same (i.e., ingroup) or different (i.e., outgroup)

treatment partners. The duration of social investigation (anogenital sniffing, body sniffing, allogroom) and play (rough/tumble play, boxing, follow/chase, evade, pinning) were measured; additionally, the frequency (over 10 min) of the play initiations (nape initiation, rump initiation), play reciprocation (half rotation, full rotation; i.e., turning halfway or completely over when initiated), and non-reciprocation (ignore, evade; i.e., either not responding when initiated or avoiding initiation) were quantified (Himmler et al., 2013; Hole, 1988).

2.2.4 Statistical analyses

Data from female and male offspring were analyzed separately, as animals were assessed at different ages. Though testing ages were chosen in order to account for sex differences in pubertal onset, the peak in play behavior appears to be pubertal independent (Meaney & Stewart, 1981). Offspring weights were analyzed using a repeated measures ANOVA [prenatal treatment (between-subject factor) × day (within-subject factor)]. To examine whether animals from the same prenatal treatment showed social preferences within a given triad, the duration/frequency subjects spent investigating an animal from the same versus different prenatal treatment group was analyzed using paired *t* tests. One-way ANOVAs were used to assess potential differences in play initiation frequency by outgroup animals across all triads. For all tests, the software package Statistica 13 (Statsoft) was used. Data are presented as mean ± *SEM*. Significance was set at $p \leq 0.05$. Effect size measurements were made using partial eta squared and Cohen's *d* as appropriate.

2.3 Results

2.3.1 Effects of prenatal treatment on offspring weight

As expected, weights increased across development for both male and female offspring in all prenatal treatments (Table 2.1; within-factor effect of age [females: $F_{4,284}$=5,242.83, $p <$

0.0001, $\eta_p^2 = 0.99$; males: $F_{4,252} = 5{,}022.21$, $p < 0.0001$, $\eta_p^2 = 0.99$]). In addition, female PF animals demonstrated slightly increased weight gain, which emerged at P22 and persisted until testing at P30 [main effect of prenatal treatment ($F_{2,71} = 270.5$, $p = 0.016$, $\eta_p^2 = 0.11$); interaction of prenatal treatment × age ($F_{8,284} = 5{,}242.83$, $p = 0.011$, $\eta_p^2 = 0.07$)]. Specifically, PF females weighed more than control females on P22, and more than both control and PAE females on P30. No other differences in weight were observed among males and females from the different prenatal treatment groups.

Table 2.1 Developmental weights

Developmental Data

	Prenatal treatment group		
	Control	PF	PAE
Female			
P1	6.2 ± 0.1	6.5 ± 0.1	6.2 ± 0.1
P8	16.1 ± 0.3	16.7 ± 0.3	16.1 ± 0.2
P15	32.0 ± 0.5	34.6 ± 0.7	33.8 ± 0.5
P22	53.2 ± 0.8	57.4 ± 1.2[a]	55.3 ± 0.9
P30	98.1 ± 1.8	104.4 ± 2.4[ab]	99.3 ± 1.9
Male			
P1	6.8 ± 0.1	6.9 ± 0.1	6.4 ± 0.2
P8	17.4 ± 0.4	17.5 ± 0.4	16.9 ± 0.3
P15	33.8 ± 0.7	35.0 ± 0.7	34.3 ± 0.5
P22	56.0 ± 1.0	58.2 ± 1.0	56.8 ± 0.9
P37	176.0 ± 3.4	177.3 ± 4.0	171.3 ± 4.1

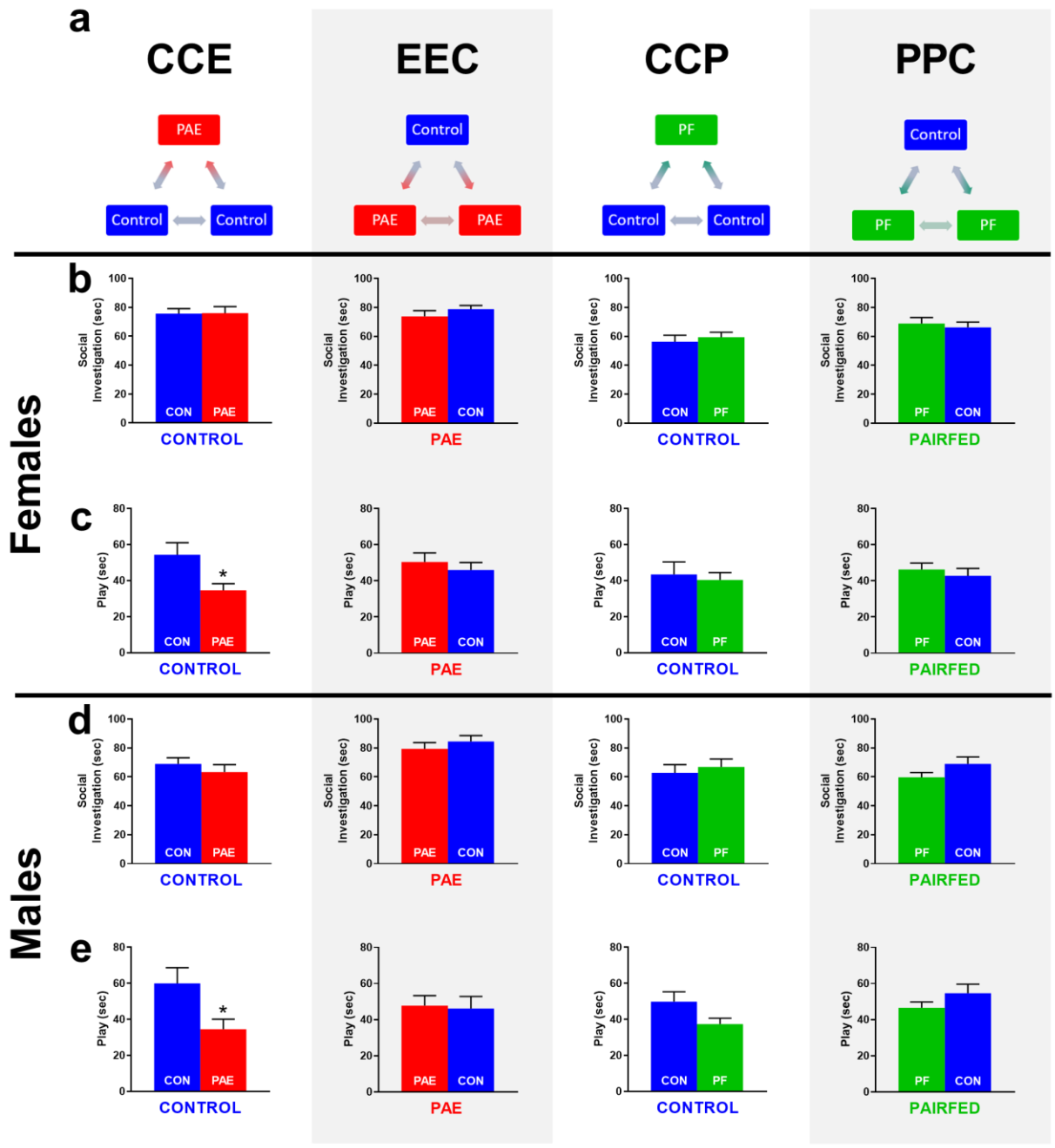
Note. Data are expressed as mean ± SEM. Superscripts indicate a significant main effect of prenatal treatment: [a] PFs are different from control or [b] PAE animals.

2.3.2 Triad composition unveils asymmetry in target of social interaction

Within each triad, assessment of behaviors directed to either the same or different prenatal treatment playmates revealed that female and male controls in CCE triads spent

significantly less time playing with a PAE playmate than with a fellow control playmate (Figure 2.1b,c [female: $t_{17} = 2.985$, $p = 0.008$, $d = 0.86$; male: $t_{15} = 2.183$, $p = 0.045$, $d = 0.87$]). Notably, play target asymmetry was exclusively observed in CCE triads, as analysis of EEC, CCP, and PPC triads revealed no differences in play duration between playmates from ingroup versus outgroup prenatal treatments. Analysis of total play time revealed no differences in play among the different triads in females, however in males, PPC triads played more than EEC triads ($F_{3,69} = 3.29$, $p = 0.025$).

Figure 2.1 Mean duration of social investigation and play behavior.

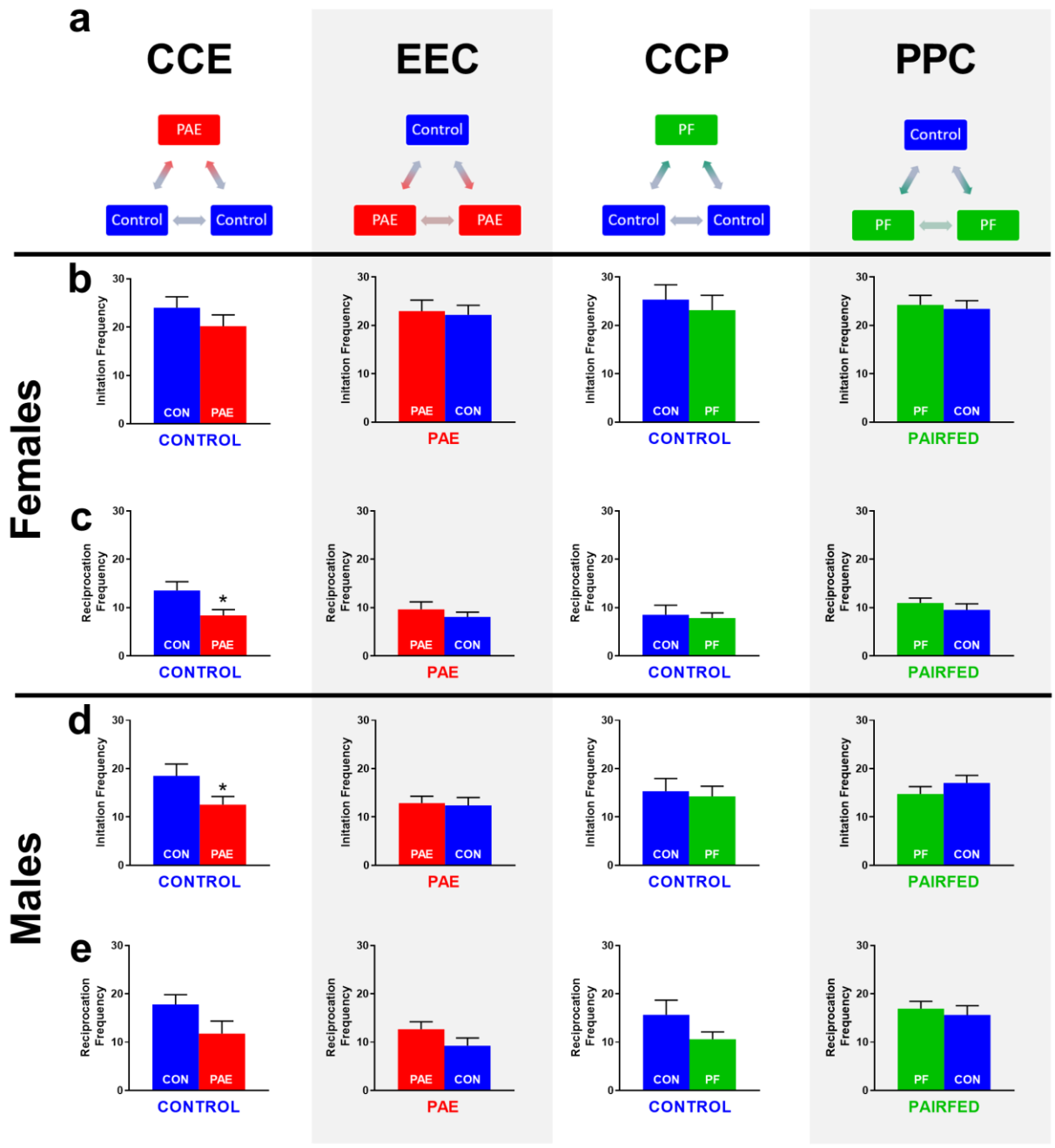


Mean duration of social investigation (rows a, c) and play behavior (rows b, d) among mixed triad groupings (columns) in females (rows a-b) and males (rows c-d). * indicates a significant difference of play behavior duration in CCE triads, such that control animals spent less time investigating a PAE vs. a fellow control playmate.

2.3.3 Play target asymmetry by control animals in CCE triads

Further assessment revealed differences in play initiation and play reciprocation in male and female CCE triads: male controls in CCE triads showed less initiation with a PAE versus a control playmate (Figure 2.2b [$t_{15} = 2.248$, $p = 0.04$, $d = 0.79$]), whereas female controls in CCE triads showed less reciprocation with a PAE versus a control playmate (Figure 2.2c [$t_{17} = 2.95$, $p = 0.009$, $d = 0.71$]). Importantly, no differences in play initiations by the outgroup animals were observed across triads (Table 2.2). Again, differences in initiation/reciprocation frequencies between ingroup and outgroup playmates were exclusive to CCE triads, as EEC, CCP and PPC triads did not show play target asymmetries in initiation/reciprocation frequencies.

Figure 2.2 Mean frequency of play initiations and reciprocations



Mean frequency of play initations (rows a, c) and play reciprocations (rows b, d) among mixed triad groupings (columns) in females (rows a-b) and males (rows c-d). * indicates a significant difference of behavioral frequency in

CCE triads, such that control females showed less reciprocations and males showed less initations with a PAE vs. a fellow control playmate.

Table 2.2 Mean frequency (#) of initiations by outgroup animals in each triad.

Mean frequency (#) of initiations by outgroup animals in each triad

	Triad			
	CCE	EEC	CCP	PPC
Female (n)	(18)	(26)	(12)	(22)
Initiations by outgroup animal (#)	17.4 ± 1.8	19.2 ± 1.5	20.7 ± 2.2	19.3 ±1.6
Male (n)	(16)	(20)	(12)	(22)
Initiations by outgroup animal (#)	16.6 ± 2.7	16.6 ± 2.4	16.4 ± 3.1	22.3 ± 2.3

Note. Data are expressed as mean ± SEM.

2.4 Discussion

Our novel approach utilizing triads of mixed-treatment composition provides insight on how play partner identity and social group composition interact to shape behavior, particularly in the context of PAE. Specifically, we found no play target asymmetry when the triad composition did not include a PAE animal (CCP or PPC), suggesting that control and PF animals do not discriminate between each other based on prenatal treatment, likely because both groups are socially competent. As well, we found no play target asymmetry within EEC triads, where PAE animals were given the choice between a fellow PAE playmate and a control playmate. However, when control animals had the option to play either with a fellow control or a PAE playmate (CCE), control animals showed play target asymmetry such that they biased their play behavior toward fellow control animals. This play bias suggests a deficit in social competence of PAE animals; that is, PAE-related social behavior deficits may not only serve to impede play with a control playmate but may also augment the relative attractiveness of a fellow control

versus a PAE playmate. Notably, these results were consistent in CCE triads of both sexes, with subtle differences observed in the frequencies of initiations vs. reciprocations of male and female animals, respectively. Taken together, our data show that PAE animals are less effective at engaging and responding in playful interactions, which demonstrates negative effects of PAE on the development of social competence.

2.4.1 Triad composition unveils play target asymmetry in play behavior duration regardless of sex

That PAE alters play behavior – and social behavior in general – has long been established, though the exact mechanisms underlying these changes are still not completely understood (for excellent review, see Marquardt & Brigman, 2016). Methods utilizing dyadic play protocols in PAE rodent models have demonstrated that PAE may reverse the sex-typical expression of play behavior (Meyer & Riley, 1986), lead to overall increases in play behavior relative to control animals (Royalty, 1990), and disrupt processing of social cues important for play behavior (Charles Lawrence et al., 2008). Importantly, these PAE-related play behavior alterations may be the root of many of the long-lasting social behavior deficits observed in PAE animals, including altered patterns of social behavior (e.g., increased transitions from sniffing to wrestling versus control rats; Donaldson et al., 2018), impaired social recognition memory (Holman, Ellis, Morgan, & Weinberg, 2018; Kelly, Leggett, et al., 2009), and reduced social interaction (Hellemans, Sliwowska, Verma, & Weinberg, 2010).

In this study, we observed play target asymmetry in both male and female CCE triads due to increased playfulness between control playmates (i.e., more time spent playing with a fellow control animal) and a subsequent attenuation in play duration with the PAE playmate. The observation that PAE animals are less desirable social targets for control playmates suggests that

PAE animals have impaired social competence, as they are less effective at engaging and responding in playful interactions. Notably, control animals only showed biased play when given a choice between a fellow control playmate and a PAE playmate, and not when choosing between a fellow control and PF playmate (i.e., CCP triads). A similar lack of play asymmetry was observed when a PF animal was given a choice between a fellow PF playmate and a control playmate (i.e., PPC triads). This lack of play asymmetry in both CCP and PPC triads supports the specificity of the PAE insult on social competence. That is, neither control nor PF animals differentially bias play when playing in heterogeneous triads, suggesting that these animals are matched in social competence and thus do not differentiate each other by prenatal treatment. Importantly, PAE animals in EEC triads also did not show play target asymmetry; that is, they did not spend differential amounts of time in play with a control than with a fellow PAE playmate. Given that control animals show play asymmetry in CCE triads – as well as the lack of play asymmetry in EEC, CCP and PPC triads – our results suggest that PAE impairs social competence to solicit and engage a playmate, which subsequently may exaggerate the attractiveness of a fellow, socially competent control playmate.

Importantly, we did not observe differences in social investigation – a proxy measure for social interest – in any triad of either sex. These results suggest that social deficits following PAE appear to be driven by impaired social competence and not from an overall lack of social motivation. This finding is also supported by previous work from our lab utilizing the 2- or 3-chambered social interaction task, which demonstrated that adolescent PAE animals do not differ from their unexposed counterparts in social motivation (Holman et al., 2018). These results are also in line with the clinical literature, which reports that individuals with FASD exhibit typical and even enhanced levels of social motivation (Nanson, 1992), and have even been described as

being inappropriately friendly (Kully-Martens et al., 2012). Attenuated social motivation is generally associated with other neurodevelopmental conditions such as autism spectrum disorder, where deficits in social motivation are thought to be a core feature (Bishop et al., 2007; Chevallier, Kohls, Troiani, Brodkin, & Schultz, 2012). Nevertheless, other studies of PAE effects on social behavior have reported reduced social motivation (Ignacio, Mooney, & Middleton, 2014; Mooney & Varlinskaya, 2011), though these studies differ from the present work in rat strain utilized, dose and pattern of PAE (e.g., single binge dose vs. chronic prenatal exposure), as well as the different behavioral paradigm utilized. Regardless, that control animals in CCE triads do not show differences in social investigation highlights the play-specific deficits resulting from PAE; control animals in CCE triads investigate PAE and fellow control playmates equally, and only show behavioral asymmetry with play likely resulting from impaired social competence of PAE animals.

2.4.2 Play target asymmetry related to differences in play initiation and reciprocation

Further assessment of play behavior allowed for a more detailed analysis of the nature of play target asymmetry, revealing that play target asymmetry in male and female CCE triads arose from differences in the frequency of initiations and reciprocations: male control animals preferentially initiated with fellow control playmates while female control animals preferentially reciprocated with control playmates. In other words, for male control animals in CCE triads, PAE animals did not appear to be attractive play targets and may instead have served to enhance the appeal of fellow control playmates. In female CCE triads, however, it seems PAE animals were not able to initiate play appropriately such that control animals were not persuaded to respond to their solicitations. We observed these differences exclusively within CCE triads and not in EEC, CCP, or PPC triads of either sex, again highlighting not only the specificity of PAE

to impair social competence, but also revealing subtle differences in the patterns of play behavior for female and male control animals playing with PAE animals.

The literature has documented sex differences in play behavior expression, such that males typically show greater frequencies than females of rough-and-tumble play (Argue & McCarthy, 2015; Auger & Olesen, 2009; Meaney & Stewart, 1981). Interestingly, previous studies have employed dyadic play protocols within a chronic model of PAE, in which each experimental animal was tested across several sessions, but playmates were alternated between control, PF, and PAE animals (Meyer & Riley, 1986). This study found that PAE resulted in a reversal of sex-typical play expression, such that PAE males exhibited play levels similar to unexposed females, while play levels of PAE females were comparable to those of unexposed males. However, play interactions were collapsed across all play sessions thus precluding the investigation of differences in play associated with play partner identity. In view of these results, it is tempting to speculate that the play target asymmetry observed in CCE triads in the present study may be driven by sex-atypical expression of social play behavior by PAE animals. However, we cannot discount the potential effects of age at testing on play behavior asymmetry, as females and males were tested at different ages in an attempt to control for the differential timing of pubertal onset in males and females. As females were tested much closer to the developmental peak of play behavior observed in rats (Spear, 2000), it would be difficult to conclude whether the different patterns of initiation/reciprocation observed between male and female CCE triads were due to sex differences or age differences. Moreover, careful inspection of the graphs indicates that the effects of PAE in both males and females – while not separately significantly different – show roughly similar patterns.

2.4.3 Possible mechanisms underlying impaired social competence following PAE

The neuropeptide oxytocin (OT) has been implicated in the regulation of various aspects of social behavior (Bredewold & Veenema, 2018; Dore et al., 2013), including social motivation (Lim & Young, 2006), social recognition (Bielsky and Young, 2004; Engelmann, Ludwig, & Landgraf, 1994; Ferguson, Young, & Insel, 2002; Veenema, Bredewold, & De Vries, 2012) and play behavior (Bredewold, Smith, Dumais, & Veenema, 2014). Though we did not assess the OT system in this study, we have previously shown that with testing at a similar early adolescent age, PAE results in impairments to social recognition memory and alterations to OT receptor binding in the amygdala and prefrontal cortex (Holman et al., 2018), brain regions that show correlated activity during play behavior (van Kerkhof et al., 2014). Both the amygdala and prefrontal cortex are critically important for the typical expression of play behavior, as lesions to either region result in severe social behavior impairments, such as decreased play behavior in infant/adolescent rats (Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009; Daenen, Wolterink, Gerrits, & Van Ree, 2002; Meaney, Dodge, & Beatty, 1981). Interestingly, research using prenatal valproic acid exposure – an animal model of autism spectrum disorders – has demonstrated reduced play behavior and OT receptor binding in the amygdala of exposed offspring (Bertelsen et al., 2017). Taken together, it is plausible that our observation of impaired social competence following PAE may be mediated, at least in part, by alterations to the OT system. Moreover, as impaired social behavior and alterations in OT binding in the amygdala have been observed in adult PAE rats, it appears that PAE has long-term consequences on social behavior development and limbic OT function (Kelly, et al., 2009).

A possible alternative explanation for play target asymmetry in CCE triads is that PAE may alter the olfactory signature such that it could be antagonistic to control playmates. Indeed,

stress-related odors emitted by conspecifics have been shown to impact on various aspects of rodent behavior (Abel & Bilitzke, 1990; Rottman & Snowdon, 1972). Of relevance to this study, predator odor has previously been shown to suppress play behavior in rats, suggesting that particularly threatening olfactory cues in the peripheral environment can impact on play behavior expression (Siviy, Harrison, & McGregor, 2006). Besides environmental odor cues, conspecific odor cues have been shown to be important for mediating attachment, social recognition memory, and sexual behavior (Camats Perna & Engelmann, 2017). Nevertheless, rodent play seems most impacted by somatosensory and – based on more recent evidence in the literature – auditory cues in the form of ultrasonic vocalizations (Charles Lawrence, et al., 2008; Wellmann, George, Brnouti, & Mooney, 2015). Indeed, striking early findings from the play literature demonstrated little to no effects of bulbectomy/anosmia on play behavior expression, suggesting that olfaction may not be as critical a sensory cue for play behavior (Beatty & Costello 1983; Thor & Holloway, 1982). While these data suggest that olfaction may not be a key factor for the typical expression of play behavior, they do not necessarily address whether olfactory cues from PAE conspecifics can modify the expression of play behavior. However, that outgroup animals – and in particular controls in EEC/PPC triads – do not differentially initiate ingroup animals does not appear to support the alternative hypothesis of different conspecific olfactory cues driving play target asymmetry in CCE triads.

2.4.4 Implications

When viewed in the context of social behavior development, the ramifications of PAE-related impairments in social competence are noteworthy. Indeed, the present data demonstrate that, in addition to PAE-related alterations in social competence – presumably due to altered processing and integration of social cues in the brain (Charles Lawrence et al., 2008; Holman et

al., 2018; Lugo et al., 2003) – PAE may levy a secondary insult by precluding age-appropriate opportunities for social experiences through the subsequent exclusion from play with competent playmates. Interestingly, an analogous situation has been described in the early neonatal period following PAE, when social interactions are restricted to the mother-pup dyad. Specifically, PAE pups show a diminished capacity to elicit retrieval by the dam as compared to unexposed pups, even when tested with a dam that never consumed alcohol (Ness & Franchina, 1990). Consequently, PAE serves as a primary insult to the pup's ability to elicit retrieval, which subsequently alters maternal responsiveness. Given the critical role of maternal behavior in shaping offspring development, this observed alteration in maternal responsiveness may further exacerbate PAE-related impairments to the development of social competence (Champagne, Diorio, Sharma, & Meaney, 2001).

Adolescence is a critical period of development encompassing significant maturational changes – including pubertal onset – that can have dramatic consequences for brain and behavioral development, including the development of social behavior, making this a unique period of increased vulnerability to social behavior dysfunction (Blakemore, 2008; Blakemore et al., 2010; McCormick & Mathews, 2007; Sisk & Zehr, 2005). Indeed, studies utilizing a model of social instability stress during adolescence – in which animals are isolated and then re-housed with a novel cagemate each day from P30-P45 – have demonstrated a wide range of negative outcomes not only on social behavior, but also on cognitive and emotional neurobehavioral development (Green, Barnes, & McCormick, 2013; Hodges et al., 2017). Impaired social competence in PAE animals may serve as a form of social instability stress, which carries additional risk for suboptimal social, emotional, and cognitive development following PAE.

The clinical literature has described PAE-related impairments in social competence, reporting that children with FASD often struggle with reading social cues, socially appropriate interactions, and establishing reciprocal peer relationships (Bishop et al., 2007; Domeij et al., 2018; Stevens et al., 2017). Importantly, deficits in social competence increase the risk for encountering additional challenges with social behavior development, as well as development in motor, cognitive, and emotional domains (Welsh & Bierman, 2001). Consequently, it is not surprising that children with FASD also experience high rates of social rejection, bullying, and later social withdrawal that may contribute to difficulties in school, further social rejection, trouble with the law, and later mental health problems (Carmichael Olson et al., 1998; Streissguth et al., 1996). Notably, FASD-related impairments in social competence extend into adulthood, as affected individuals continue to exhibit deficits in social responsiveness and interpersonal relationship skills (Kelly et al., 2000; Kully-Martens et al., 2012), and consistently lag behind peers in social behavior function (Streissguth et al., 1991). The present data highlight the impact of the social environment in the context of impaired social competence, which has important implications for understanding the complete impact of PAE on social behavior development.

Results from this study also raise important questions related to designing appropriate interventions for ameliorating PAE-related social behavior deficits. For example, several preclinical studies have investigated whether social enrichment can attenuate PAE-related social behavior deficits. In one study utilizing a moderate/low alcohol exposure paradigm, PAE and control rats were housed in mixed pairs from weaning until adulthood, when they were assessed using tests of social, motor, and cognitive behavior (Rodriguez et al., 2016). Not only did social enrichment (i.e., mixed housing) fail to ameliorate PAE-related social behavior deficits, it instead

led to behavioral impairments in the control cagemates of PAE animals for all behavioral domains assessed. Conversely, another study of social enrichment performed in an acute binge model of PAE found that group housing a PAE rat with control animals positively impacted some but not all aspects of social behavior in a social interaction test. However, in addition to the different exposure paradigm, this study also utilized a slightly altered method of social enrichment, in which each PAE animal was housed with 2-3 control animals from weaning until adolescent behavioral testing (Middleton, Varlinskaya, & Mooney, 2012).

Though both earlier studies observed social behavior deficits following PAE, there are several caveats to understanding the contrasting results of social enrichment on PAE-related social behavior deficits. First, the two reference studies utilized different PAE paradigms, which induced unique deficits in social behavior; indeed, even within the same binge exposure paradigm, the timing of exposure can result in vastly different outcomes for social behavior (Mooney & Varlinskaya, 2011). Second, the social enrichment protocols between these two studies differed significantly in their duration, as the first utilized mixed housing of animal pairs over a ~13-week period while the other employed mixed housing of groups of animals for a relatively protracted period (~20 days) during adolescence. Finally, in the Middleton et al. study, PAE animals were compared to control animals housed with other control animals, and not to their control cagemates – as was done in the Rodriguez et al. study. Thus, while social enrichment using group housing with controls appears to improve PAE-related social behavior deficits, it is not clear if this positive effect is specific to the unique effects of G12 binge exposure and/or is limited to adolescent animals; moreover, the potential impact of mixed housing on control animals housed with a PAE rat is not addressed. Nevertheless, that control animals reared with PAE cagemates show aberrant social behavior would be predicted by our

present results, and presumably arises due to impaired social competence of PAE animals during social development and subsequent asymmetry of social targeting by control animals.

2.5 Conclusions

Taken together, asymmetries in play behavior by control animals in male and female CCE triads, together with the lack of play target asymmetry in EEC, CCP, and PPC triads, suggests that PAE compromises social competence, which may in turn be exacerbated by reduced play opportunities with socially competent conspecifics. Though social behavior is dramatically different between the rat and human, the parallels between our results and findings from the clinical FASD literature are striking. Indeed, our results highlight the complexity of addressing social behavior impairments following PAE and indicate that behavioral interventions should consider the value of the social environment in promoting meaningful improvements to social behavior function in individuals prenatally exposed to alcohol.

Chapter 3: Prenatal alcohol exposure disrupts male adolescent social behavior and oxytocin receptor binding in rodents

3.1 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; S. E. 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 & 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 & Franchina, 1990). Social deficits persist and often worsen during adolescence, with PAE rats showing disrupted play behavior and changes in social investigation (Charles Lawrence et al., 2008; Mooney and Varlinskaya, 2011), which in themselves may have long-lasting effects on normal social development (Auger & 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, Verma, 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 & 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 & 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 & Veenema, 2018; Dore et al., 2013), including social motivation (Lim & 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, Beiderbeck, Lukas, & Neumann, 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 male and female rats (Kelly, Leggett, 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 (F. Q. 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 & Renard, 2018). Additionally, previous work from our laboratory indicates that PAE alters sensitivity of central AVP pathways to testosterone in adult males (Lan, Hellemans, et al., 2009; Lan et al., 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, Levy, Nixon, & Wilson, 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.

3.2 Methods

3.2.1 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 ± 1 ºC) 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.

3.2.2 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 G15 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 a 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 maltose-dextrin 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 1 h 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 & 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 >1 male per litter was used 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.

3.2.3 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.

3.2.4 Buried food olfactory testing

Olfactory testing was performed in early (~P30) or late (~P40) adolescence using a modified buried food olfactory test (Yang & 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.

3.2.5 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 h 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 s each, separated by 10 s 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 & 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).

3.2.6 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, Kuti, & Sur, 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.

3.2.7 Social recognition memory testing

Immediately following social motivation testing, animals were singly housed for 24 h to increase salience of the social stimulus animals (Niesink & 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. trail 5).

3.2.8 Social discrimination testing

On the day following social motivation testing, animals were singly housed for 4 h 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, Hädicke, & Noack, 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.

3.2.9 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 ^{125}I 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 ≤10% for both assays.

3.2.10 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 (representative images can be found in Appendix A). 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.

3.2.11 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; $V1_aR$ 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 (50 mM; pH 7.4). The slides were then exposed to tracer buffer (Tris + 10 mM $MgCl_2$, 0.1% BSA, and tracer) for 60 min, and then washed four times in Tris + $MgCl_2$. 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 to allow for comparisons by age.

3.2.12 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 & Richter, 1984; W. S. Young, Mezey, & Siegel, 1986). Sense oligos for OT and AVP mRNA were used as negative controls; moreover, high stringency hybridization and wash conditions of our protocol, as well as our observation of typical expression patterns ensure probe specificity (Laurent, Hindelang, Klein, Stoeckel, & Felix, 1989). 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 h 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.

3.2.13 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 (s) animals spent in each chamber (social vs. non-social) was analyzed using repeated measures ANOVA [prenatal treatment × 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 × 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 $p \leq 0.05$.

3.3 Results

3.3.1 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 3.1; group×day interaction ($F_{6,327} = 47.53$, $p < 0.0001$, $\eta_p^2 = 0.47$)]. 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 in 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 3.1) 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 3.1; within-factor comparison of weigh date ($F_{3,330} = 7430$, $p < 0.0001$, $\eta_p^2 = 0.99$)].

Table 3.1 Litter size and gestational and developmental weights

Developmental Data

	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[a]	305.6 ± 3.1 [a]
GD14	376.3 ± 3.5	347.5 ± 3.8 [a]	348.2 ± 3.4 [a]
GD21	485.5 ± 4.7	443.4 ± 5.0 [a]	432.0 ± 4.5 [a]
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

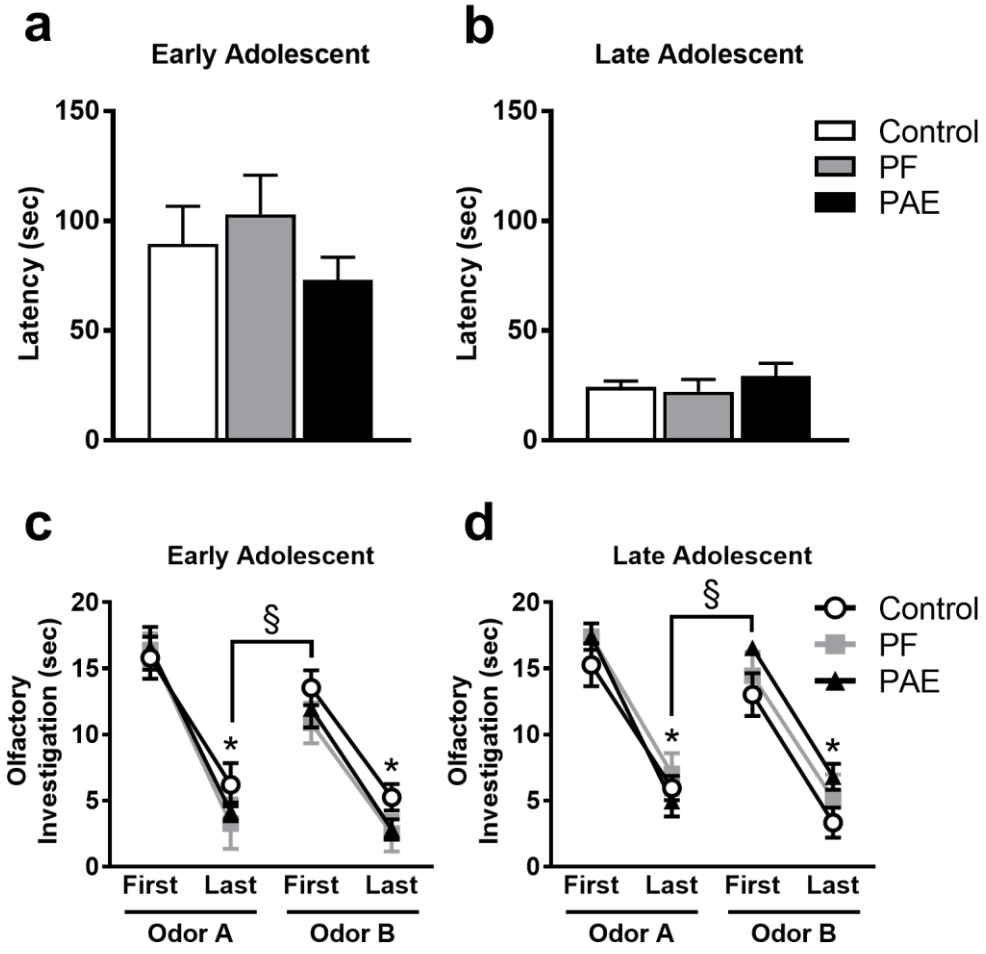
Note: Data are expressed as mean ± SEM. [a] indicates a significant main effect of prenatal treatment, where PAE and/or PF are different from control animals.

3.3.2 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 3.1a,b). Likewise, investigation patterns in the social odor habituation/discrimination were indistinguishable among prenatal treatment groups in both early and late adolescence [Figure 3.1c,d; within-factor comparison of olfactory investigation of odors across trials (early adolescent: $F_{1,57} = 76.67$, $p < 0.0001$, $\eta_p^2 = 0.80$; late adolescent: $F_{3,84} = 74.82$, $p < 0.0001$, $\eta_p^2 = 0.73$)]. 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).

Figure 3.1 General olfaction and social odor habituation/discrimination testing

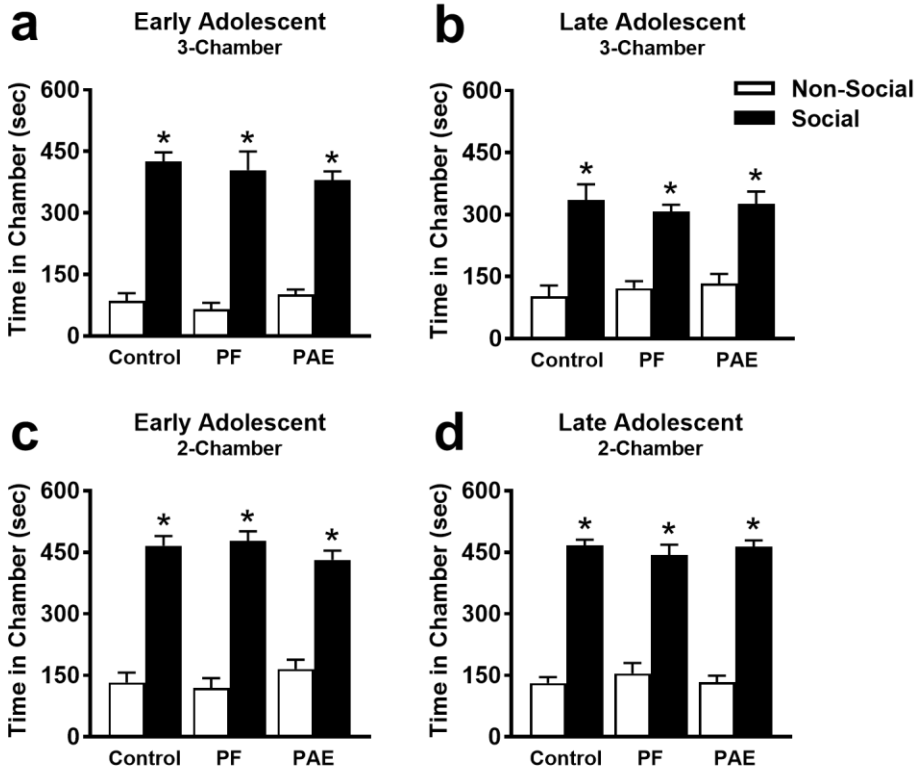


Tests of olfaction using the buried food olfactory (a-b) and social odor habituation/discrimination (c-d) tests in early (a, c) and late (b, d) adolescence. Data are expressed as mean ± SEM of latency to find the buried food or duration of the first and last investigation of each social odor. * indicates 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).

3.3.3 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 3.2; [within-factor comparison of time in each chamber (early adolescent: $F_{1,20} = 148.9$, $p < 0.0001$, $\eta_p^2 = 0.88$; late adolescent: $F_{1,26} = 54.19$, $p < 0.0001$, $\eta_p^2 = 0.68$)]. A similar pattern was observed in the 2-chamber apparatus [within-factor comparison of time in each chamber (early adolescent: $F_{1,26} = 141.4$, $p < 0.0001$, $\eta_p^2 = 0.84$; late adolescent: $F_{1,26} = 203.3$, $p < 0.0001$, $\eta_p^2 = 0.89$)].

Figure 3.2 Social motivation testing



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 ± 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).

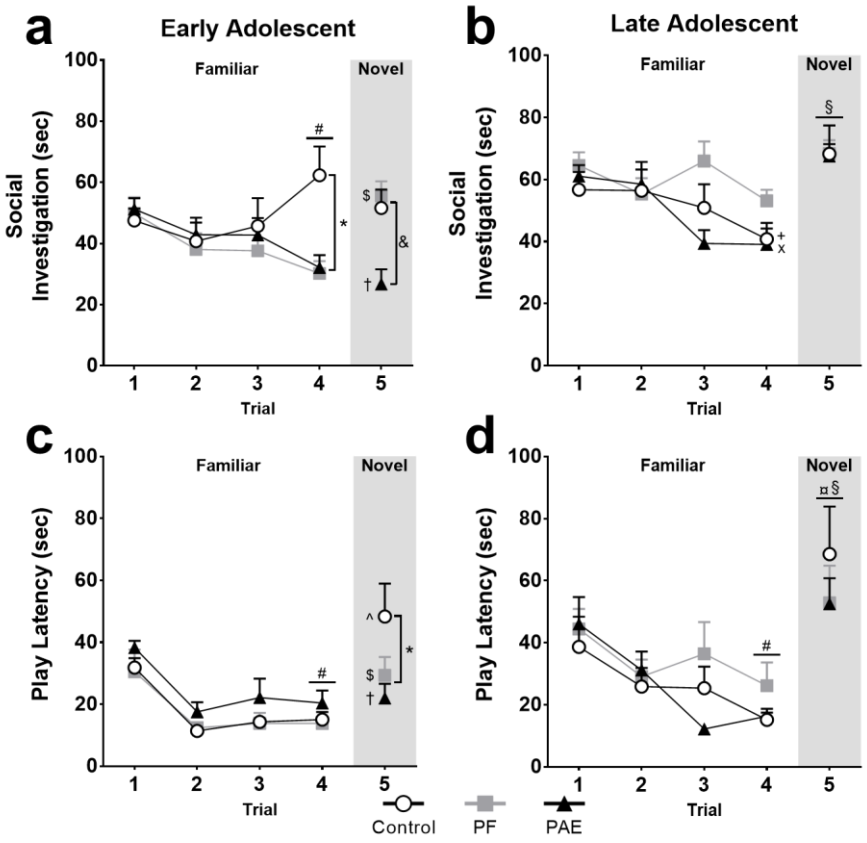
3.3.4 PAE impairs social recognition memory in early adolescence

In early adolescence, we observed markedly different olfactory investigation patterns among animals in the three prenatal treatments across social recognition testing trials [Figure 3.3a,b; interaction of prenatal treatment × trial ($F_{4,42} = 6.697$, $p < 0.001$, $\eta_p^2 = 0.39$)]. 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 & Stewart, 1981), we used the latency to initiate play as an age-relevant measure of social recognition memory (Figure 3.3c,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 × trial ($F_{3.46,31.53} = 3.462$, $p < 0.03$, $\eta_p^2 = 0.25$); calculated using Greenhouse-Guisser correction ($\varepsilon = 0.75$). 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 ($F_{2,52} = 20.404$, $p < 0.0001$, $\eta_p^2 = 0.44$)]. 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 ($F_{1.47,38.14} = 12.933$, $p < 0.001$, $\eta_p^2 = 0.33$); calculated using Greenhouse-Geisser correction ($\varepsilon = 0.73$)].

Figure 3.3 Social recognition memory testing



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 ± 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).

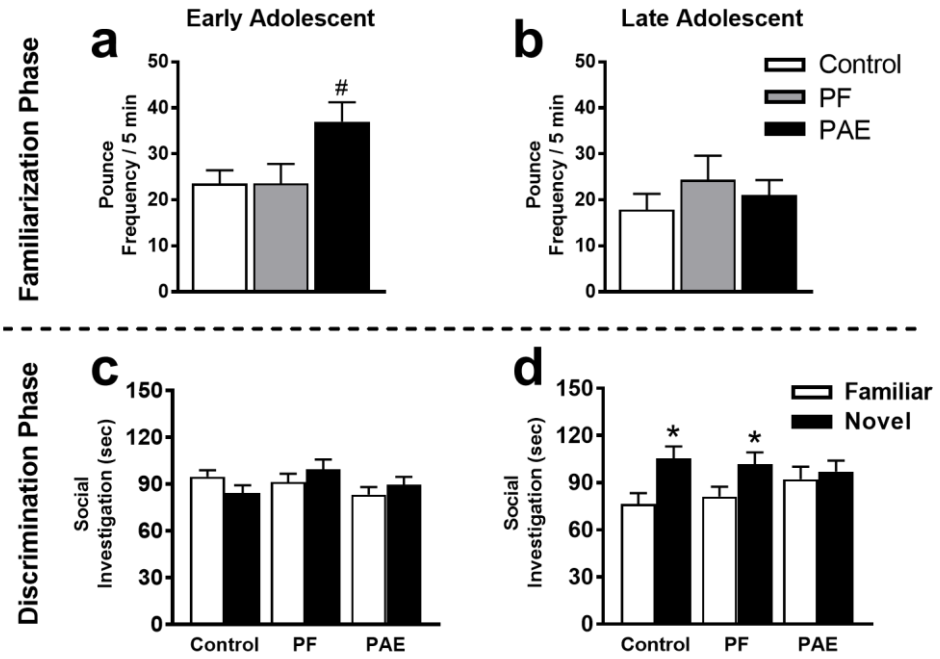
Importantly, PAE can produce general deficits in learning and memory; however, clinical data frequently demonstrate that social behavior deficits are more profound following PAE – even when accounting for more general deficits in learning and memory (e.g., IQ). Moreover, as shown in chapter 4, PAE-related deficits in learning and memory appear to be specific to social recognition memory and not for non-social object recognition memory.

3.3.5 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 3.2). PAE resulted in significantly higher play initiation (i.e., pounce) frequency in early adolescence as compared to control and PF animals (Figure 3.4a; $F_{2,26} = 4.10$, $p < 0.05$, $\eta_p^2 = 0.24$). Additional comparisons of social behavior are included in Appendix B.

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 3.2). 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 ($t_9 = 0.42$, $p = 0.69$).

Figure 3.4 Social discrimination testing



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 ± 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).

Table 3.2 Total investigation during the social discrimination test

Social Investigation Data

	Prenatal treatment group		
Familiarization Phase	Control	PF	PAE
Early Adolescent	149.6 ± 7.8	159.8 ± 6.7	152.4 ± 7.4
Late Adolescent	144.3 ± 10.1	164.6 ± 8.8	160.2 ± 8.4
Discrimination Phase (Familiar + Novel)			
Early Adolescent	178.9 ± 8.5	190.7 ± 6.7	172.7 ± 5.3
Late Adolescent	184.5 ± 7.0	182.8 ± 10.8	189.2 ± 9.5

Note: Data are expressed as mean ± SEM.

3.3.6 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 3.3). 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 3.3). Furthermore, tracking of preputial separation (a gross physical marker of pubertal onset) was not different among groups (Control: P41.8 ± 0.6; PF: P40.8 ± 0.6, PAE: P41.0 ± 0.7).

Table 3.3 Hormone assays

Hormone Data

	Prenatal treatment group		
Corticosterone (μg/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	33.5 ± 5.0	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

Note: Data are expressed as mean ± SEM.

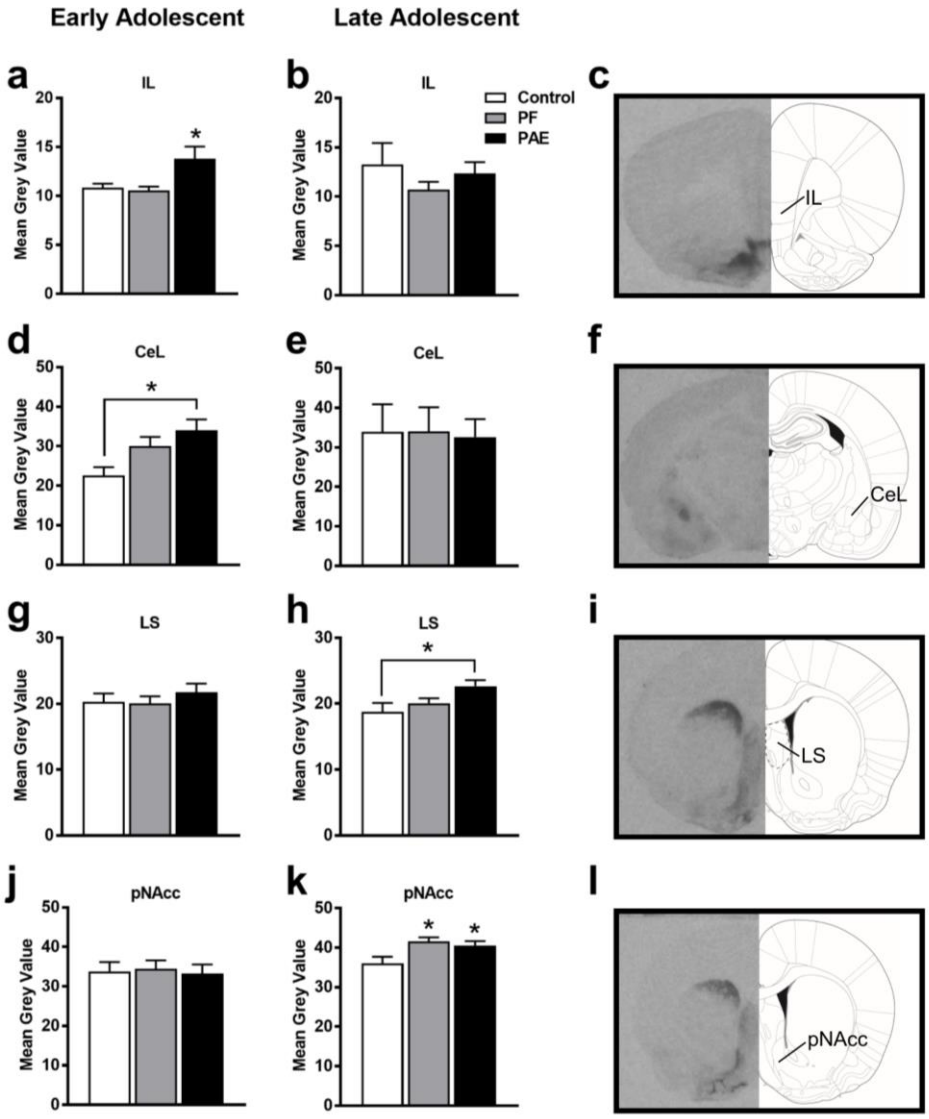
3.3.7 Oxytocin receptor binding is altered in age-dependent manner following PAE

Early adolescence. PAE animals exhibited increased OTR binding in the IL subdivision of the mPFC as compared to control and PF animals [(Figure 3.5a; $F_{2,26} = 3.503$, $p < 0.05$, $\eta_p^2 = 0.21$); 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 3.5d; $F_{2,26} = 5.36$, $p < 0.05$, $\eta_p^2 = 0.29$). 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 3.5h; $F_{2,24} = 3.76$, $p < 0.05$, $\eta_p^2 = 0.24$). OTR receptor binding in the posterior NAcc (pNAcc) increased from early to late adolescence ($F_{1,52} = 13.3$, $p < 0.001$, $\eta_p^2 = 0.22$), and was also increased in PAE and PF animals relative to controls

(Figure 3.5k; $F_{2,23} = 4.79$, $p < 0.05$, $\eta_p^2 = 0.29$). No other differences in OTR binding among prenatal treatment groups were observed.

Figure 3.5 Oxytocin receptor binding



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 ± SEM (mean grey 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).

3.3.8 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.

3.3.9 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 (data included in Appendix C).

3.4 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.

3.4.1 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 (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 & Varlinskaya, 2011). Importantly, in these previous studies, experimental animals were able to interact freely with age-matched social stimulus animals, 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, Phan, Clipperton-Allen, Kavaliers, & Choleris, 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 & 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, Terranova, & Laviola, 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 & 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-h 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 & Engelmann, 2017). 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, Leggett, 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.

3.4.2 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 & Young, 2004; Ferguson et al., 2002; Ross & Young, 2009; Veenema & 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 & 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 PFC (Charles Lawrence et al., 2012; Hamilton et al., 2010; 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, PrL, ACC and orbitofrontal cortices comprising the PFC (Kita & 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 & 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., 2010; 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, Knobloch-Bollmann, Eliava, Busnelli, & Chini, 2016; Wacker & 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 & Landgraf, 1994) or OT (Popik & 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 & Albers, 2016; Dölen & 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 (Charles Lawrence et al., 2008), but combined prenatal alcohol and nicotine exposure also result in increased OTR binding in the NAcc of adult male offspring (S. K. 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 are 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. 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 occurs during puberty (Blakemore et al., 2010; Grinevich, Desarménien, Chini, Tauber, & Muscatelli, 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, $V1_aR$ binding was not altered in PAE relative to control animals in any of the regions examined. Our results demonstrating increased OTR but not $V1_aR$ binding suggest that the OT system is more vulnerable to PAE insult. Nevertheless, the OT/AVP systems have significant crosstalk (Song & 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, Slattery, & Neumann, 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 & Perkeybile, 2012; Veenema, 2012). For example, prenatal stress results in increased CeA OTR binding in adult offspring (Lee, Brady, Shapiro, Dorsa, & Koenig, 2007), and acute prenatal exposure to valproic acid potentiates OTR mRNA expression in the mPFC and CeA in adulthood that is associated with increased sociability on the social interaction test (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, Schlosser, Hassan, & Almeida, 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.

3.5 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.

Chapter 4: Effects of prenatal alcohol exposure and early life adversity on social recognition neurobiology in adolescent male and female rats

4.1 Introduction

Prenatal alcohol exposure (PAE) alters social behavior development resulting in lifelong impairments in social functioning, making it a particularly pervasive feature across the entire spectrum of cognitive, physiological and behavioral sequelae typically associated with PAE (Doyle et al., 2019; Kully-Martens et al., 2012; Mattson et al., 2019). Importantly, individuals prenatally exposed to alcohol are at an increased risk of experiencing early-life adversity (ELA; Price, Cook, Norgate, & Mukherjee, 2017; Streissguth et al., 2004), which itself can lead to impaired social behavior development (Alink et al., 2012; Conaway & Hansen, 1989; Ometto et al., 2016). Dissociating the contribution of each insult to long-term outcomes is extremely difficult, if not impossible, in clinical studies, due not only to the fact that they often co-occur but also to an inability to control for the many PAE and ELA exposure parameters. Relatively few clinical studies have systematically investigated social behavior function following combined PAE and ELA exposure during adolescence. However there is some evidence that, perhaps not unexpectedly, alcohol-exposed children who also experience ELA have poorer social function than children with ELA alone (Henry et al., 2007). Animal models of PAE and ELA have separately investigated the contribution of each insult to social behavior development, with results closely paralleling neurobehavioral phenotypes observed in clinical populations, including altered play behavior, impaired social learning and memory, increased aggression, as well as multiple changes in the underlying neurocircuitry and neurotransmitter systems known to support typical social behavior function (Marquardt & Brigman, 2016; Walker et al., 2017).

While several studies have combined models of PAE and ELA in an effort to examine the contributions of each insult to development of stress systems (Alberry & Singh, 2016; Raineki et al., 2017), none have investigated social behavior development.

In the context of PAE, social behavior dysfunction emerges with increasing complexity of the social environment; indeed, we previously reported that PAE rats did not differ from controls on a social motivation/interaction test, but were impaired on tests of social recognition memory (Holman et al., 2018). Social recognition memory – or the ability to learn and distinguish between familiar and novel conspecifics – is a key component of social behavior function (Bielsky & Young, 2004). To evaluate social recognition, behavioral assays such as the social discrimination test exploit the natural tendency of rodents to investigate a novel social stimulus more than a familiar social stimulus (Engelmann et al., 2011; Gabor et al., 2012). Intact social recognition memory involves the coordinated activity of the highly conserved "social behavior neural network," which is composed of interconnected brain regions including the amygdala, lateral septum (LS), hypothalamus, and prefrontal cortex (PFC; Camats Perna & Engelmann, 2017; Newman, 1999). Moreover, the hypothalamic neuropeptides oxytocin (OT) and vasopressin (AVP) are key signaling molecules involved in regulating social recognition memory (Albers, 2012; Dantzer et al., 1987; Ross & Young, 2009), and their receptors are widely distributed within the social behavior neural network (Stoop, 2014). Importantly, social recognition memory appears to be mediated in a sexually dimorphic manner, such that males and females show distinct activity within the social behavior neural network and OT/AVP systems (Bredewold & Veenema, 2018). Interestingly, PAE animal models have shown deficits in social recognition memory and altered OT system function during adolescence (Holman et al., 2018) and in adulthood (Kelly, Leggett, et al., 2009). Given the important role of the OT/AVP systems

in typical social behavior function, these results suggest the possibility that PAE-related social behavior impairments may involve impaired OT function. Preclinical research on ELA showing social behavior deficits associated with alterations in OT and AVP systems provide further support for this possibility (Veenema, 2012). However, relatively little is known about how PAE and ELA may interact to shape social neurobehavioral development.

Here, we use our well-established model of PAE in combination with a model of ELA to assess the unique and potentially interactive neurobehavioral effects on social recognition memory in male and female rats during adolescence. We chose adolescence as it is a period characterized by numerous physical, physiological, and neurobiological maturational changes – including changes in the OT/AVP systems – as well as marked alterations in social behavior such as a peak in play behavior (Meaney & Stewart, 1981). This makes adolescence a unique period of increased vulnerability to social behavior dysfunction (Andersen & Teicher, 2008; Cushing, 2013; Spear, 2000), when the transition to a more complex social environment may exacerbate existing deficits in social behavior. To model ELA, we utilized a naturalistic ELA paradigm whereby dams are provided with limited nest bedding from postnatal day (P)8-12, which results in increased abusive-like maternal behaviors such as rough handling of and stepping on pups as well as reduced arched-backed nursing (Raineki et al., 2017; Walker et al., 2017). To capture a fuller picture of adolescent social behavior development, we evaluated animals in early (P30) and late (P45) adolescence and also used repeated testing across two days at each age. Furthermore, we also assessed novel object recognition memory in a separate cohort of animals to establish specificity of PAE and ELA effects on the social domain (S. E. Thomas et al., 1998). To understand the implications of PAE-/ELA-induced alterations for neural activity of the social behavior neural network, we assessed mRNA expression of the immediate early gene

c-fos – a marker of neural activity – within key regions known to support social recognition memory, as well as hypothalamic OT and AVP expression.

4.2 Methods

4.2.1 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 ± 1 ºC) 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.

4.2.2 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. PAE dams (n = 29) 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 = 13) were taken on G15 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 a mean of 176.9 ± 67.2 mg/dL (max BAL = 252.8 mg/dL; min BAL = 68.7 mg/dL). For reference, most jurisdictions set 80-100 mg/dL as the legal limit of intoxication. Pair-fed dams (n = 31) were offered a liquid control diet with maltose-dextrin 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 = 31) 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 1 h 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 & 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 – P1), 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 (P1, P7, P15, 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 >1 male or female per litter was used at each age of testing. Behavioral testing occurred in early (P30) or late (P45) 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 discrimination testing.

4.2.3 Early life adversity

ELA rearing condition: Within 2 h of lights off on P7, half the dams/litters from each prenatal treatment (PAE, n = 16; pair-fed, n = 16; or ad libitum-fed control, n = 15) were transferred to clean cages with limited nesting/bedding material that consisted of 300 mL of Beta Chip® bedding (Northeastern Products Corp, Warrensburg, NY). The animals remained in this limited bedding environment until the afternoon of P12 (within 2 h of lights off). *Normal rearing condition:* The remaining dams/litters (PAE, n = 15; pair-fed, n = 15; or ad libitum-fed control, n = 17) were transferred to clean cages with abundant nesting/bedding material (3000 mL) within 2 h of lights off on P7 and remained in this environment until the afternoon of P12.

4.2.4 Experimental design

For all experiments, male and female offspring were tested in either early (~P30) or late (~P45) adolescence. Social discrimination and object recognition behavioral testing was performed using separate experimental cohorts to reduce repeated testing effects (n = 8-10 per prenatal treatment/rearing condition/sex/age). Brain analyses were performed only for the social discrimination cohort.

4.2.5 Social discrimination testing

On two consecutive days of testing in early and late adolescence, animals were singly housed for 4 h prior to social discrimination testing to increase salience of the social stimulus animals. Testing occurred in the isolation cage (17″L × 10.5″W × 7.3″H, Allentown, Inc., Allentown, NJ) 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. Preliminary analysis (agnostic to prenatal treatment/rearing condition) revealed peak social discrimination during the first two minutes of testing (i.e. animals habituated to the novel social stimulus by minute 3 of testing), so only the first two minutes of testing were analyzed. The percentage of time investigating the novel rat (time investigating novel rat/time investigating familiar + novel social stimulus × 100) was measured. Social discrimination occurred when the percentage of time the subject spent investigating the novel rat differed significantly from chance level (50%). The absolute time investigating either the familiar or novel social stimulus was measured to verify that treatments did not alter social investigation behavior (Veenema et al., 2012). Animals were decapitated 30-min after the end of testing on the second day for collection of trunk blood and brains.

4.2.6 Object recognition testing

On two consecutive days of testing in early and late adolescence, animals were singly housed 30 min prior to object recognition testing, which occurred in a standard cage (17″L × 10.5″W × 7.3″H, Allentown, Inc., Allentown, NJ) filled with clean bedding. Testing consisted of a 5-min familiarization phase with two identical objects (LEGO™ Duplo® block, 25 mL plastic medicine bottle, 15 mL plastic Falcon™ tube, or 3.75″ Nylabone®) and a 5-min discrimination phase (novel vs. familiar object) separated by a 15-min retention period (Bevins & Besheer, 2006). Objects were presented as pairs (block and bottle vs. tube and bone) on each day, and

presentation order and identity of the familiar/novel object were counterbalanced each day and across testing days. Testing was filmed and scored later by a trained observer blind to prenatal treatment and familiar/novel object identity using a computer-assisted data acquisition system (Noldus Observer, Netherlands). The duration of olfactory investigation for each object was recorded. Preliminary analysis (agnostic to prenatal treatment/rearing condition) revealed peak object recognition during the first three minutes of testing (i.e. animals habituated to the novel object by minute 4 of testing), so only the first three minutes of testing were analyzed. The percentage of time investigating the novel object (time investigating novel object/time investigating familiar + novel object × 100) was measured. Object recognition occurred when the percentage of time the subject spent investigating the novel object differed significantly from chance level (50%). The absolute time investigating either the familiar or novel objects was measured to verify that treatments did not alter investigation behavior. Animals spending less than 2 sec investigating objects were excluded from analysis. Of excluded animals, all were among animals tested at P30, however, binomial logistic regression confirmed that exclusion of animals was not different among the different prenatal treatment/rearing conditions (day 1: 15 animals excluded of 98 tested; day 2: 16 animals excluded of 98 tested).

4.2.7 Tissue collection

30-min after the end of social discrimination testing, 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 *c-fos*/OT/AVP *in situ* hybridization (ISH) assays. Regions of interest for *c-fos* ISH assays included the amygdala [lateral (LA), basal (BA), medial (CeM) and lateral (CeL) divisions of the central amygdala (CeA), cortical (CoA), and medial (MeA) subnuclei], hypothalamus [paraventricular (PVN; magnocellular and

parvocellular divisions) and supraoptic nuclei (SON)], lateral septum (LS), medial PFC [(mPFC): anterior cingulate (ACC), prelimbic (PrL), and infralimbic (IL) cortices], olfactory regions [olfactory bulb (OB) and piriform cortex (PCX)], and orbitofrontal cortex (OFC). Only the PVN and SON were assayed using OT/AVP ISH. Brain tissue from early and late adolescent animals were run simultaneously by brain region to be able to compare groups.

4.2.8 *in situ* hybridization for c-fos, hypothalamic OT and AVP

A ribonucleotide probe was used to measure *c-fos* and was prepared using a rat *c-fos* 2116 bp template provided by Dr. Victor Viau (Department Cellular and Physiological Sciences, The University of British Columbia, Canada). Probes were labeled with 35S-UTP (Amersham Biosciences, NJ, USA) using Polymerase T7 and Promega Riboprobe System (Promega Corporation, Madison, WI, USA). All probes were purified using Micro Bio-Spin 30 Columns (Bio-Rad, CA, USA). One molar of DTT was added to prevent oxidation. Representative images can be found in Appendix D.

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 & Richter, 1984; W. S. Young et al., 1986). Sense oligos for OT and AVP mRNA were used as negative controls moreover, high stringency hybridization and wash conditions of our protocol, as well as our observation of typical expression patterns ensure probe specificity (Laurent et al., 1989). 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: ***c-fos***: 1 × PBS twice for 10 min each, proteinase K (100 μg/L; at 37°C) for 9 min, 0.1 M triethanolamine-hydrochloride (TEA) for 10 min, 0.1 M TEA with 0.25% acetic anhydride for 10 min, 2 × SSC twice for 10 min each, dehydration by a graded series of ethanol, chloroform for 5 min, and finally 100% ethanol before being air dried. Hybridization buffer (75% formamide, 3 × SSC, 1 × Denhardt's solution, 200 μg/mL yeast tRNA, 50 mM sodium phosphate buffer (pH 7.4), 10% dextran sulfate, and 10 mM DTT) was applied (1 × 106 cpm/slide) and covered with HybriSlips (Sigma-Aldrich, ON, Canada). Sections were incubated overnight at 55°C in chambers humidified with 75% formamide. HybriSlips were removed and the slides were rinsed as follows: 2 × SSC twice for 20 min, 2 × SSC for 30 min, 50 μg/L RNAse A solution (at 37°C) for 60 min, 2 × SSC with 0.01 M DTT for 10 min, 1 × SSC for 10 min, 0.5 × SSC with 0.01 M DTT for 10 min, 0.1 × SSC with 0.01 M DTT (at 60°C) for 60 min, and 0.1 × SSC for 5 min. Sections were dehydrated by a graded series of ethanol and air dried overnight. **OT/AVP**: 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.

Kodak BioMax autoradiography film was exposed to hybridized slides of the all brain regions except for amygdala and hypothalamus. Amygdala and hypothalamus 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 h 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).

Autoradiographic films were scanned and analyzed with Image J 1.50i software (National Institutes of Health, Bethesda, MD). The left and right mPFC, LS, OB (mitral cell layer), PCX, and OFC were traced freehand according to a stereotaxic rat brain atlas (Paxinos & Watson, 2005) in two sections per animal to determine mean gray density levels. Background was measured from the corpus callosum, and corrected gray levels were obtained by subtracting the background level from each of the four measurements. Left and right levels in each measured area were averaged together for analysis. For emulsion dipped slides (amygdala, hypothalamus), ISH 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 corpus callosum from

corresponding sections (amygdala) or the optic tract immediately adjacent to each side of the area of interest (hypothalamus). 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.

4.2.9 Statistical analyses

All data are expressed as mean ± SEM. Outliers were identified and removed using the Robust regression and Outlier removal (ROUT) method with Q= 0.05. Dam and offspring weights were analyzed using a repeated measures ANOVA [dams: diet group (between-subject factor) × day (within-subject factor); offspring: sex × prenatal treatment × rearing condition (between-subject factors) × day (within subject factor)]. Behavioral (total investigation, % novel investigation) and brain data were first analyzed using four-way ANOVAs (sex × age × prenatal treatment × rearing condition). Results indicated that for the majority of measures, significant main or interactive effects of sex and age were detected (Appendix E). Accordingly, subsequent three-way ANOVA analyses (treatment × rearing condition × testing day) were performed independently by sex and age. Social discrimination and object recognition and were assessed by testing whether the percent time investigating the novel object/social stimulus differed from chance (50%) using one-sample t tests. c-fos and OT/AVP mRNA expression were assessed by 2-way ANOVAs, separate for sex and age, for the factors of prenatal treatment and rearing condition. Where appropriate, Newman-Keuls post hoc tests were used to test for differences among groups. Further analyses utilized planned comparisons to test the *a priori* hypotheses that: (1) PAE will alter behavioral and neural activity responses (i.e., normally reared PAE animals will show differential behavioral/neural activity as compared to normally reared control animals); and (2) ELA rearing will differentially alter behavioral and neural activity responses

(i.e., ELA control animals will show differential behavioral/neural activity as compared to normally reared control animals). For all tests, the software packages Statistica 13 (Statsoft, USA) and Graphpad Prism 8.0 (USA) were used. Significance was set at $p \leq 0.05$.

4.3 Results

4.3.1 Prenatal treatment and ELA impact on dam and offspring weight

As expected, dam weights increased across gestation regardless of treatment group, with PF and PAE dams showing attenuated weight gain relative to controls [Table 4.1; treatment group × day interaction ($F_{6,252} = 26.25$, $p < 0.0001$, $\eta_p^2 = 0.38$)]. Specifically, dam weights were not different on G1, but PF and PAE dam weighed less than controls from G7-21 and PAE dams weighed less than control and PF dams on G21. We did not identify any differences in offspring viability or mean litter size among dams from the three treatment groups (Table 4.1). Offspring weights increased across the pre-weaning period; however, this weight gain was reduced for PAE ELA animals from P12-22 and for control ELA animals on P22 relative to their normally reared counterparts. PF ELA animals were not different from their normally reared counterparts on P22 but weighed more than control ELA and PAE ELA animals [Table 4.1; prenatal treatment × rearing condition × day interaction ($F_{6,513} = 4.18$, $p < 0.001$, $\eta_p^2 = 0.05$)]. Expected sex differences in offspring weight emerged at P30 and increased at P45 [Table 4.2; age × sex interaction ($F_{2,270} = 11.94$, $p < 0.0001$, $\eta_p^2 = 0.44$)]. Separate ANOVAs revealed distinct effects of prenatal treatment and rearing condition among male and female animals in early and late adolescence. Specifically, P30 ELA males weighed slightly less than their normally reared counterparts [Table 4.2; main effect of rearing condition ($F_{1,102} = 3.91$, $p = 0.05$, $\eta_p^2 = 0.04$)], while P30 female weights did not differ by prenatal treatment or rearing condition [prenatal treatment × rearing condition interaction ($F_{2,102} = 6.56$, $p < 0.01$, $\eta_p^2 = 0.11$); post hoc

comparisons were not significantly different]. At P45, PF ELA males weighed more than all other animals [prenatal treatment × rearing condition interaction ($F_{2,100} = 4.70$, $p < 0.05$, $\eta_p^2 = 0.09$)], while P45 female weights did not differ by prenatal treatment or rearing condition. Importantly, weight differences observed among dams and offspring from different treatments/rearing conditions were small – ranging from 4-11% reductions – and in the same direction as previously reported (Holman, Baglot, Morgan, & Weinberg, 2019; Holman et al., 2018; Walker et al., 2017).

Table 4.1 Litter size and gestational and developmental weights

Developmental Data

	Prenatal treatment					
	Control		PF		PAE	
Dam N	31		31		30	
Litter size	15.1 ± 0.5		14.5 ± 0.5		14.5 ± 0.6	
Dam weight (g)						
G1	312.3 ± 3.8		310.9 ± 2.7		311.7 ± 3.0	
G7	350.8 ± 4.3		329.4 ± 3.5[a]		327.3 ± 3.4[a]	
G14	406.4 ± 5.4		373.3 ± 3.9[a]		370.2 ± 4.1[a]	
G21	508.3 ± 8.4		464.1 ± 4.7[a]		449.6 ± 5.3[ab]	
Pup weight (g)						
	Control		PF		PAE	
	Normal	ELA	Normal	ELA	Normal	ELA
P1	6.6 ± 0.1	6.6 ± 0.1	6.9 ± 0.2	6.9 ± 0.1	6.8 ± 0.1	6.6 ± 0.1
P7	13.9 ± 0.2	14.2 ± 0.4	14.0 ± 0.3	14.1 ± 0.2	14.7 ± 0.5	14.1 ± 0.4
P12	25.3 ± 0.4	23.7 ± 0.5	24.6 ± 0.5	25.3 ± 0.4	26.5 ± 0.6	23.4 ± 0.7[c]
P22	57.6 ± 0.8	55.3 ± 0.9[c]	57.6 ± 0.9	58.4 ± 0.7[d]	58.7 ± 1.1	53.6 ± 1.3[c]

Note: Data are expressed as mean ± SEM. [a] indicates a significant main effect of prenatal treatment, where PAE and/or PF are different from control animals; [b] indicates significant main effect of prenatal treatment, where PAE is different from PF animals; [c] indicates a significant interaction of prenatal treatment by rearing condition , where ELA animals weighed less than their respective normally reared counterparts; [d] indicates a significant interaction of

prenatal treatment by rearing condition , where among ELA-reared animals PF animals weighed more than control and PAE animals.

Table 4.2 Weight of animals on first day of behavioral testing

Weight at Testing (g)

	Prenatal treatment					
Males	Control		PF		PAE	
	Normal	ELA	Normal	ELA	Normal	ELA
P30	106.9 ± 2.0	101.1 ± 2.9[a]	103.4 ± 3.0	104.7 ± 3.0[a]	109.6 ± 1.8	101.3 ± 2.9[a]
n	18	18	18	18	18	18
P45	259.8 ± 5.7	251.6 ± 7.1	254.2 ± 3.8	274.9 ± 4.2[b]	257.1 ± 4.5	250.5 ± 4.3
n	18	18	18	18	16	18
Females	Control		PF		PAE	
	Normal	ELA	Normal	ELA	Normal	ELA
P30	101.8 ± 1.8	95.3 ± 2.5	95.7 ± 2.3	103.3 ± 2.4	100.8 ± 1.6	94.5 ± 2.4
n	18	18	18	18	16	20
P45	199.7 ± 5.5	190.4 ± 4.6	190.4 ± 3.0	200.0 ± 5.1	195.4 ± 4.1	194.7 ± 4.5
n	18	18	20	18	16	18

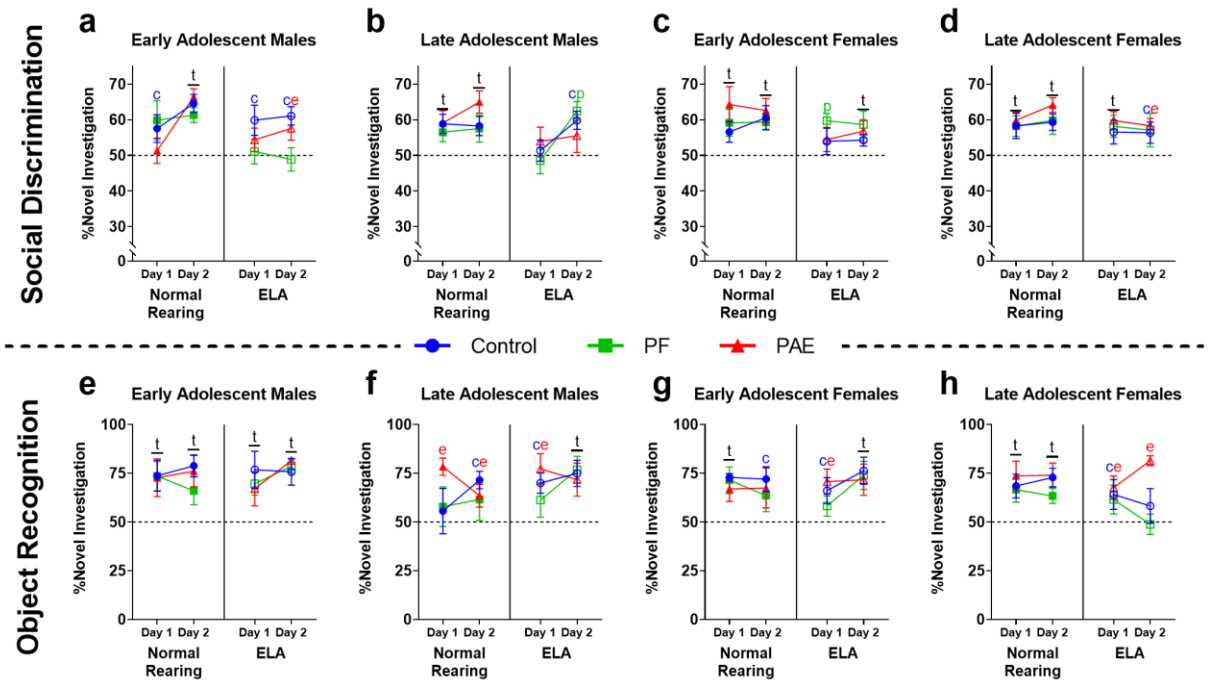
Note: Data are expressed as mean ± SEM. [a] indicates a significant main effect of rearing condition, where ELA animals are different from normally reared animals; [b] indicates a significant interaction of prenatal treatment by rearing group, where PF ELA animals are different from all other animals.

4.3.2 Social discrimination

Subtle behavioral differences were observed during the learning phases for males (Appendix F) and females (Appendix G), as well as in total social investigation (Appendix H) during the discrimination phase across both days of testing. Overall, ELA led to reduced % novel investigation relative to normally reared animals regardless of prenatal treatment in early adolescent females and in males at both ages [main effect of rearing condition: early adolescent females ($F_{1,52} = 4.73$, $p < 0.05$, $\eta_p^2 = 0.08$), early adolescent males ($F_{1,53} = 4.93$, $p < 0.05$, $\eta_p^2 =$

0.09), late adolescent males ($F_{1,52} = 4.22$, $p < 0.05$, $\eta_p^2 = 0.08$). Additionally, all males showed improved performance over days of testing [effect of testing day: early adolescence ($F_{1,53} = 5.46$, $p < 0.05$, $\eta_p^2 = 0.09$), late adolescence ($F_{1,52} = 6.89$, $p < 0.05$, $\eta_p^2 = 0.12$)]. Importantly, during early adolescence, control males showed social discrimination on both days of testing regardless of rearing condition, whereas PAE males showed social discrimination only on the second day of testing regardless of rearing condition (Figure 4.1a; see Table 4.3 for t-test results). Interestingly, normally reared PF males exhibited social discrimination abilities on the second day of testing, whereas ELA PF males failed to discriminate between social stimuli on either day of testing. By contrast, early adolescent females that were normally reared showed social discrimination across both days of testing, while control and PAE animals that experienced ELA showed social discrimination only on the second day of testing (Figure 4.1c). During late adolescence, all normally reared animals showed social discrimination across both days of testing regardless of sex or prenatal treatment. Among animals that experienced ELA, however, control males showed social discrimination on the second day of testing while PAE males failed to show social discrimination on either day of testing (Figure 4.1b). In late adolescent females, however, both control and PAE animals showed social discrimination across both days of testing regardless of rearing condition (Figure 4.1d).

Figure 4.1 Social discrimination & novel object recognition



Social discrimination (a-d) and novel object recognition (e-h) testing in early and late adolescent males and females. Data are expressed as mean ± SEM of percentage of time investigation the novel social stimulus or object over total investigation time (%Novel Investigation). **t** indicates %Novel Investigation is significantly higher than chance (50%). **c** indicates control %Novel Investigation is significantly higher than chance (50%). **p** indicates PF %Novel Investigation is significantly higher than chance (50%). **e** indicates PAE %Novel Investigation is significantly higher than chance (50%).

Table 4.3 Social discrimination statistical results

Statistical Results

		Prenatal treatment					
Males		Control		PF		PAE	
		Normal	ELA	Normal	ELA	Normal	ELA
P30							
	Day 1	t_9=1.93[a]; d=0.61	t_9=2.35[a]; d=0.74	t_8=1.81; d=0.60	t_9=0.31; d=0.10	t_9=0.37; d=0.12	t_9=1.33; d=0.42
	Day 2	t_9=5.65[c]; d=1.79	t_9=4.27[b]; d=1.35	t_8=5.43[c]; d=1.81	t_9=-0.34; d=-0.11	t_9=7.17[c]; d=2.27	t_9=2.28[a]; d=0.72
P45							
	Day 1	t_9=3.32[b]; d=1.05	t_9=0.45; d=0.14	t_9=2.44[a]; d=0.77	t_9=-0.41; d=-0.13	t_7=2.61[a]; d=0.92	t_9=0.98; d=0.31
	Day 2	t_9=3.07[b]; d=0.97	t_9=3.90[b]; d=1.23	t_9=1.99[a]; d=0.63	t_9=4.70[c]; d=1.48	t_7=4.83[c]; d=1.71	t_9=1.17; d=0.37
Females		Control		PF		PAE	
		Normal	ELA	Normal	ELA	Normal	ELA
P30							
	Day 1	t_9=2.27[a]; d=0.72	t_9=1.06; d=0.34	t_9=2.42[a]; d=0.77	t_9=4.66[c]; d=1.48	t_7=2.86[a]; d=1.01	t_9=1.30; d=0.41
	Day 2	t_9=3.09[b]; d=0.98	t_9=2.59[a]; d=0.82	t_9=4.64[c]; d=1.47	t_9=2.30[a]; d=0.73	t_7=3.74[b]; d=1.32	t_9=2.14[a]; d=0.68
P45							
	Day 1	t_9=2.33[a]; d=0.74	t_9=1.31; d=0.41	t_9=2.85[b]; d=0.9	t_8=2.64[a]; d=0.88	t_7=4.26[b]; d=1.51	t_9=3.94[b]; d=1.25
	Day 2	t_9=4.02[b]; d=1.27	t_9=2.14[a]; d=0.68	t_9=2.50[a]; d=0.79	t_8=1.51; d=0.50	t_7=6.94[c]; d=2.45	t_9=4.05[b]; d=1.28

Note: Statistical results of object recognition and social discrimination testing (% novel investigation) using one-sample t-test (one-tailed) against chance (50%). [a] % novel investigation significantly higher than chance at $p \leq 0.05$; [b] % novel investigation significantly higher than chance at $p \leq 0.01$; [c] % novel investigation significantly higher than chance at $p \leq 0.001$.

Table 4.4 Novel object recognition statistical results

Statistical Results

		Prenatal treatment					
Males		Control		PF		PAE	
		Normal	ELA	Normal	ELA	Normal	ELA
P30							
	Day 1	$t_7=2.99^a$; d=1.06	$t_5=2.81^a$; d=1.15	$t_7=3.13^b$; d=1.11	$t_7=3.34^b$; d=1.18	$t_5=2.36^a$; d=0.96	$t_6=1.95^a$; d=0.74
	Day 2	$t_5=5.15^b$; d=2.10	$t_5=3.77^b$; d=1.54	$t_5=2.22^a$; d=0.91	$t_6=8.03^c$; d=3.03	$t_6=3.24^b$; d=1.22	$t_6=18.00^b$; d=6.80
P45							
	Day 1	$t_7=0.48$; d=0.17	$t_7=3.83^b$; d=1.35	$t_7=0.77$; d=0.27	$t_7=1.28$; d=0.45	$t_7=6.45^c$; d=2.28	$t_7=3.48^b$; d=1.23
	Day 2	$t_7=4.73^b$; d=1.67	$t_7=3.71^b$; d=1.31	$t_7=1.07$; d=0.38	$t_7=3.90^b$; d=1.38	$t_7=2.31^a$; d=0.82	$t_7=2.58^a$; d=0.91
Females		Control		PF		PAE	
		Normal	ELA	Normal	ELA	Normal	ELA
P30							
	Day 1	$t_3=11.52^c$; d=5.76	$t_6=2.40^a$; d=0.91	$t_7=3.14^b$; d=1.11	$t_7=1.57$; d=0.56	$t_5=2.66^a$; d=1.09	$t_8=3.23^b$; d=1.08
	Day 2	$t_7=3.45^b$; d=1.22	$t_6=3.79^b$; d=1.43	$t_7=1.64$; d=0.58	$t_7=3.31^b$; d=1.17	$t_7=1.71$; d=0.60	$t_8=2.69^a$; d=0.90
P45							
	Day 1	$t_7=3.03^b$; d=1.07	$t_7=1.86$; d=0.66	$t_9=2.60^a$; d=0.82	$t_7=1.55$; d=0.55	$t_7=3.17^b$; d=1.12	$t_7=2.95^a$; d=1.04
	Day 2	$t_7=4.84^c$; d=1.71	$t_7=0.92$; d=0.33	$t_9=3.46^b$; d=1.09	$t_7=-0.24$; d=-0.08	$t_7=3.87^b$; d=1.37	$t_7=12.60^c$; d=4.45

Note: Statistical results of object recognition and social discrimination testing (% novel investigation) using one-sample t-test (one-tailed) against chance (50%). [a] % novel investigation significantly higher than chance at $p \leq 0.05$; [b] % novel investigation significantly higher than chance at $p \leq 0.01$; [c] % novel investigation significantly higher than chance at $p \leq 0.001$.

4.3.3 Object recognition memory

During early adolescence, all males exhibited object recognition memory on both days of testing regardless of prenatal treatment or rearing condition (Figure 4.1e; see Table 4.4 for statistical results). By contrast, control females consistently exhibited object recognition across both days of testing regardless of rearing condition, whereas normally reared PAE and PF females exhibited object recognition on the first but not second day of testing. ELA only affected PF females, as they showed object recognition on the second but not first day of testing (Figure 4.1g). Interestingly, during late adolescence, only PAE animals demonstrated object recognition memory on both days of testing regardless of sex or rearing condition; normally reared control males showed object recognition on the second day of testing, while ELA-reared control and PF females showed object recognition on the first but not second day of testing. (Figure 4.1f,h). Normally reared PF males failed to show object recognition on either testing day during late adolescence, while ELA PF males exhibited object recognition on the second day of testing.

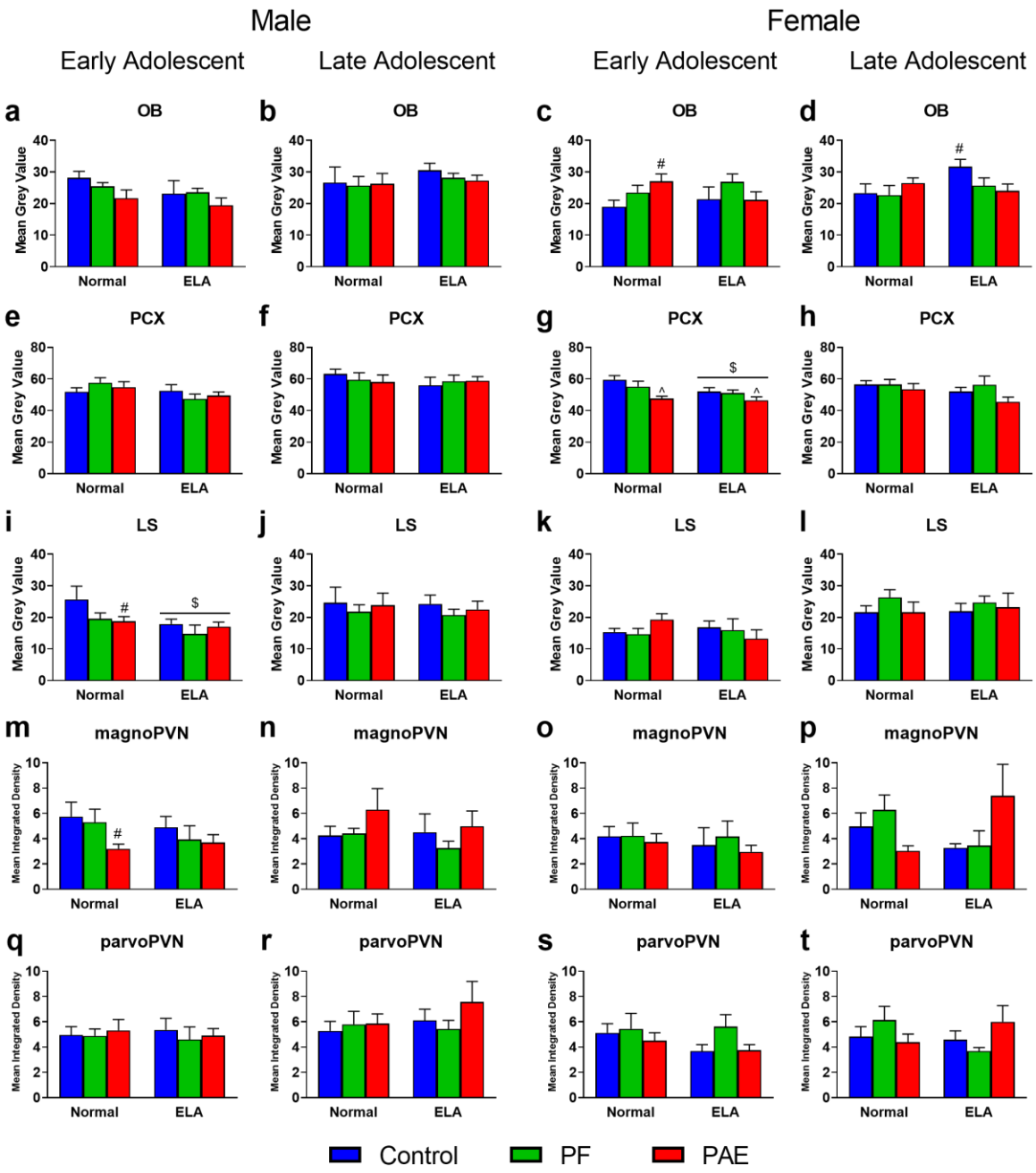
4.3.4 c-fos mRNA expression

Olfactory bulb (OB) and piriform cortex (PCX). ANOVA analysis of OB *c-fos* expression found no significant differences by prenatal treatment and/or rearing condition (Figure 4.2). Nevertheless, *a priori* analysis indicated that relative to normally reared controls, OB *c-fos* expression was higher in normally reared PAE females during early adolescence (Figure 4.2c; $p < 0.05$, $d = 1.56$) and in control ELA females in late adolescence (Figure 4.2d; $p < 0.05$, $d = 1.22$) relative to normally reared control females. For the PCX, ANOVAs revealed that during adolescence, PAE reduced PCX *c-fos* expression overall in females relative to their control and PF counterparts [Figure 4.2g; main effect of prenatal treatment ($F_{2,42} = 6.95$, $p < 0.01$, $\eta_p^2 = 0.25$)]. In addition, ELA reduced PCX *c-fos* expression relative to normally reared

females regardless of prenatal treatment [main effect of rearing condition ($F_{1,42} = 4.54$, $p < 0.05$, $\eta_p^2 = 0.10$)].

Lateral septum (LS) and hypothalamus. ELA attenuated LS *c-fos* expression in early adolescent males regardless of prenatal treatment [Figure 4.2i; main effect of rearing condition ($F_{1,42} = 5.84$, $p < 0.05$, $\eta_p^2 = 0.12$)]. Moreover, *a priori* analysis indicated that, relative to normally reared controls, LS *c-fos* expression was attenuated in normally reared PAE ($p < 0.05$, $d = -0.78$) as well as ELA control males ($p < 0.05$, $d = -0.88$). No differences by prenatal treatment and/or rearing condition were observed in the magnoPVN nor the parvoPVN, however, *a priori* analysis for magnoPVN revealed that normally reared early adolescent PAE males showed reduced *c-fos* expression relative to normally reared control males (Figure 4.2m; $p < 0.05$, $d = -1.12$).

Figure 4.2 OB, PCX, LS, & hypothalamic *c-fos* expression

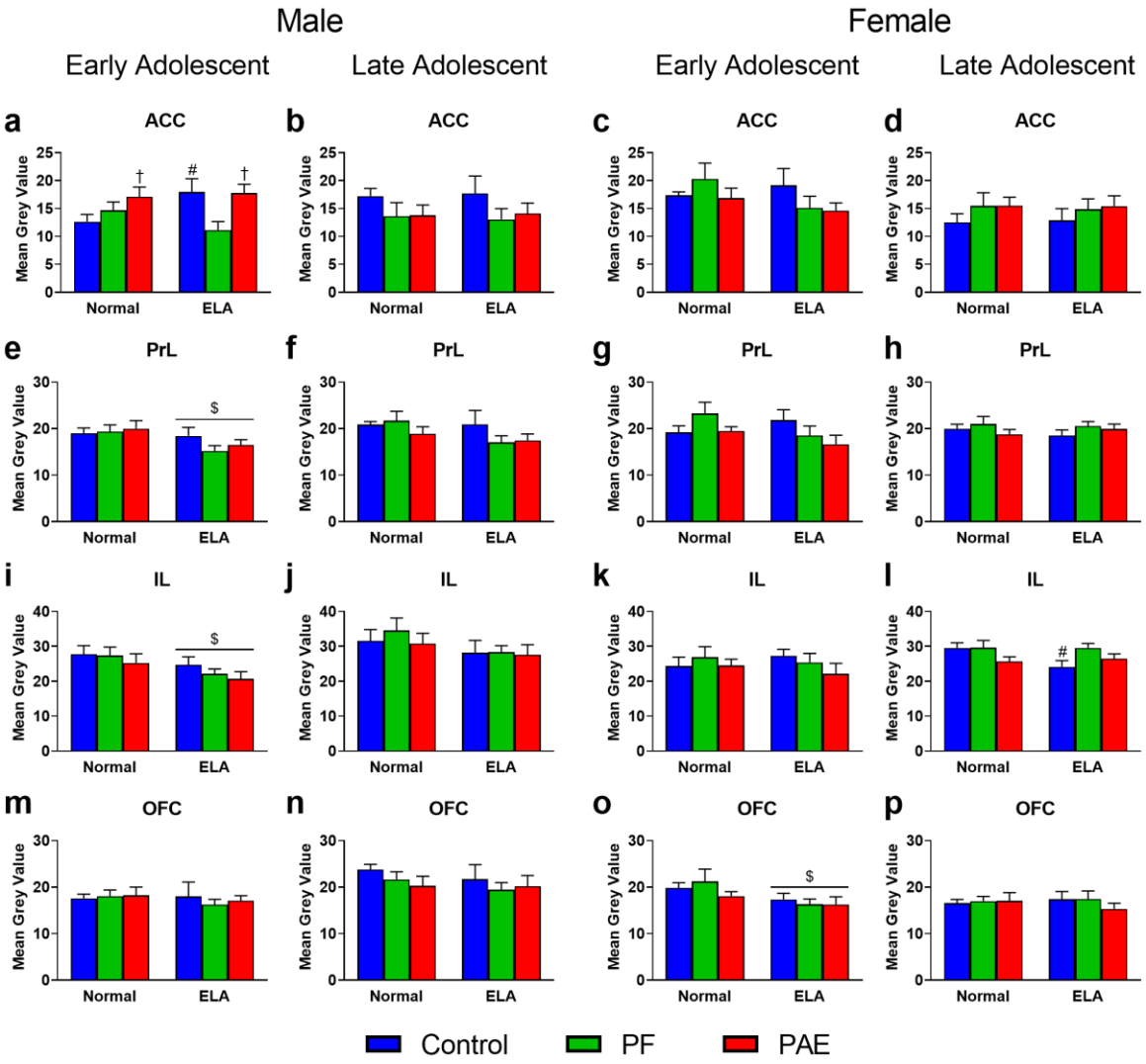


c-fos expression in OB (a-d), PCX (e-h), LS (i-l), and magno- (m-p) and parvoPVN (q-t). Data are expressed as mean ± SEM of mean gray value (OB, PCX, and LS) or mean integrated density (PVN). ^ indicates PAE *c-fos* expression is significantly lower than control and PF animals regardless of rearing condition. $ indicates ELA *c-fos*

expression is significantly different from normally reared animals regardless of prenatal treatment. # indicates an *a priori* difference from normally reared control animals.

Medial prefrontal (mPFC) and orbitofrontal cortices (OFC). During early adolescence, PAE males exhibited increased ACC *c-fos* expression relative to PF males regardless of rearing condition [Figure 4.3a; main effect of prenatal treatment ($F_{2,42} = 3.45$, $p < 0.05$, $\eta_p^2 = 0.14$)]. Additionally, *a priori* analysis indicated that ELA increased ACC *c-fos* expression in control males relative to normally reared controls ($p < 0.05$, $d = 0.98$). ELA attenuated PrL and IL *c-fos* expression in early adolescent males regardless of prenatal treatment [Figure 4.3e; main effect of rearing condition ($F_{1,42} = 5.58$, $p < 0.05$, $\eta_p^2 = 0.12$); Figure 4.3i; main effect of rearing condition ($F_{1,42} = 5.52$, $p < 0.05$, $\eta_p^2 = 0.12$)]. In late adolescence, *a priori* analysis indicated ELA decreased IL *c-fos* expression in control females relative to their normally reared counterparts (Figure 4.3l; $p < 0.05$, $d = -1.15$). ELA reduced OFC *c-fos* expression in females regardless of prenatal treatment [Figure 4.3o; main effect of rearing condition ($F_{1,42} = 5.68$, $p < 0.05$, $\eta_p^2 = 0.12$)].

Figure 4.3 mPFC & OFC *c-fos* expression

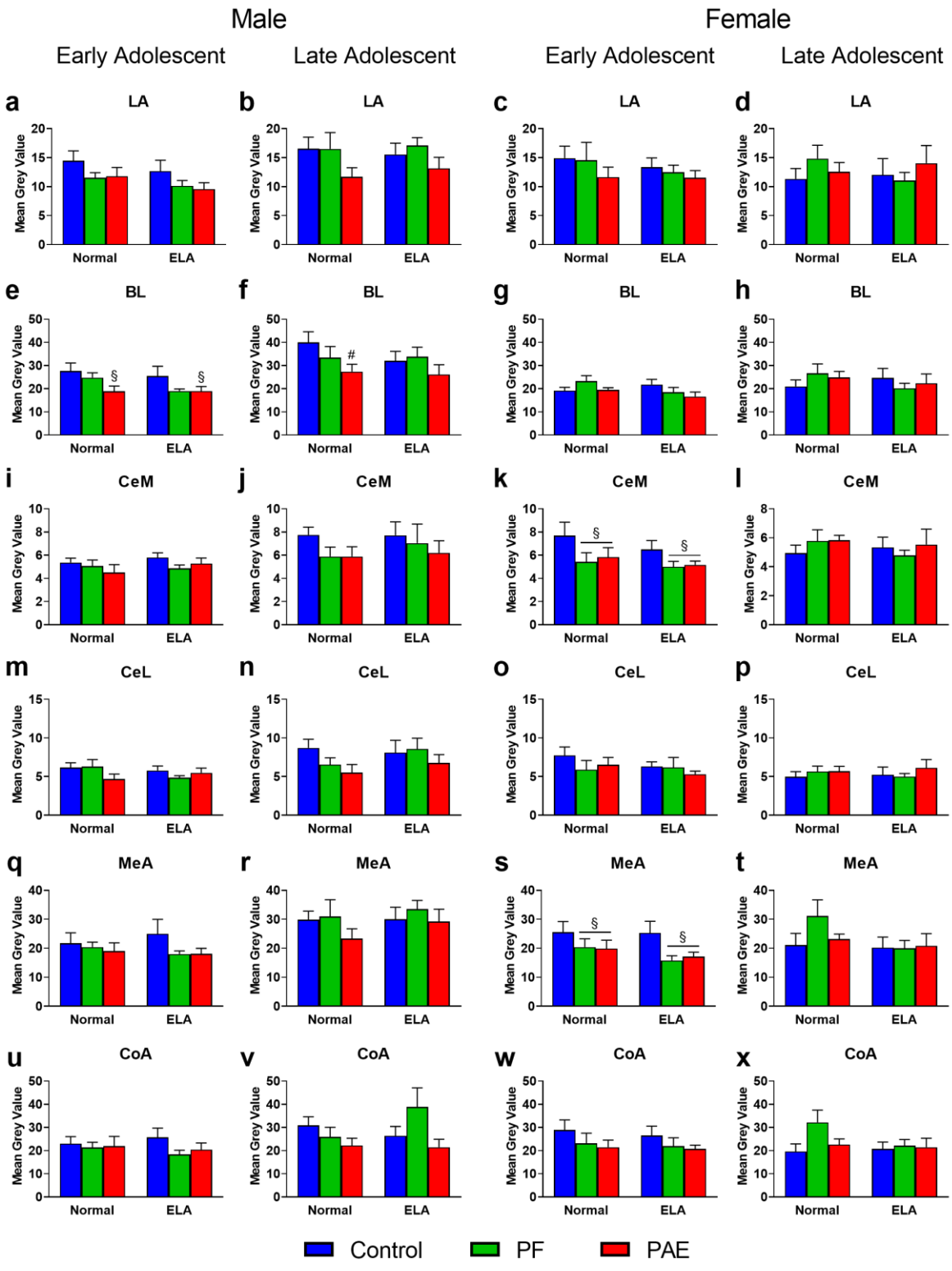


c-fos expression in mPFC (ACC: a-d; PrL: e-h; IL:i-l) and OFC (m-p). Data are expressed as mean ± SEM of mean gray value. † indicates PAE *c-fos* expression is significantly different than PF animals regardless of rearing condition. $ indicates *c-fos* expression is significantly different from normally reared animals regardless of prenatal treatment. # indicates an *a priori* difference from normally reared control animals.

Amygdala. In early adolescence, PAE males had reduced BL *c-fos* expression relative to controls regardless of rearing condition [Figure 4.4e; main effect of prenatal treatment ($F_{2,42}$ = 4.04, $p < 0.05$, $\eta_p^2 = 0.16$)]. *A priori* analysis indicated that in late adolescence, normally reared

PAE males had reduced BL *c-fos* expression relative to normally reared control males (Figure 4.4f; $p < 0.05$, $d = -1.12$). In early adolescent females, PAE and PF animals had attenuated CeM *c-fos* expression relative to controls regardless of rearing condition [Figure 4.4k; main effect of prenatal treatment ($F_{2,42}= 3.49$, $p < 0.05$, $\eta_p^2 = 0.14$)]. No significant differences by prenatal treatment and/or rearing condition were observed in the CeL. In early adolescence, PAE and PF females had attenuated MeA *c-fos* expression relative to control females regardless of rearing condition [Figure 4.4s; main effect of prenatal treatment ($F_{2,42}= 3.97$, $p < 0.05$, $\eta_p^2 = 0.16$)]. In the CoA, no significant differences by prenatal treatment and/or rearing condition.

Figure 4.4 Amygdala *c-fos* expression

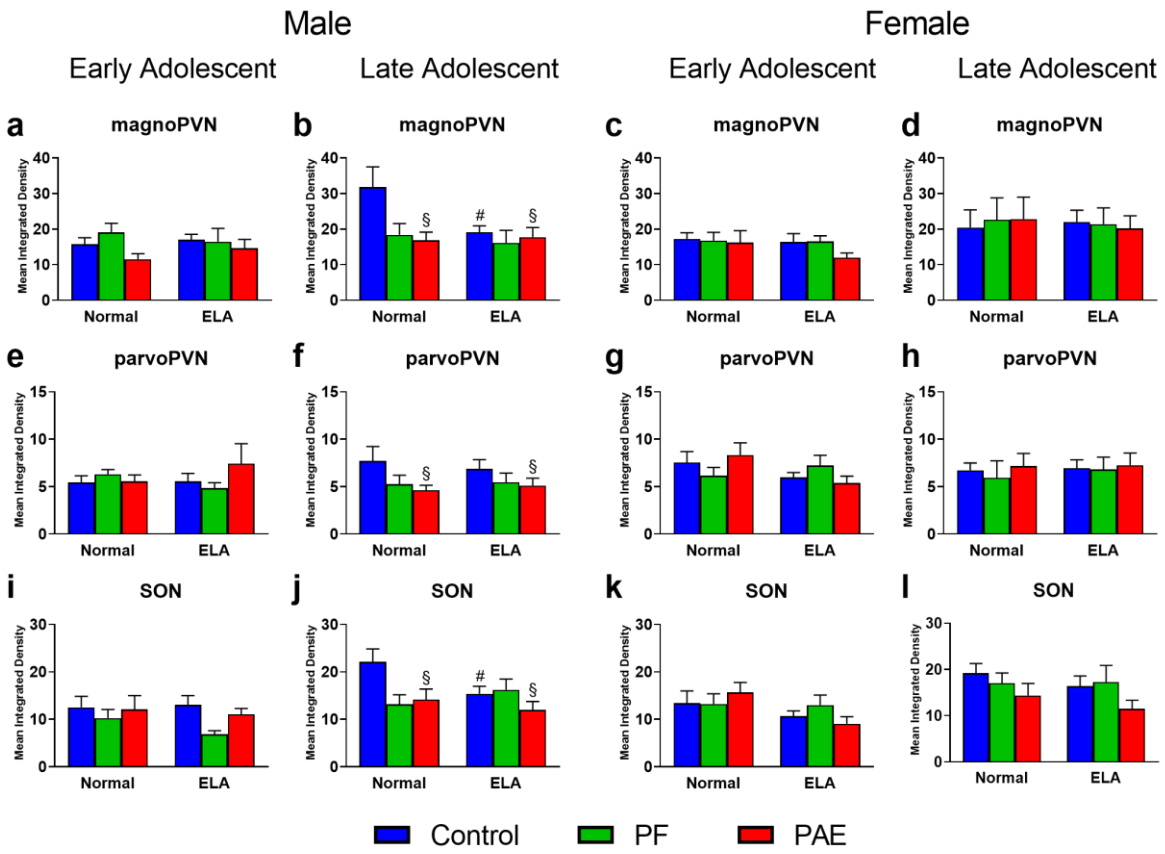


c-fos expression in amygdala (LA: a-d; BL: e-h; CeM:i-l; CeL: m-p; MeA: q-t; CoA: u-x). Data are expressed as mean ± SEM of mean gray value. § indicates *c-fos* expression is significantly different than control animals regardless of rearing condition. # indicates an *a priori* difference from normally reared control animals.

4.3.5 OT and AVP mRNA expression

OT mRNA expression. In late adolescence, PAE attenuated OT expression in the magno- and parvoPVN as well as the SON relative to that in control males regardless of rearing condition [magnoPVN: Figure 4.5b, main effect of treatment ($F_{1,41} = 3.73$, $p < 0.05$, $\eta_p^2 = 0.15$); parvoPVN: Figure 5f, main effect of treatment ($F_{1,41} = 3.20$, $p < 0.05$, $\eta_p^2 = 0.14$); SON: Figure 4.5j, main effect of treatment ($F_{1,40} = 3.75$, $p < 0.05$, $\eta_p^2 = 0.16$)]. Moreover, *a priori* analysis indicated that relative to normally reared control males, magnoPVN and SON OT expression was decreased in control males that experienced ELA (magnoPVN: $p < 0.05$, $d = -1.05$; SON: $p < 0.05$, $d = -1.08$).

Figure 4.5 Hypothalamic OT expression

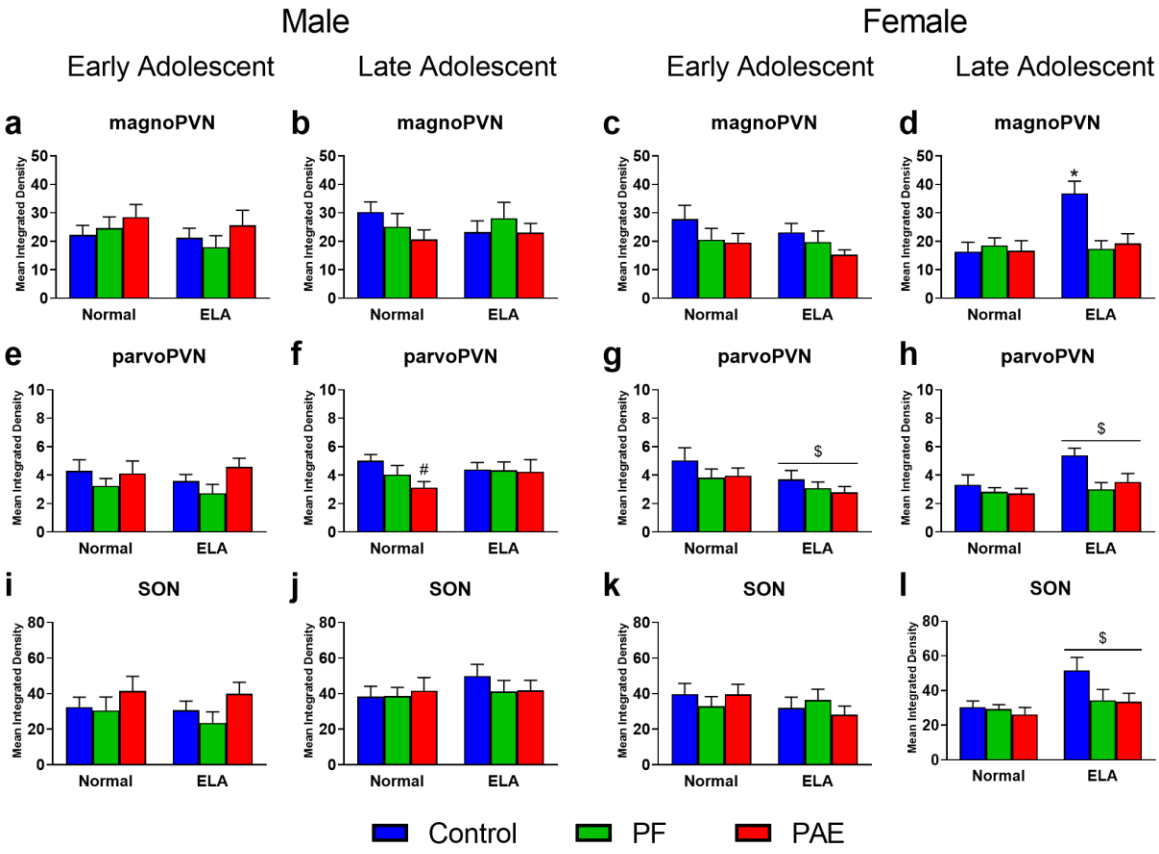


OT expression in magnoPVN (a-d), parvoPVN (e-h) and SON (i-l). Data are expressed as mean ± SEM of mean integrated density. § indicates OT expression is significantly different than control animals regardless of rearing condition. # indicates an *a priori* difference from normally reared control animals.

AVP mRNA expression. In early adolescence, ELA attenuated AVP expression relative to that observed in normally reared females regardless of prenatal treatment [Figure 4.6g; main effect of rearing condition ($F_{1,42} = 4.75$, $p < 0.05$, $\eta_p^2 = 0.10$)]. During late adolescence, ELA control females showed the highest AVP expression in the magnoPVN compared to all other females [Figure 4.6d; prenatal treatment × rearing condition interaction ($F_{1,42} = 5.85$, $p < 0.01$, $\eta_p^2 = 0.22$)]. SON AVP expression was also increased in late adolescent ELA females relative to their normally reared counterparts regardless of prenatal treatment [Figure 4.6l; main effect of

treatment ($F_{1,40} = 3.75$, $p < 0.05$, $\eta_p^2 = 0.16$)]. *A priori* analysis indicated PAE also reduced parvoPVN AVP expression in late adolescent control males relative to their normally reared counterparts (Figure 4.6f; $p < 0.05$, $d = -1.01$).

Figure 4.6 Hypothalamic AVP expression



AVP expression in magnoPVN (a-d), parvoPVN (e-h) and SON (i-l). Data are expressed as mean ± SEM of mean integrated density. $ indicates AVP expression is significantly different from normally reared animals regardless of prenatal treatment. * indicates significant difference from all other animals.

4.4 Discussion

Overall, our results show that the typical neurobehavioral expression of adolescent social recognition memory is impaired by PAE and ELA compared to that in normally reared controls. Importantly, we found sex differences in the effects of PAE and ELA on the development of

social discrimination, with more pronounced neurobehavioral changes observed in males than in females in both early and late adolescence. Specifically, females regardless of prenatal treatment showed social behavioral deficits only in early adolescence and only following ELA, while males showed deficits in both early and late adolescence in response to unique and interactive effects of PAE and ELA. Interestingly, deficits in social discrimination resulting from PAE and/or ELA were independent of performance on non-social object recognition, suggesting that the effects of PAE and ELA on social learning and memory are specific to the social domain and not simply due to a more general deficit in learning and memory (S. E. Thomas et al., 1998). PAE- and/or ELA-related deficits in social discrimination appear to be associated with changes in neural activity (e.g., *c-fos* expression) in brain regions that support social recognition memory, including the PFC, LS and amygdala. Importantly, different brain regions appear to show differential sensitivity to each insult, with PAE generally leading to hypoactivity of the amygdala and ELA suppressing mPFC neural activity. Finally, PAE and ELA also resulted in unique alterations in the typical hypothalamic expression of OT/AVP mRNA in a sex-specific manner, as females exhibited lower levels of AVP as compared to controls while males showed lower levels of OT expression relative to controls. Taken together, our results demonstrate not only how PAE and ELA differentially affect the neurobehavioral expression of social behavior, but also highlight the importance of examining sex differences to understand fully how early-life insults impact social behavior function across adolescent development.

4.4.1 Unique and interactive effects of PAE and ELA on social discrimination depend on sex and age of testing

Results from the two-day social discrimination test demonstrated that PAE impacted males more than females. PAE females were similar to controls in being able to discriminate

between novel and familiar social stimuli regardless of test day and age of testing. In contrast to their control counterparts, however, normally reared PAE males tested in early adolescence were unable to discriminate between novel and familiar social stimuli on the first day of testing but showed discrimination like that in controls on the second day. In late adolescence, however, PAE males exhibited typical social discrimination on both days of testing. Together, these results suggest (1) males appear to be more vulnerable to PAE-related deficits in social recognition memory as compared to females; (2) PAE-induced impairments in social recognition memory during early adolescence appear to represent an experience-dependent effect, as typical social discrimination abilities emerged when given an additional day of testing; (3) evidence for delayed development in PAE males is provided by the finding that deficits in social discrimination were present in early but not late adolescence. These results corroborate our previous findings that demonstrated PAE-related deficits in adolescent social recognition memory using habituation-dishabituation and social discrimination testing (Holman et al., 2018). However, results of the previous and the present study differ in terms of the age at which we detected deficits. In our previous study, early adolescent animals were unable to show social discrimination regardless of prenatal treatment; in late adolescence, however, controls showed the expected social discrimination whereas PAE animals continued to show impairments. It is possible that the test environments utilized contributed to these differential results. Previously, we utilized an unfamiliar testing apparatus, which may represent a more stressful environment for the animals (Camats Perna & Engelmann, 2017). As PAE animals are known to be more sensitive to stressors, both behaviorally and physiologically (Hellemans, Sliwowska, et al., 2010; Weinberg, Sliwowska, Lan, & Hellemans, 2008), it is possible that an unfamiliar test environment may have affected PAE animals more than controls. Here, we extend these findings

to show that when animals are tested in a more familiar home cage-like environment, we can overcome the deficit shown previously by early adolescent controls while PAE animals are still unable to discriminate between novel and familiar social stimuli. Observation of intact social discrimination in females following PAE was not entirely unexpected, given the extensive literature demonstrating a general female advantage over males, as females generally show social recognition over much longer retention intervals than males (Engelmann et al., 2011; Markham & Juraska, 2007). Moreover, our results are consistent with studies showing PAE-related sex difference (i.e., males but not females show deficits) in social recognition in adult rats using a three-trimester equivalent PAE model (Kelly, Leggett, et al., 2009) as well as previous results from our laboratory (Holman et al., 2018).

ELA also impacted social recognition memory in an age- and sex-specific manner. Specifically, ELA impaired social discrimination on the first but not second day of testing in early adolescent control females and late adolescent control males. Thus, in contrast to the male-specific effects of PAE on social discrimination, ELA impacts both males and females, albeit in an age-dependent manner. Our results mirror and extend the ELA literature, including rodent studies employing low bedding (Kohl et al., 2015), maternal separation (Franklin, Linder, Russig, Thöny, & Mansuy, 2011; Hulshof et al., 2011; Lukas, Bredewold, Landgraf, Neumann, & Veenema, 2011), and maternal deprivation (Kentrop et al., 2018), as well as paternal deprivation studies using biparental mandarin voles (Cao et al., 2014; Z. He et al., 2018). Indeed, the robust effects observed across different species, age of assessment, and testing protocols for the negative impacts of ELA on social recognition memory highlight the importance of the early environment for the development of social behavior. Interestingly, our observation of intact social discrimination ability in early but not late adolescent control males following ELA

suggests a possible incubation period for some of the effects of ELA. These findings partially replicate results in rats tested at a similar early adolescent age and in adulthood following maternal separation, where adult animals showed impaired social discrimination, but early adolescent animals did not – and suggest that pubertal maturation may further shape development of social recognition memory following ELA (Lukas et al., 2011). In contrast, ELA in females seems to delay development of social recognition memory, given that deficits in social discrimination were present in early but not late adolescence. The differential effects of ELA on social recognition in males and females may stem from (1) the inherent sexually dimorphic neurobiology underlying social behavior and/or (2) sex differences in how ELA affects development of these systems (Gabor et al., 2012; Veenema, 2012).

By testing at two ages, we were able to assess the roles of development and experience in shaping social behavior function over time; moreover, the two-day testing protocol revealed a potential synergistic effect of PAE and ELA, at least in males, as both early and late adolescent PAE ELA males showed greater impairment of social discrimination abilities relative to their normally reared control counterparts. However, the finding that early adolescent PAE ELA males exhibited social discrimination following a second day of testing suggests delayed development rather than a more permanent deficit. Interestingly, however, we also found that in late adolescence, control ELA males could discriminate between social stimuli on the second but not first day of testing, whereas PAE ELA males failed to discriminate between novel and familiar social stimuli on either day of testing. Taken together, these results suggest that in males, the effects of PAE and ELA are additive, and at least in late adolescence, prevented experience-related improvements in social discrimination, presumably due to their synergistic effects. Furthermore, our finding that social experience across the two testing days improved

social discrimination performance for most PAE and ELA animals suggests that – despite some initial deficits in social recognition memory – animals with these insults could show improved performance when given additional opportunities to learn.

4.4.2 PAE and ELA effects on neural activity

Altered patterns of *c-fos* mRNA expression following social discrimination testing revealed that PAE and ELA resulted in an overall attenuation of neural activity in specific nuclei within the social behavior neural network known to support social recognition memory. Overall, our results suggest that the effects of PAE and ELA on *c-fos* expression are age-specific, sexually dimorphic and are consistent with observed behavioral deficits. Moreover, brain regions supporting social recognition memory appear to be differentially sensitive to PAE and ELA, such that the amygdala is particularly vulnerable to PAE while the mPFC is more vulnerable to ELA.

The social behavior neural network comprises a complex and interconnected circuitry that is involved in multiple aspects of social behavior function, including social recognition memory formation and expression (Adolphs, 2003; Camats Perna & Engelmann, 2017). As the dominant sensory system in rodents, the OB and PCX detect and provide the initial processing of socially relevant olfactory stimuli during social discrimination (Richter et al., 2005; Young, 2002). These olfactory areas project to limbic and cortical areas – such as the amygdala, LS, and PFC – which provide higher-order processing and integration that support social recognition memory and subsequent behavioral responses (Dias et al., 2016). The amygdala is a critical hub of the social behavior neural network, processing socioemotional stimuli to mediate social recognition memory (Adolphs, 2001, 2010; Camats Perna & Engelmann, 2017; Garrido Zinn et al., 2016; Tanimizu et al., 2017). Despite a more prominent role for the MeA, other nuclei, including the BL, LA, CeA and CoA are also implicated in social behavior (Insel & Shapiro,

1992; Katayama et al., 2009; Maaswinkel et al., 1996). The amygdala has reciprocal connections with the LS, a forebrain region associated with social motivation and essential for intact social recognition memory formation, particularly in males; indeed, silencing of the LS can block social recognition memory and agonism or antagonism of the OT/AVP systems within the LS can enhance or impair, respectively, social recognition memory (Engelmann et al., 1995; Lukas et al., 2013; Mesic et al., 2015; Popik & van Ree, 1999; Sheehan & Numan, 2000; Veenema et al., 2012). The amygdala has reciprocal connections with ACC, IL, PRL and OFC subdivisions of the PFC (Kita & Kitai, 1990; McDonald, 1998; Swanson, 2003), a brain region generally associated with higher cognitive tasks (Arnsten, 1998; Diamond, 2011; Floresco et al., 1997) but also essential for social behavior (Adolphs, 2001; Anderson et al., 2010). Involved in olfactory processing, the OFC is thought to encode information about odor identity and reward value to guide social and non-social behavioral responses (Bell et al., 2009; Brenhouse & Andersen, 2011; Rolls, 2000). For example, the OFC can shape behavioral responses based on play partner identity (Himmler et al., 2018; Pellis et al., 2006); however, lesions of the OFC do not block adult social recognition memory (Rudebeck et al., 2007). Importantly, the ACC has been hypothesized to process the motivational states of social conspecifics during social interactions (Apps et al., 2016), and lesion studies have demonstrated an important role for the ACC in social recognition memory in rodents and non-human primates (Rudebeck et al., 2006, 2007), including responsiveness to social stimuli, such that rats with ACC lesions failed to habituate to repeated presentations of a social stimulus. Finally, all of the above regions are innervated by OT/AVP projections from the PVN and SON, which influence their activity due to the high density of OT/AVP receptors in these regions (Althammer & Grinevich, 2018).

Our data suggest that adverse effects of PAE and ELA on social recognition memory are consistent with altered signaling within the social behavior neural network. Early adolescent PAE males exhibited attenuated *c-fos* expression of BL, LS and magnoPVN, as well as potentiated expression in the ACC relative to controls – which occurred in the context of impaired social discrimination. Decreased activity of the magnoPVN following PAE suggests that altered OT/AVP signaling may contribute to the impaired social discrimination observed in this group. Given that we observed PAE-related increases in ACC *c-fos* expression, it is tempting to speculate that this increased activity may be a compensatory mechanism in the face of generally depressed activity in other areas of the social behavior neural network. In late adolescent males, PAE reduced *c-fos* expression only in the BL, suggesting that PAE-related changes in neural activity observed in early adolescence appear to resolve across adolescent development except for those in the BL. This interpretation is supported by our finding that late adolescent males exhibited social discrimination on both days of testing. Nevertheless, continued suppression of BL activity in early and late adolescent males does not preclude later social behavior dysfunction and/or poor performance on other tests of social behavior function, particularly with increasing complexity of the social environment (Hellemans, Verma, et al., 2010; Holman et al., 2018).

ELA induced widespread changes in male mPFC neural activity during early adolescence, including reduced activity in the PrL and IL, but increased activity in the ACC; ELA also decreased LS *c-fos* expression. ELA-related changes in mPFC circuitry/development are well-documented in the clinical literature (Fan et al., 2014) as well as in preclinical models using low bedding (e.g., as used here; Fan et al., 2014; Rincón-Cortés & Sullivan, 2016) and maternal separation (Brenhouse et al., 2013; Chocyk et al., 2013; Holland et al., 2014;

Muhammad & Kolb, 2011; Sandi & Haller, 2015). In contrast to our PrL and IL data, our finding of increased ACC activity contrasts with reports of decreased ACC activity following social interaction in ELA male and female adolescent rats (Rincón-Cortés & Sullivan, 2016). An important difference between this earlier study and the present study, however, is their use of a social interaction test completed in a two-chamber apparatus in which one chamber contained a social stimulus animal behind a wire mesh barrier to prevent physical interaction with the experimental animal. Social discrimination testing as performed in the present study is arguably a more complex social context and places stronger demands on the experimental rat to discriminate between novel and familiar social stimuli through physical interaction. Importantly, the ACC has been hypothesized to process the motivational states of social conspecifics during social interactions (Apps et al., 2016); in view of these data, it is possible that increased ACC activity observed in ELA animals represents more effortful processing of social stimulus animals within the complex social context of social discrimination testing. Finally – and somewhat surprisingly given the strong effect of ELA to impair social discrimination behavior – we did not detect any ELA-related alterations in *c-fos* expression among late adolescent males in any of the brain areas analyzed. Nevertheless, we cannot rule out altered function of these neural circuits in the absence of differential *c-fos* expression; indeed, despite the immense value of *c-fos* expression as a tool to interrogate changes in neural activation patterns, *c-fos* expression alone cannot account for the specific identity (e.g., glutamatergic, GABA-ergic, etc.) and or connectivity of individual activated neurons. Thus, equivalent *c-fos* expression among groups does not necessarily indicate equivalent neural processing (Kovács, 2008). Moreover, ELA-related social discrimination deficits in late adolescent males may result from earlier

developmental changes induced by altered circuit function or may be mediated by other brain regions not analyzed here.

By contrast, despite the increased OB activity and reduced activity in the amygdala (CeM and MeA) and PCX of PAE females during early adolescence, we did not observe any PAE-related alteration in female social recognition memory. Previous work has shown that the encoding of social stimuli was impaired in adult PAE females only when the learning phase was reduced from 5 to 2 min (Kelly, Leggett, et al., 2009). Thus, the 5 min learning phase in the present study may have allowed females to overcome any behavioral impairments in the context of altered activity in olfactory/amygdala regions. In late adolescence, however, social discrimination and *c-fos* expression in PAE females did not differ from controls in any of the brain areas analyzed. Alternatively, our observation of PAE-related alterations in olfactory/amygdala activation in females during early adolescence suggests that atypical function of these brain regions during development may contribute to later social processing impairments in adulthood despite equivalent overall activity within the social behavior neural network.

Like PAE, ELA also resulted in reduced *c-fos* expression in the PCX of early adolescent females, suggesting the possibility of impaired encoding of olfactory stimuli that could contribute to deficits in social recognition memory; related models of ELA have also shown altered activity of PCX (e.g., increased activity of anterior PCX during olfactory learning during the sensitive period; Roth & Sullivan, 2005). ELA also resulted in attenuated OFC activity in early adolescent females. Studies using maternal separation to model ELA have reported altered development of PFC subregions (e.g., increased dendritic spine density), including the OFC, in addition to increased anxiety-like behavior (Muhammad & Kolb, 2011). Given the impaired social discrimination of early adolescent ELA females in our study, the observed alterations in

PCX and OFC suggest that ELA disrupts encoding of social information necessary for social recognition memory. In late adolescent females, ELA increased neural activity in the OB, but decreased IL neural activity in the absence of social discrimination deficits. Our results of decreased IL activity replicate previous findings in late adolescent rats using the same ELA model and following social interaction testing, indicating a robust effect of ELA on mPFC function (Rincón-Cortés & Sullivan, 2016).

4.4.3 PAE and ELA effects in the OT/AVP systems

OT/AVP are critical for regulating various aspects of social behavior, and are particularly important for social recognition memory (Bielsky & Young, 2004; Engelmann et al., 1994). Assessment of OT/AVP mRNA expression following social discrimination testing revealed that PAE and/or ELA resulted in an overall suppression of hypothalamic neuropeptide transcription in an age- and sex-specific manner. In general, alterations in OT/AVP expression in males were driven by PAE while changes in females resulted from ELA. Moreover, differences in AVP expression were present in both males and females, while altered expression of OT was exclusive to males. Sex differences in the OT/AVP systems are well documented (Bredewold & Veenema, 2018), and our results add to this literature by demonstrating sex-specific responses to prenatal and early-life insults that may support the unique pattern of subsequent social recognition memory deficits. Moreover, as areas showing altered *c-fos* expression also express high levels of OT/AVP receptors, our finding of altered hypothalamic OT/AVP expression suggest that changes in neural activity may result from PAE-/ELA-related impairments in social neuropeptide signaling within the social behavior neural network.

In males, we observed differential neuropeptide expression only in late adolescence, as both PAE and ELA blunted OT expression in the PVN and SON and PAE alone reduced AVP

expression in the parvoPVN. These changes in neuropeptide expression parallel our behavioral results, where ELA animals with reduced OT expression showed social discrimination impairments. Use of receptor knockout mice, OT antagonists, and ICV OT infusion have demonstrated an important role for the OT system in social recognition memory (Bielsky & Young, 2004; Dore et al., 2013). Furthermore, PAE has previously been shown to reduce hypothalamic OT in adult voles (Feng et al., 2019; F. Q. He et al., 2012) but not in adult rats with both PAE and nicotine exposure (S. K. Williams et al., 2009). The present finding of reduced hypothalamic OT expression extends these previous data and further supports a role for altered OT function in the etiology of PAE-related social behavior deficits. Additional support for a role for OT comes from a previous study, where we observed PAE-related alterations in OTR binding of adolescent male rats, including the mPFC, LS and amygdala (Holman et al., 2018). Interestingly, we now show altered neural activity within these same regions following social discrimination testing. Studies in rats that have utilized maternal separation during the pre-weaning period to model ELA have shown similar decreases in hypothalamic OT (Oreland, Gustafsson-Ericson, & Nylander, 2010). However, depending on the species, ELA paradigm, sex or age of the animal, OT expression following ELA has been shown to increase or decrease (Veenema, 2012). To our knowledge, we are the first to investigate hypothalamic OT expression using the more chronic low bedding model of ELA (Walker et al., 2017).

We found no PAE-related differences in hypothalamic OT expression in early or late adolescent females, however, ELA resulted in suppressed parvoPVN AVP expression in early adolescent females and increased AVP expression in the magno/parvoPVN and SON of late adolescent females and most dramatically in controls. As with males, these results are consistent with our behavioral results. Manipulations of the AVP system have demonstrated the important

role this neuropeptide system plays in in social recognition memory, with $V1_aR$ antagonism disrupting and central AVP infusions enhancing social recognition (Gabor et al., 2012). Interestingly, this AVP-enhancing effect on social recognition appears to be limited to females during adolescence (Veenema et al., 2012). Taken together, our data suggest that ELA-related deficits in social discrimination and suppressed amygdala activity in early adolescent females may result from impaired AVP signaling. Moreover, ELA-induced increases in AVP in late adolescent females demonstrate that ELA effects on the AVP system are not static, but rather change across adolescent development. As relatively few studies have investigated the effects of ELA on the OT/AVP systems, particularly in females, our results add insight into how the early environment impacts social behavior development in a sexually dimorphic fashion (Veenema, 2012).

4.4.4 PAE and ELA-related social behavior deficits are specific to social domain

Social behavior deficits following PAE have often been described as a secondary effect of general cognitive deficits in learning and memory (Mattson & Riley, 2000). While deficits in learning and memory likely mediate aspects of social cognition and other behaviors, comparisons of individuals with FASD to unexposed, IQ-matched controls often demonstrate more profound deficits in the social domain than can be explained by general intelligence, suggesting that PAE directly impacts social behavior function (Greenbaum, Stevens, Nash, Koren, & Rovet, 2009; S. E. Thomas et al., 1998). For example, when compared to children with similar verbal IQs, children with FAS scored significantly lower on general social skills (S. E. Thomas et al., 1998). Utilization of novel object recognition testing with testing parameters (other than objects) identical to those of social discrimination testing allowed us to examine whether PAE- and/or ELA-related deficits in social recognition memory were associated with an

impairment in a non-social form of learning and memory. Importantly, our finding of no relationship between social recognition and object recognition memory, other than some impairment in early adolescent PAE females on day 2 of testing, is consistent with our previous findings that, at least in adulthood, PAE animals show comparable object recognition memory as compared to controls (Berman & Hannigan, 2000; Kim et al., 1997).

The preclinical literature on ELA and its effects on object recognition memory is mixed, with some studies identifying deficits in aged rodents (Brunson, 2005; Rice, Sandman, Lenjavi, & Baram, 2008) and others showing no effect in adult rats (Maniam, Antoniadis, Le, & Morris, 2016). Our results extend this literature by including adolescent testing using a relatively short retention period (e.g., 15 min) and are the first using the low-bedding ELA paradigm in female animals. Interestingly, ELA appeared to impair object recognition memory only in late adolescent female controls, and we found no interactive effects of PAE and ELA among early or late adolescent animals, suggesting that – at least under these testing protocols – short-term memory for non-social objects is basically intact in PAE animals regardless of rearing condition. An important caveat, however, involves the specific testing conditions of animals in the present study, as other studies – using different testing protocols and or models of PAE/ELA – have identified object recognition memory deficits following PAE (Shirasaka et al., 2012) or ELA (e.g., maternal separation; Hulshof et al., 2011).

Dissociated performance on social discrimination and object recognition testing following PAE and/or ELA supports our suggestion that the deficits observed in social recognition memory are specific to the social domain. Moreover, we suggest that the underlying memory systems supporting these behaviors are differentially sensitive to PAE and ELA insults.

Taken together, our results add to this literature and highlight the importance of considering how the timing, amount, and other parameters of PAE/ELA can impact later life outcomes.

4.4.5 Unique effects of pair-feeding

Pair feeding resulted in a number of unique effects, such that PF animals appeared different from both control and PAE animals on several measures. For example, unlike PAE and control ELA animals, early adolescent ELA PF females showed no deficits on social discrimination, while ELA completely blocked social recognition in early adolescent PF males. Interestingly, PF animals showed significantly more impaired object recognition memory relative to control and PAE animals, in a pattern that did not align with social recognition impairments. For brain measures, PF animals generally exhibited expression profiles more similar to PAE animals or intermediate to control and PAE animals.

The finding that PF animals differ from both control and PAE animals or are similar to PAE on some measures is not entirely surprising. Pair-feeding is intended to control for the reduced food intake of PAE dams (Gallo & Weinberg, 1981; Zhang et al., 2012). However, while PAE dams are fed *ad libitum*, PF dams receive a reduced ration of food and typically consume their entire daily ration within a few hours such that they are essentially food deprived until the next feeding. In this way, pair-feeding can be considered a treatment in itself; indeed, moderate food restriction during pregnancy is often used as a model to study intrauterine growth restriction (Akitake et al., 2015; Vieau et al., 2007). The underlying mechanisms of PF effects are still not fully understood, but it is possible that similar phenotypes in PAE and PF offspring may be differentially mediated (e.g., direct/indirect effects of alcohol vs. effects of food/nutrient restriction and/or mild prenatal stress due to the hunger that accompanies food restriction) rather than occurring along a continuum. Of relevance to the present results, research on prenatal stress

has demonstrated a number of adverse effects on offspring social neurobehavioral outcomes (de Souza et al., 2013; Grundwald, Benítez, & Brunton, 2016; Lee et al., 2007).

4.5 Conclusions and implications

Here we show that PAE and ELA result in age- and sex-specific impairments in social recognition memory, revealing the complex way these insults shape social neurobehavioral development. Specifically, males appear to be more sensitive than females to the effects of both PAE and ELA. These results are supported by previous work in adult PAE animals suggesting that social recognition memory is impaired primarily in males (Kelly, Leggett, et al., 2009). Our results also indicate that, in general, PAE and ELA target nodes of the social behavior neural network in unique ways, perhaps due to differential vulnerability of these brain regions to early life insults. Our neural activity data suggest that the effects of PAE and ELA on brain systems supporting social recognition memory are somewhat dissociable, such that the amygdala appears to be particularly sensitive to PAE while the mPFC seems to be more vulnerable to ELA; indeed, amygdala subnuclei were affected by PAE in all but late adolescent females while ELA-related *c-fos* expression changes were more prominent in the mPFC. Moreover, behavioral sex differences were associated with sex-specific alterations in hypothalamic neuropeptide expression, such that ELA perturbed the female AVP system whereas PAE resulted in alterations of both OT and AVP expression in males. Together, these data suggest that PAE and ELA uniquely and interactively impact the development of the OT/AVP systems, which supports future investigation of these neuropeptide systems as potential therapeutic targets for impaired social behavior resulting from these deleterious pre- and postnatal environments.

Recent estimates indicate a 1-5% prevalence of Fetal Alcohol Spectrum Disorders (FASD) among American children, making PAE a leading cause of developmental disability

(May et al., 2018). Impaired social behavior associated with PAE is known to have widespread implications for function in other domains and may contribute to difficulties within the school environment, social rejection, trouble with the law, and later mental health problems (Kully-Martens et al., 2012). Given the high occurrence of ELA in individuals with PAE, it is difficult from a clinical standpoint to separate effects of PAE from those of ELA on outcomes (Henry et al., 2007). The relevance of the current research stems from the need for establishing a more specific social neurobehavioral profile that could support the development of strategies for earlier diagnoses and more targeted interventions for FASD. Our results add to the literature demonstrating how PAE directly impacts social behavior and its underlying neurobiology and underscores the role of the early environment in influencing social neurobehavioral development. Though we show that ELA can exacerbate PAE-related social behavior deficits, we also suggest that early interventions that mitigate adverse early life experiences have the potential to improve neurobehavioral outcomes.

Chapter 5: Discussion

5.1 Overview of research findings

Results from my dissertation research demonstrate the deleterious effects of PAE on social behavior and contribute to the literature a more distinctive social neurobehavioral profile to support the development of specific strategies for earlier diagnoses and more targeted interventions for individuals with FASD. By characterizing the development of impaired social behavior function within a well-established rodent model of PAE, we were able to investigate attendant neurobiological correlates to a degree not possible in clinical studies. Moreover, our experimental design incorporated modifications to the social context during behavioral tests – including progressively increasing the social complexity of testing conditions – to uncover how the social environment may shape PAE-related social behavior deficits. Importantly, we focused on the adolescent developmental period, when the transition to a more complex social environment may exacerbate existing deficits in social behavior function. In addition to social behavior testing, we also assessed olfaction, social odor discrimination, and non-social object recognition memory to further address the specificity of the PAE insult to the social behavior domain and provide evidence that social behavior deficits are not simply a downstream effect of impaired sensory or general cognitive function. Finally, inclusion of both male and female animals provided important insight into sex differences in social function following PAE. Overall, our data indicate that PAE impairs adolescent social behavior – especially with increasing complexity of the social context or the experience of ELA – and that impairments are associated with alterations in the social behavior neurocircuitry and altered development of the oxytocin (OT) and vasopressin (AVP) systems.

In Chapter 2, we assessed play partner preference utilizing a novel approach in which adolescent male and female rats interacted within same-sex triads comprised of conspecifics from mixed prenatal treatments to determine how play partner identity and social group composition interact to shape play behavior. When triads included one PAE animal and two control animals (i.e., control animals had the option to play either with a fellow control or a PAE playmate), we observed play target asymmetry whereby controls preferentially played with fellow controls. Notably, these results were consistent for triads of both males and females, with subtle differences in frequency of initiations versus reciprocations. We found no play target asymmetry, however, when triads included two PAE animals and one control animal or different configurations of control and pair-fed animals. Taken together, play target asymmetry resulting from ineffective social interactions – including a failure to engage with, respond to, and/or solicit play from control play partners appropriately – suggests that PAE has direct negative impacts on the development of social competence; moreover, PAE may levy a secondary insult by precluding age-appropriate opportunities for social experiences through the subsequent exclusion from play with competent playmates.

We next (Chapter 3) performed 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 OT and AVP systems following PAE, given their well-documented role in mediating social behavior. Results from Chapter 3 demonstrated that PAE impairs and delays development of adolescent social recognition memory, particularly in a complex social context, which is associated with

specific age-dependent changes in OT receptor (OTR) binding within the social behavior neural network. Specifically, early adolescent PAE males showed impairments on the social recognition memory test and increased OTR binding in limbic networks, while late adolescent PAE males exhibited impairments on the social discrimination test and increased OTR binding in forebrain reward systems.

Building on these results, Chapter 4 experiments involved combining animal models of PAE and ELA to investigate their unique and/or interactive effects on social neurobehavioral function in early and late adolescent male and female rats. Importantly, individuals with PAE are more likely to experience ELA, which alone can lead to a variety of social behavior deficits. Behavioral testing was followed by assessment of expression of the immediate early gene *c-fos*, a marker of neural activity, within key regions of the social behavior neural network as well as the expression of hypothalamic OT and AVP, key neuropeptides in the regulation of social behavior. Our results indicated that PAE and ELA have unique sex- and age-specific effects on social recognition memory, neural activity and OT/AVP expression, with more pronounced neurobehavioral changes observed in males than in females in both early and late adolescence. Specifically, ELA impaired social recognition in early adolescent females regardless of prenatal treatment, while males showed deficits in both early and late adolescence in response to unique and interactive effects of PAE and ELA. Neurobiological data suggested that specific brain regions show differential vulnerability to perinatal insults, such that the amygdala and OT system appear to be particularly sensitive to PAE while the mPFC and AVP system seem to be more vulnerable to ELA.

Our data collectively provide novel insight into how the early environment may mediate outcomes of PAE as well as the power of animal models to interrogate this relationship

systematically. Additionally, though we show that ELA can exacerbate PAE-related social behavior deficits, our data also suggest that early interventions that mitigate adverse early life experiences have the potential to improve neurobehavioral outcomes.

5.1.1 Specificity of PAE insult on the social domain

Social behavior deficits following PAE have been suggested to result simply as a secondary consequence of upstream sensory deficits (e.g., olfaction) or of general cognitive deficits in learning and memory (Barron et al., 1988; Mattson & Riley, 2000). Given that sensory processing as well as learning and memory are important components of social behavior function, this hypothesis is not without merit (Adolphs, 2003). To address this possibility and assess the specificity of the PAE insult to the social behavior domain, we included additional cohorts for assessments of olfaction and social odor discrimination (Chapter 3) as well as non-social object recognition memory (Chapter 4). Results from these experiments demonstrated that PAE rats did not differ from controls in general olfaction, ability to discriminate between two different social odors, or novel object recognition, suggesting that the PAE-related social behavior deficits we identified appear to stem from specific insult to the social behavior domain. Though these additional tasks were arguably less cognitively demanding relative to our play and social recognition assessments, we suggest that – rather than simply reflecting purely an effect of task difficulty – our collective results demonstrate that PAE-related social behavior deficits are more likely a result of global impairments to the integration of multi-sensory stimuli and recruitment of the social behavior neural network subserving the diverse neurocognitive processes required for intact social behavior function (Stevens et al., 2017). Indeed, our data are somewhat reminiscent of findings in the clinical literature showing that social behavior deficits are poorly correlated with overall intelligence (Doyle et al., 2019; S. E. Thomas et al., 1998).

Nevertheless, previous research has also indicated an important role for somatosensory and/or auditory (e.g., ultrasonic vocalizations) cues in mediating social behavior (Pellis et al., 2018; Siviy & Panksepp, 1987), and that processing of these cues can be altered by PAE (Charles Lawrence, et al., 2008; Mooney & Varlinskaya, 2011; Waddell et al., 2016)

5.2 Adolescent social behavior profile following PAE

The relevance of my dissertation research stems from the need for establishing a more specific social neurobehavioral profile that could support the development of strategies for earlier diagnoses and more targeted behavioral, social, and/or pharmacological interventions for FASD. Our profile of adolescent social behavior development encompassed three broad subprocesses of social function: social motivation, play behavior, and social recognition memory. To assess social motivation (Chapter 3), we utilized the social interaction test – which was originally designed as an assay to test social deficits in animal models of autism spectrum disorders (ASD; Ricceri, Moles, & Crawley, 2007). Social interaction testing was performed in 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 by eliminating the ambiguity of a "neutral" start chamber (Chaumont et al., 2012). Because social behavior inherently involves the actions of, at minimum, two actors, identifying specific social behavior deficits of the experimental animal can be challenging given that the behavior of social stimuli can influence that of the experimental animal. Support for this comes from our data utilizing play triads, where we found that control animals preferentially bias their play away from PAE animals and towards fellow controls, which then impacts the behavior of the test animal and the overall quality of social interactions (Pellis & McKenna, 1992). Accordingly, to isolate the specific social motivation of the experimental animal to interact with

the stimulus animal during the social interaction test, in both the three- and two-chambered test apparatuses, social stimulus animals were retained behind a clear Plexiglas barrier to prevent physical contact, and thus, any behavioral influence of the social stimulus animal while retaining key visual and olfactory social cues. Regardless of age or the specific testing apparatus (three- versus two-chambered), we found no PAE-related differences in social motivation during adolescence, suggesting that social motivation is not impaired by PAE. In fact, our finding that PAE animals show increased play initiation during the learning phase of the social discrimination task (Chapter 3) also indicates that, not only is social motivation not attenuated following PAE, it may actually be increased among PAE animals. This is consistent with previous research using similarly aged animals in a modified social motivation test, where PAE animals showed shorter isolation-induced approach latencies to a cagemate contained in a goal box at the end of an alley (Lugo et al., 2003). Nevertheless, other studies of PAE effects on social behavior have reported reduced social motivation (Ignacio et al., 2014; Mooney & Varlinskaya, 2011), though these studies differ in rat strain utilized, dose and pattern of PAE (e.g., single binge dose vs. chronic prenatal exposure), as well as the different behavioral paradigm utilized (e.g., experimental animals were able to interact freely with age-matched social stimulus animals).

Our finding that PAE did not affect social motivation provides important insight into the specific nature of social behavior deficits associated with PAE, particularly when considering other neurodevelopmental disorders characterized by impaired social function. For example, given the considerable social behavior impairments observed in individuals across the fetal alcohol spectrum, it is not surprising that the clinical literature has documented some overlap in social behavior deficits between FASD and ASD. Though both neurodevelopmental disorders

are characterized by impaired social behavior functioning – and particularly in the quality of social interactions – the behavioral profiles of the two disorders diverge significantly with regards to social motivation; whereas deficits in social motivation are a hallmark of ASD (Chevallier et al., 2012), children with FASD are generally described as having a high degree of social motivation (Bishop et al., 2007; Stevens et al., 2017; Stevens, Nash, Koren, & Rovet, 2013). Our data further support this conclusion and suggest that FASD-related deficits arise from distinct neurobehavioral changes in the social domain – including social recognition memory and processing of social cues. We also did not observe differences in social investigation – a proxy measure for social interest – in PAE animals from our play triad assessments (Chapter 2), suggesting that social deficits following PAE appear to be driven by impaired social competence and not from an overall lack of social motivation.

We next tested social recognition memory using two different testing paradigms, The social recognition memory test (i.e., habituation-dishabituation paradigm; Gheusi et al., 1994) was used to assess the ability of PAE animals to remember a repeatedly presented social stimulus and also recognize the presentation of a novel social stimulus (Chapter 3). Given the relative simplicity of this social recognition memory test paradigm (i.e., only one social stimulus presented per trial), we also used the social discrimination test to investigate further the effects of PAE on social recognition memory in a more complex social context (Chapters 3-4). 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 more direct measure of social recognition memory in a complex social context (Engelmann et al., 2011). Social recognition deficits following PAE have previously been documented in adult animals (Kelly,

Leggett, et al., 2009; Kelly & Tran, 1997), and here we add to this literature by showing social recognition deficits are present in adolescence. Specifically, PAE animals showed delayed development of social recognition memory relative to controls, as PAE-related impairments on the simpler "habituation-dishabituation" test were present in early but not late adolescence. In the more complex social discrimination test (Chapter 3), all early adolescent animals failed to discriminate between novel and familiar social stimuli, regardless of prenatal treatment; however, in late adolescent animals, only PAE animals were unable to show social discrimination. Taken together, our social recognition memory data using two different testing paradigms suggest that deficits in social recognition memory are still present in late adolescence but are only be uncovered under conditions of increased complexity of the testing protocol. These findings parallel accounts from the clinical literature, which report not only that children with FASD perform relatively well on simple tasks but are more impaired with more demanding or complex tasks (Kodituwakku, 2007), but also that they consistently lag behind peers in social behavior function (Streissguth et al., 1991). Importantly, though we report delayed development of social recognition memory – perhaps as a positive indication that at least some impaired subprocesses of social function may improve with age – data in adult animals suggest that overall delays in social behavior function may eventually plateau (Stevens et al., 2017), highlighting the need for earlier interventions that may shift developmental trajectories to result in continued growth and improved outcomes for social behavior function.

We were somewhat surprised by the failure of any early adolescent animals to exhibit social discrimination (Chapter 3), as previous research has demonstrated social discrimination abilities in early adolescent rats following much longer retention intervals between the familiarization and discrimination phases (e.g., 1 hr; Veenema et al., 2012). However, an

important difference between these earlier studies and our results in Chapter 3 is that we tested animals in an unfamiliar testing apparatus rather than in the home cage, which likely represents a more stressful environment for early adolescent animals (Camats Perna & Engelmann, 2017). Accordingly, we opted to perform our next set of social discrimination tests in a more familiar home cage environment (Chapter 4) in an attempt to mitigate this stress. We found that – like our "habituation-dishabituation" results – when tested in a less stressful, familiar home cage, PAE-related social discrimination deficits were observed in early adolescence but had resolved in late adolescence. By utilizing two different testing environments for social discrimination testing, our results further enhance our social neurobehavioral profile by showing that in addition to the complexity of the social context, PAE-related social behavior deficits also appear to be sensitive to the relative stressfulness of the testing context. This interpretation is consistent with previous work from our laboratory and others demonstrating that PAE offspring are typically behaviorally and physiologically hyperresponsive to stressors (Hellemans, Sliwowska, et al., 2010; Weinberg et al., 2008; Weinberg, Taylor, & Gianoulakis, 1996; Wieczorek, Fish, O'Leary-Moore, Parnell, & Sulik, 2015), and suggests altered stress reactivity following PAE may further mediate social behavior deficits (Kelly, Goodlett, et al., 2009).

Finally, our results from social recognition memory testing also revealed sex differences in the effects of PAE on social behavior development, as social recognition memory was impaired in male but not female animals. Our observation of intact social discrimination in females following PAE was not entirely unexpected, given the extensive literature demonstrating superior recognition memory among females, as they exhibit social recognition over much longer retention intervals than males (Engelmann et al., 2011; Markham & Juraska, 2007). Moreover, our results corroborate previous studies showing PAE-related sex differences (i.e.,

males but not females show deficits) in social recognition in adult rats using a three-trimester equivalent PAE model (Kelly, Leggett, et al., 2009). Importantly, sex differences are relatively poorly investigated in the clinical and preclinical neuroscience literature (Beery & Zucker, 2011); moreover – with respect to studies involving human subjects with FASD – many studies collapse subjects across gender or are underpowered to investigate these differences (Kully-Martens et al., 2012). Thus, the use of males and females in this dissertation research helps to fill a gap in the literature by demonstrating sex differences in the effects of PAE on social behavior function and, in particular, enhanced vulnerability of males to PAE-related social behavior deficits. Despite intact social recognition in PAE females presented here, our finding of impaired social competence in both males and females (Chapter 2) in addition to results from previous research suggest that females are not completely resistant to PAE-related social behavior impairments; rather, our data of sex-specific outcomes in social behavior function following PAE provide for a more comprehensive and inclusive social neurobehavioral profile by demonstrating the potential for sex-specific effects of PAE on social behavior expression and development. Our data suggest that PAE-related sex differences in social behavior function arise from sex convergence (i.e., similar behavioral expression but different underlying neurobiology in males and females) or divergence (i.e., sex difference uncovered only under specific situations for each sex; (McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012).

5.3 Role of postnatal experience in mediating PAE-related social behavior impairments

Social behavior development is an ongoing process involving ongoing maturation of underlying neurocognitive processes that support social competence across the lifespan. Inherent in this maturation of social competence is the role of experience, which continually shapes and refines social behavior to meet the demands of an increasingly complex social environment that

unfolds across development – from attachment and play behavior in early life to sexual behavior in adulthood (Galef & LaLand, 2005; Insel, 2000; Meaney & Stewart, 1981). Results from our assessments of play triads demonstrated that PAE impairs the development of social competence, as we observed play target asymmetries in triads with two controls and one PAE animal (Chapter 2). When viewed in the context of experience-dependent social behavior development, the ramifications of PAE-related impairments in social competence are noteworthy, as deficient social competence not only prevents appropriate social functioning in the moment, but also imposes a secondary insult by depriving PAE animals of age-appropriate opportunities for social experiences through the subsequent exclusion from play with competent playmates. A similar situation arises during the early postnatal period, as PAE pups show a diminished capacity to elicit retrieval by the dam (Ness & Franchina, 1990), such that PAE targets pup social competence (i.e., the ability to elicit retrieval), which subsequently alters maternal responsiveness. In turn, altered maternal responsiveness feeds back to the pup, with the potential to further exacerbate PAE-related impairments to the development of social competence (Champagne et al., 2001). In a sense, PAE animals appear perpetually out of sync with the developmental context of their social environment, which may explain why social impairments often become more profound with age (Kully-Martens et al., 2012).

Results from our combined models of PAE and ELA further demonstrate the importance of early experience for mediating social behavior outcomes following PAE; moreover, our use of the two-day testing protocol allowed us to investigate further the role of adolescent social experience to improve social recognition. Specifically, our finding that social experience across the two testing days improved social discrimination performance for most PAE and ELA animals suggests that – despite some initial deficits in social recognition memory – animals with these

insults could show improved performance when given additional opportunities to learn. One of the most critical findings from our two-day testing protocol was that the effects of PAE and ELA resulted in longer lasting behavioral impairments, preventing experience-related improvements in social discrimination – at least in late adolescence – possibly due to additive effects. That is, due to the differential impacts of these two insults within the social behavior neural network, the combined exposures led to more profound behavioral deficits on the social discrimination test than were observed separately for each insult.

Our data related to experience-dependent improvements in social recognition as well as synergistic effects of ELA have important implications for designing interventions that target the social environment. In the absence of targeted brain-based therapies, modulating the social environment presents a tractable and potentially high-impact avenue for ameliorating impaired social behavior resulting from PAE. As an example, social skills training in children aged 6-12 with FASD has previously been shown to improve children's knowledge of appropriate social behavior as well as parent ratings of social skills (O'Connor et al., 2006). Likewise, preclinical research has shown that providing enriched social environments during adolescence can rescue social behavior deficits following PAE and, notably, PAE-related epigenetic and neurotransmitter changes in the amygdala (Ignacio et al., 2014; Middleton et al., 2012).

5.4 Impact of PAE on social behavior neurobiology

Assessments of the adolescent social neuropeptide systems following PAE revealed robust age-dependent changes in the OT system – particularly in males – including increased OT receptor binding in key regions of the social behavior neural network (Chapter 3) as well as reduced hypothalamic OT expression (Chapter 4), which were also aligned with our behavioral results. Additionally, regions of the social behavior neural network appeared to be differentially

sensitive to PAE and ELA as revealed by alterations in neural activity following social discrimination testing (Chapter 4), such that the amygdala and lateral septum were particularly vulnerable to PAE while the mPFC was more vulnerable to ELA. There is an extensive literature on the importance of OT acting within the social behavior neural network for mediating social behavior function (Bielsky & Young, 2004; Bredewold & Veenema, 2018), and our results provide further insight into how perinatal insults impact on OT system development in the context of social recognition memory (Veenema, 2012). Moreover, our assessment of activity-dependent *c-fos* expression following social discrimination testing (Chapter 4) revealed attenuated activity in many of the same brain regions where we identified increased OTR binding, including the mPFC and amygdala – which both receive OTergic axonal projections from the PVN – as well as the lateral septum (Johnson & Young, 2017).

Previous studies investigating the role of OT in mediating social behavior have described how OT functions in specific nodes of the social behavior neural network within the context of the particular neurocognitive function subserved by the region (Bredewold & Veenema, 2018); however, more recent network-based approaches have begun to show how OT mediates social behavior by activating OTRs distributed within the social behavior neural network to facilitate multi-sensory integration of socially relevant stimuli for processing and generation of appropriate behavioral responses (Johnson & Young, 2017; Marlin & Froemke, 2017). For example, OT has also been implicated in promoting cross-modal, experience-dependent cortical development in mice, as deprivation induced deficits in sensory cortex plasticity can be rescued by OT treatment (Zheng et al., 2014), Additionally, male prairie voles have been shown to exhibit correlated neural activity (Fos protein expression) thought to represent functional coupling of the social behavior neural network following sociosexual interactions as compared to

isolated males. Importantly, this functional coupling was blocked by ICV infusion of an OT antagonist (Johnson et al., 2016). In view of these data and our observation of reduced hypothalamic OT expression as well as increased OTR binding and neural activity within the social behavior neural network, it is tempting to speculate that PAE-related social behavior deficits arise from direct effects of alcohol on the OT system development; however, more causal experiments, particularly manipulation of central OT levels will be required to establish this relationship more directly. Nevertheless, our data provide a strong rationale for the investigation of OT as a potential therapeutic intervention for treating PAE-related social behavior impairments.

5.5 Future directions: Oxytocin treatment as a crosscutting intervention for PAE-related social behavior deficits

OT administration is increasingly being investigated as a crosscutting intervention to alleviate social behavior deficits (Born et al., 2002). Our findings build on the well-documented role of OT in regulating social behavior and its clinical availability, which make it an excellent candidate for therapeutic use in disorders with social behavior impairments (Insel, 2010). The clinical and preclinical literature support a role for OT treatment in enhancing social behavior in healthy subjects (Benarroch, 2013; Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003; Litvin & Pfaff, 2013), and in ameliorating social behavior deficits associated with ASD (Andari et al., 2010), frontotemporal dementia (Jesso et al., 2011), and other psychiatric disorders (Neumann & Landgraf, 2012). fMRI studies suggest that improved social behavior function following OT treatment is correlated with activation of the amygdala (Domes et al., 2007), and animal studies confirm an effect of exogenous OT to enhance social recognition (Cushing, 2013) and to rescue social behavior deficits in a mouse model of ASD (Teng et al., 2013). Given our findings of

increased OTR binding and neural activity in the amygdala following PAE, OT's effects on amygdala function and resulting enhancements in social behavior function provide support for investigating OT as a potential therapeutic tool for ameliorating PAE-related social behavior deficits. Furthermore, OT has been shown to reduce inter-male aggression (Wesson, 2013), enhance sociability (Bowen, Carson, Spiro, Arnold, & McGregor, 2011), and promote hippocampal neurogenesis (Leuner, Caponiti, & Gould, 2012). Neumann et al., utilizing microdialysis, observed central increases in OT levels following i.p. injection in mice (Neumann, Maloumby, Beiderbeck, Lukas, & Landgraf, 2013). Nevertheless, a recent meta-analysis indicated equivocal to modest results across 16 randomized-control trials (RCT) among children with ASD (Y. Wang, Wang, Rong, He, & Yang, 2019). Rather than indicating a lack of OT efficacy in treating social behavior impairments in humans, these negative data may instead be an indication of important sex/gender or developmental differences in OT system function, as well as limits on intranasal delivery of OT to the central nervous system across the blood-brain barrier (Leng & Ludwig, 2016). As a potential solution to overcome these limitations, however, more recent research utilizing nanoparticle encapsulation of OT to increase brain bioavailability has shown promising results (Oppong-Damoah, Zaman, D'Souza, & Murnane, 2019). Interestingly, intranasal AVP has shown favorable results among individuals with ASD, suggesting that AVP treatment may be another potential therapeutic avenue for ameliorating social behavior deficits related to FASD. Nevertheless, despite the potential for OT/AVP treatment to ameliorate social behavior deficits across a broad range of disorders, neither have been assessed for their therapeutic value in clinical or preclinical research in the context of PAE-related social behavior impairments. Because of the sexually dimorphic roles of OT/AVP on social behavior, as well as their sex-specific alterations following PAE, it is possible that

OT/AVP may only be effective in one sex versus the other. Nevertheless, examination of neuropeptide effects on social behavior in the context of PAE would help delineate OT/AVP specificity in rescuing PAE-related social behavior deficits and provide guidance for effective sex-specific treatment of impaired social behavior. Notably, there are caveats of AVP treatment given AVP's facilitatory effects on the hypothalamic-pituitary-adrenal (HPA) or stress axis in the context of HPA hyperresponsivity following PAE (Neumann & Landgraf, 2012; Weinberg et al., 2008). Finally, OT treatment does not have uniformly positive effects: neonatal exposure, for example, may adversely affect development (Carter, 2003; Rault et al., 2013) whereas adolescent exposure has generally shown beneficial effects (Bowen et al., 2011). Future research on OT/AVP will be invaluable for clarifying the effects of adolescent social neuropeptide treatment on development and evaluating the specificity of OT/AVP in mediating PAE-related social behavior deficits. Based on the data presented in my dissertation, we expect that OT treatment would rescue PAE-related social behavior deficits by increasing central OT levels (Neumann et al., 2013) presumably by normalizing patterns of neural activity in PAE animals such that they show *c-fos* activation patterns similar to controls, including increased activity within the amygdala, prefrontal cortex and lateral septal nodes of the social behavior neural network.

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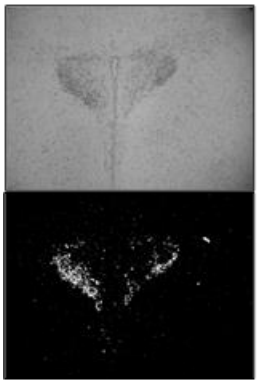
Appendices

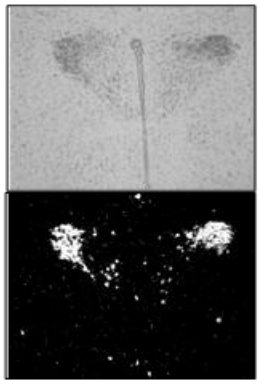
Appendix A Representative images of OT/AVP *in situ* hybridization in the hypothalamus (Chapter 3)

Toluidine Blue counterstain of PVN

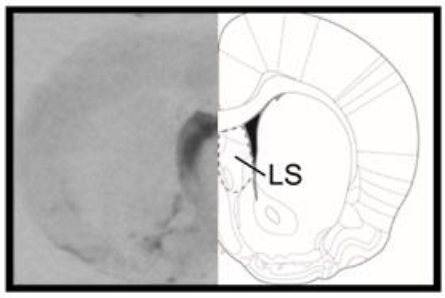


Dark-Field image of OT *in situ* signal

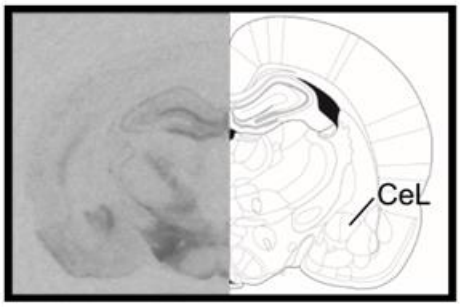
Toluidine Blue counterstain of PVN



Dark-Field image of AVP *in situ* signal



Autoradiograph of $V1_aR$ binding with corresponding atlas reference for LS (above) and CeL (below; Paxinos & Watson 2005).



Appendix B Additional behavioral measures (Chapter 3)

Duration and frequency of behaviors during familiarization phase

	Prenatal treatment group		
Early Adolescence	Control	PF	PAE
Rearing	18.6 ± 3.0	18.3 ± 2.5	11.2 ± 1.4
Self Groom	6.5 ± 1.8	3.3 ± 1.1	2.3 ± 1.0
Follow/Chase	10.0 ± 2.2	8.2 ± 2.5	10.0 ± 1.7
Evade	3.5 ± 1.8	3.0 ± 0.9	4.0 ± 1.9
Pin Frequency	1.8 ± 0.8	2.2 ± 1.2	3.2 ± 2.0
Late Adolescence			
Rearing[1]	40.8 ± 6.6	16.1 ± 2.4[a]	19.2 ± 3.4[a]
Self Groom	4.9 ± 1.5	1.3 ± 0.5[b]	2.8 ± 0.7
Follow/Chase	8.7 ± 1.7	9.6 ± 1.1	6.3 ± 1.7
Evade	2.7 ± 1.9	4.5 ± 2.2	1.8 ± 0.8
Pin Frequency	0.2 ± 0.1	1.9 ± 1.3	1.3 ± 0.9

Note: Data are expressed as mean ± SEM. [1] Behavior is significantly different between early and late adolescent animals ($p \leq 0.05$). [a] Behavior of PF and PAE males significantly greater than control males ($p \leq 0.05$); [b] Behavior of PF males significantly less than control males ($p \leq 0.05$).

Appendix C Hypothalamic OT/AVP mRNA expression (Chapter 3)

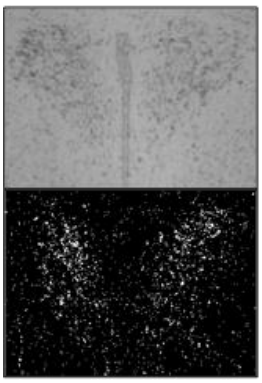
OT & AVP mRNA Expression (mean integrated density)

	Prenatal treatment group		
Early Adolescence	Control	PF	PAE
magnoPVN (OT)	17.2 ± 2.5	27.5 ± 5.6	20.8 ± 2.8
parvoPVN (OT)	5.6 ± 0.9	8.7 ± 1.1	2.3 ± 1.0
SON (OT)	47.5 ± 3.7	45.7 ± 3.2	47.1 ± 2.0
magnoPVN (AVP)	32.1 ± 6.2	28.9 ± 4.9	25.8 ± 4.6
parvoPVN (AVP)	5.4 ± 0.7	5.3 ± 0.9	5.1 ± 0.9
SON (AVP)	44.5 ± 3.7	45.8 ± 3.7	44.8 ± 3.0
Late Adolescence			
magnoPVN (OT)	20.8 ± 4.5	22.3 ± 4.3	27.7 ± 4.7
parvoPVN (OT)	5.7 ± 0.6	6.9 ± 1.4	5.8 ± 0.4
SON (OT)	45.0 ± 2.5	43.5 ± 2.8	44.7 ± 4.2
magnoPVN (AVP)	29.4 ± 2.2	22.0 ± 2.4	39.9 ± 6.6
parvoPVN (AVP)	6.2 ± 0.8	5.7 ± 1.0	8.0 ± 1.8
SON (AVP)	48.4 ± 3.3	47.9 ± 2.2	48.3 ± 2.4

Note: Data are expressed as mean ± SEM.

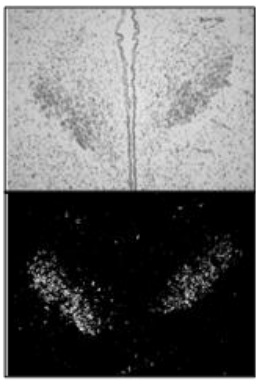
Appendix D Representative images of *c-fos*, OT, & AVP *in situ* hybridization (Chapter 4)

Toluidine Blue counterstain of PVN



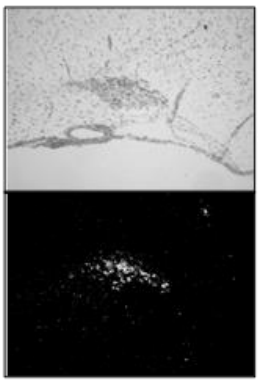
Dark-Field image of *c-fos in situ* signal

Toluidine Blue counterstain of PVN



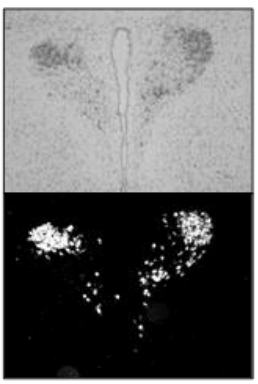
Dark-Field image of OT *in situ* signal

Toluidine Blue counterstain of SON

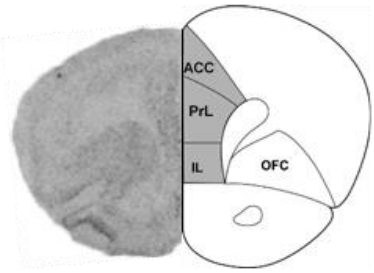


Dark-Field image of OT *in situ* signal

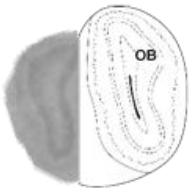
Toluidine Blue counterstain of PVN

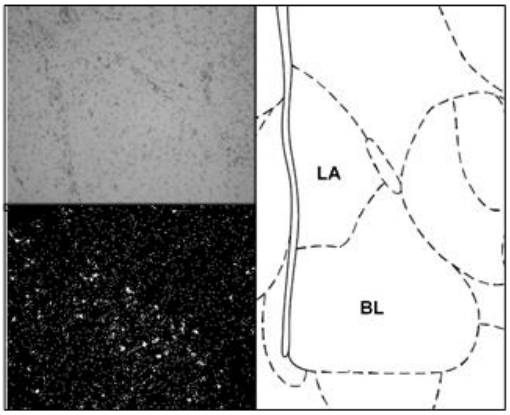


Dark-Field image of AVP *in situ* signal

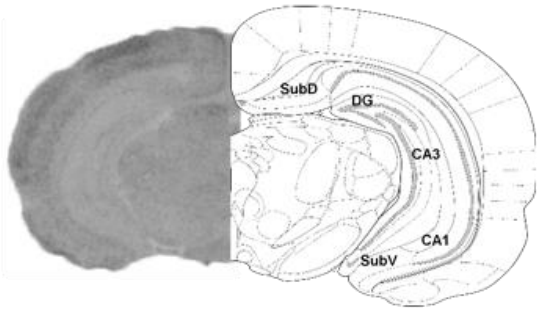


Autoradiograph of c-fos *in situ* with corresponding atlas reference for PFC (left) and OB (right; Paxinos & Watson 2005).





Toluidine Blue counterstain of the lateral amygdala (LA; upper left); dark-Field image of c-fos *in situ* signal (bottom left) with corresponding atlas reference (right; Paxinos & Watson 2005).



Autoradiograph of c-fos ISH with corresponding atlas reference (Paxinos & Watson 2005).

Appendix E Sex- and age-related changes in *c-fos*, OT, and AVP mRNA expression

Statistical Results

	Effect		
c-fos	Sex	Age	Interaction
OB		$F_{1,126} = 8.33$, $p < 0.01$ P30 < P45	
PCX			$F_{1,168} = 3.85$, $p = 0.05$ P45 ♂ > all groups
ACC			
PrL			
IL		$F_{1,168} = 7.10$, $p < 0.01$ P30 < P45	
OFC			$F_{1,167} = 12.85$, $p < 0.001$ P45 ♂ > all groups
LS		$F_{1,164} = 26.68$, $p < 0.001$ P30 < P45	
LA			$F_{1,168} = 6.04$, $p < 0.05$ P30 ♂ < P45 ♂
BL			$F_{1,168} = 15.61$, $p < 0.001$ P45 ♂ > all groups
CeM			$F_{1,168} = 11.48$, $p < 0.001$ P30 ♂ = P45 ♀ < P45 ♂
CeL			$F_{1,168} = 12.91$, $p < 0.001$ P45 ♂ > all groups
MeA			$F_{1,168} = 6.13$, $p < 0.05$ P45 ♂ > all groups
CoA			$F_{1,168} = 4.17$, $p < 0.05$ P30 ♂ < P45 ♂
magnoPVN			
parvoPVN			
CA1			$F_{1,164} = 9.89$, $p < 0.01$ P45 ♀ > all groups
DG			$F_{1,164} = 4.08$, $p < 0.05$ P30 ♀ < P45 ♀
CA3			$F_{1,164} = 20.55$, $p < 0.001$ P30 ♀ > P30 ♂ = P45 ♂ > P45 ♀
SubD			$F_{1,164} = 11.53$, $p < 0.001$ P45 ♂ = P30 ♀ > P45 ♀
SubV		$F_{1,164} = 7.23$, $p < 0.01$ P30 > P45	
OT			
magnoPVN		$F_{1,166} = 13.04$, $p < 0.001$ P30 < P45	
parvoPVN	$F_{1,166} = 4.60$, $p < 0.05$ ♀ > ♂		
SON		$F_{1,163} = 20.39$, $p < 0.001$ P30 < P45	
AVP			
magnoPVN	$F_{1,168} = 4.40$, $p < 0.05$ ♀ < ♂		
parvoPVN			
SON			

Appendix F Duration and frequency of behaviors during learning phase (Chapter 4)

Additional Behaviors (Males)

Prenatal Treatment	**Behaviors**							
Early Adolescence (Day 1)	Social Invest	Play	Rearing	Self Groom	Follow/Chase	Evade	Pounce (#)	Pin (#)
Control Normal Rearing	138.2 ± 10.0	12.2 ± 4.2	36.4 ± 8.3	2.9 ± 1.4	4.6 ± 0.8	1.0 ± 0.7	1.5 ± 0.8	2.2 ± 0.9
PF Normal Rearing	159.2 ± 6.8	12.1 ± 4.1	23.1 ± 4.1	6.3 ± 1.1	3.4 ± 1.0	0.7 ± 0.3	3.1 ± 1.0	1.8 ± 0.8
PAE Normal Rearing	158.9 ± 7.7	10.2 ± 3.4	18.1 ± 4.4	5.1 ± 1.5	4.2 ± 1.2	0.5 ± 0.2	4.0 ± 1.5	1.8 ± 0.6
Control ELA	150.4 ± 7.2	18.4 ± 4.3	24.2 ± 5.0	6.3 ± 2.2	6.1 ± 1.3[a]	0.7 ± 0.3	3.4 ± 0.8	2.6 ± 0.8
PF ELA	155.7 ± 11.2	17.9 ± 6.1	26.0 ± 5.9	5.6 ± 1.6	6.9 ± 1.5[a]	0.9 ± 0.3	2.3 ± 0.8	3.3 ± 1.3
PAE ELA	138.8 ± 9.9	13.7 ± 6.0	22.1 ± 4.2	9.7 ± 2.9	6.3 ± 1.1[a]	0.8 ± 0.4	2.5 ± 0.6	2.4 ± 1.8
Early Adolescence (Day 2)								
Control Normal Rearing	165.7 ± 9.0	23.0 ± 6.6	10.1 ± 1.2	6.4 ± 1.1	6.6 ± 1.4	0.1 ± 0.1	1.5 ± 0.5	3.4 ± 1.2
PF Normal Rearing	174.5 ± 11.6	13.3 ± 4.0	10.5 ± 2.5	7.1 ± 2.0	6.2 ± 0.7	0.3 ± 0.2	1.0 ± 0.3	1.7 ± 0.5
PAE Normal Rearing	162.7 ± 7.4	10.7 ± 3.9	13.6 ± 2.4	5.0 ± 1.7	6.2 ± 1.0	0.0 ± 0.0	1.6 ± 0.6	1.4 ± 0.6
Control ELA	161.8 ± 8.6	12.1 ± 3.4	15.3 ± 3.3	5.7 ± 1.7	5.7 ± 0.8	0.1 ± 0.1	1.5 ± 0.3	1.7 ± 0.6
PF ELA	150.7 ± 7.7	17.8 ± 5.1	19.8 ± 5.3	6.9 ± 1.4	5.2 ± 1.0	0.1 ± 0.1	1.4 ± 0.5	2.2 ± 0.9
PAE ELA	156.6 ± 9.1	14.5 ± 5.3	11.2 ± 2.8	7.9 ± 2.1	7.1 ± 0.8	0.0 ± 0.0	1.1 ± 0.4	2.4 ± 1.0
Late Adolescence (Day 1)								
Control Normal Rearing	181.5 ± 8.3	3.8 ± 1.8	13.5 ± 3.3	4.4 ± 1.1	1.9 ± 0.7	0.1 ± 0.1	1.0 ± 0.4	0.9 ± 0.4
PF Normal Rearing	168.2 ± 10.2	7.2 ± 2.7	10.8 ± 3.4	5.5 ± 2.2	3.4 ± 0.6	0.2 ± 0.1	3.0 ± 0.8	1.7 ± 0.7
PAE Normal Rearing	168.3 ± 9.3	21.9 ± 7.3[b]	8.8 ± 3.2	3.7 ± 1.3	4.3 ± 1.3	0.4 ± 0.2	2.3 ± 1.0	5.5 ± 1.9
Control ELA	174.8 ± 4.9	12.7 ± 4.0	9.1 ± 1.8	4.5 ± 1.2	4.3 ± 1.1	0.3 ± 0.2	2.8 ± 0.8	3.8 ± 1.2
PF ELA	170.3 ± 10.2	7.5 ± 2.3	8.8 ± 1.7	3.3 ± 1.0	2.7 ± 0.7	0.3 ± 0.2	2.7 ± 0.4	2.4 ± 0.9
PAE ELA	162.9 ± 7.3	14.2 ± 6.4[b]	11.8 ± 3.4	3.4 ± 1.0	2.9 ± 0.6	0.3 ± 0.3	2.6 ± 0.7	4.2 ± 2.0
Late Adolescence (Day 2)								
Control Normal Rearing	172.0 ± 7.5	17.7 ± 5.3	14.1 ± 3.0	5.4 ± 0.6	8.0 ± 1.6	0.8 ± 0.5	2.3 ± 0.7	2.9 ± 1.0
PF Normal Rearing	160.8 ± 5.0	23.0 ± 3.6	18.0 ± 4.1	7.6 ± 1.8	5.6 ± 1.2	0.6 ± 0.2	0.8 ± 0.3	2.1 ± 0.5
PAE Normal Rearing	172.4 ± 10.9	28.2 ± 5.6	8.8 ± 2.2	7.5 ± 2.2	5.0 ± 1.1	1.1 ± 0.6	1.1 ± 0.4	2.9 ± 1.0
Control ELA	147.4 ± 16.3	20.3 ± 6.3	16.7 ± 6.8	4.2 ± 1.3[a]	8.2 ± 2.0	0.7 ± 0.4	1.6 ± 0.8	2.3 ± 0.8
PF ELA	169.3 ± 10.6	17.8 ± 5.4	19.7 ± 2.9	3.1 ± 0.6[a]	5.6 ± 0.8	1.2 ± 0.5	2.1 ± 0.6	2.1 ± 0.7
PAE ELA	147.8 ± 8.8	19.5 ± 4.5	12.6 ± 2.1	3.6 ± 1.6[a]	5.5 ± 1.5	1.1 ± 0.6	2.4 ± 0.7	1.9 ± 0.4

Note: Data are expressed as mean ± SEM. [a] Behavior of ELA animals significantly different from Normal Rearing animals ($p \leq 0.05$);

[b] Behavior significantly different from Controls ($p \leq 0.05$)

Appendix G Duration and frequency of behaviors during learning phase (Chapter 4)

Additional Behaviors (Females)

Age & Day	**Behaviors**							
Early Adolescence (Day 1)	Social Invest	Play	Rearing	Self Groom	Follow/Chase	Evade	Pounce (#)	Pin (#)
Control Normal Rearing	143.0 ± 7.4	12.9 ± 4.2	39.7 ± 5.3	8.7 ± 2.6	2.6 ± 0.7	2.6 ± 0.9	11.1 ± 3.2	1.7 ± 0.6
PF Normal Rearing	179.8 ± 9.9[a]	8.4 ± 2.9	29.5 ± 5.3	4.0 ± 0.8	2.1 ± 0.6	1.3 ± 0.4	14.8 ± 2.8	1.0 ± 0.5
PAE Normal Rearing	183.8 ± 10.9[a]	7.9 ± 2.5	19.6 ± 4.2[a]	6.9 ± 1.8	1.8 ± 0.4	1.6 ± 0.4	11.8 ± 2.2	1.3 ± 0.6
Control ELA	179.4 ± 6.8[a]	15.2 ± 3.3	16.1 ± 3.7[a]	9.0 ± 1.6	2.1 ± 0.6	2.7 ± 0.6	16.9 ± 3.8	1.5 ± 0.4
PF ELA	160.4 ± 8.8	9.7 ± 2.3	24.3 ± 3.6	6.4 ± 2.0	1.6 ± 0.4	4.5 ± 0.4[b]	9.1 ± 1.7	0.8 ± 0.2
PAE ELA	161.5 ± 7.2	14.8 ± 4.3	23.3 ± 4.3	6.4 ± 1.7	0.9 ± 0.3	1.9 ± 0.5	11.7 ± 2.4	1.5 ± 0.5
Early Adolescence (Day 2)								
Control Normal Rearing	171.2 ± 8.9	16.2 ± 3.0	28.7 ± 5.2	8.4 ± 2.0	1.4 ± 0.5	2.1 ± 0.6	7.8 ± 1.4	1.8 ± 0.5
PF Normal Rearing	177.3 ± 9.0	16.9 ± 4.5	22.7 ± 6.7	8.5 ± 2.1	1.3 ± 0.2	0.9 ± 0.3	10.4 ± 2.1	1.4 ± 0.5
PAE Normal Rearing	174.2 ± 8.4	9.6 ± 4.5	21.9 ± 3.6	6.9 ± 2.6	0.6 ± 0.4	0.9 ± 0.4	8.1 ± 4.7	0.9 ± 0.5
Control ELA	197.0 ± 10.2	8.8 ± 2.9	13.8 ± 2.5	8.4 ± 2.3	1.4 ± 0.5[c]	1.1 ± 0.5	6.1 ± 1.3	0.6 ± 0.3
PF ELA	164.5 ± 8.2	14.7 ± 5.4	30.1 ± 4.4	8.4 ± 1.9	2.1 ± 0.4[c]	1.7 ± 0.7	8.2 ± 3.1	1.3 ± 0.6
PAE ELA	163.9 ± 9.2	13.9 ± 3.1	19.8 ± 2.8	9.3 ± 2.4	2.1 ± 0.5[c]	2.9 ± 1.2	5.4 ± 1.1	1.6 ± 0.5
Late Adolescence (Day 1)								
Control Normal Rearing	145.9 ± 6.8	1.2 ± 0.4	47.0 ± 4.0	3.8 ± 1.0	0.8 ± 0.3	1.9 ± 0.7	2.5 ± 1.0	0.0 ± 0.0
PF Normal Rearing	153.0 ± 10.5	5.1 ± 1.8	30.5 ± 3.8[a]	3.0 ± 0.7	1.1 ± 0.3	4.8 ± 1.5	5.5 ± 2.2	0.3 ± 0.2
PAE Normal Rearing	154.1 ± 10.5	9.8 ± 3.9	19.3 ± 3.5[a]	3.6 ± 1.3	0.3 ± 0.2	5.5 ± 2.0	8.8 ± 3.7	1.4 ± 0.9
Control ELA	160.5 ± 8.4	3.4 ± 1.5	25.0 ± 4.1[a]	5.6 ± 1.2	0.7 ± 0.2	3.0 ± 1.0	7.0 ± 1.8	0.0 ± 0.0
PF ELA	162.7 ± 6.7	5.7 ± 3.5	25.1 ± 3.0[a]	2.6 ± 0.8	0.7 ± 0.2	2.9 ± 0.8	5.2 ± 1.8	0.7 ± 0.6
PAE ELA	152.9 ± 7.2	4.7 ± 1.2	22.1 ± 3.8[a]	5.2 ± 1.3	0.8 ± 0.3	4.5 ± 1.3	5.2 ± 1.2	0.4 ± 0.2
Late Adolescence (Day 2)								
Control Normal Rearing	142.2 ± 9.0	7.8 ± 4.8	44.7 ± 6.7	7.3 ± 1.8	0.4 ± 0.1	2.8 ± 1.2	2.5 ± 1.0	0.7 ± 0.3
PF Normal Rearing	136.4 ± 10.4	10.9 ± 4.6	41.7 ± 9.4	3.2 ± 0.8	1.1 ± 0.5	6.5 ± 2.2	3.2 ± 1.2	1.1 ± 0.5
PAE Normal Rearing	150.2 ± 12.6	12.5 ± 3.9	22.6 ± 5.6	7.2 ± 2.8	0.4 ± 0.2	4.4 ± 2.5	5.4 ± 1.6	1.4 ± 0.6
Control ELA	147.8 ± 8.4	5.6 ± 1.9	35.6 ± 6.4	6.6 ± 2.2	0.5 ± 0.2	2.4 ± 1.1	2.3 ± 0.5	0.8 ± 0.2
PF ELA	152.7 ± 6.3	10.1 ± 3.5	31.7 ± 6.4	4.0 ± 1.1	0.6 ± 0.2	3.7 ± 1.1	1.8 ± 0.7	0.5 ± 0.3

PAE ELA	139.5 ± 8.6	8.5 ± 4.5	34.8 ± 9.4	4.2 ± 1.6	0.3 ± 0.1	4.1 ± 1.2	3.7 ± 1.2	0.8 ± 0.6

Note: Data are expressed as mean ± SEM. [a] Behavior significantly different from Normal Controls ($p \leq 0.05$); [b] Behavior significantly different from all groups except Normal Control ($p \leq 0.05$); [c] Behavior of ELA animals significantly different from Normal Rearing animals ($p \leq 0.05$)

Appendix H Total social investigation duration (sec)

Total Social Investigation (Familiar + Novel)

	Prenatal treatment					
Males	Control		PF		PAE	
	Normal	ELA	Normal	ELA	Normal	ELA
P30						
Day 1	75.5 ± 3.5	81.9 ± 4.3	82.7 ± 5.2	78.4 ± 4.4	73.4 ± 3.4	73.9 ± 4.2
Day 2[a]	87.7 ± 5.8	80.6 ± 5.1	92.0 ± 2.9	85.3 ± 2.5	84.9 ± 3.2	82.8 ± 2.2
P45						
Day 1	83.8 ± 3.4	87.1 ± 1.8	91.0 ± 1.4	88.8 ± 3.2	83.7 ± 2.8[b]	76.3 ± 2.9[b]
Day 2[a]	88.5 ± 1.1	88.7 ± 2.4	90.0 ± 3.3	88.4 ± 3.1	89.7 ± 1.7[b]	86.1 ± 2.1[b]

Females	Control		PF		PAE	
	Normal	ELA	Normal	ELA	Normal	ELA
P30						
Day 1	72.7 ± 3.4	78.2 ± 4.1	82.1 ± 4.4	79.4 ± 3.4	79.6 ± 1.9	75.9 ± 2.3
Day 2	89.4 ± 2.6	94.3 ± 2.5[c]	87.7 ± 3.2	77.5 ± 3.8	77.5 ± 4.5	83.0 ± 4.1
P45						
Day 1	75.3 ± 4.2	76.3 ± 2.4	70.8 ± 5.8	74.2 ± 4.5	69.4 ± 4.5	71.5 ± 3.7
Day 2	65.9 ± 4.2	76.2 ± 2.4	75.7 ± 5.6	75.4 ± 4.6	77.4 ± 5.1	72.2 ± 3.0

Note: Data are expressed as mean ± SEM. [a] Total social investigation on Day 1 < Day 2 (effect of testing day [$F_{1,53} = 14.7$, $p < 0.001$]); [b] Total social investigation of PAE < PF (main effect of treatment group [$F_{2,52} = 5.32$, $p \leq 0.05$]); [c] Total social investigation of C ELA Day 2 > all groups across testing days (treatment group × day interaction [$F_{2,52} = 7.91$, $p \leq 0.001$]