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Consequences of Prenatal Exposure to Valproic Acid in the Socially Monogamous Praire Vole

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CONSEQUENCES OF PRENATAL EXPOSURE TO VALPROIC ACID IN THE SOCIALLY MONOGAMOUS PRAIRE VOLE

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I dedicate this work to my mother, brother, sister, and husband. I would not have been able to persevere without their unconditional love, encouragement, and support.

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ABSTRACT

Autism spectrum disorder (ASD) embodies a spectrum of complex, pervasive, and heterogeneous neurodevelopmental disorders. There is not one single identified cause, but is instead composed of dynamically diverse etiologies and developmental directions that ultimately influence the degree of core symptom severity. Valproic acid (VPA), clinically known as Depakote, is a popular anticonvulsant with teratogenic properties. Indeed, fetal exposure to valproic acid during the first trimester is associated with a seven-fold occurrence risk for the development of ASD. Likewise, prenatal VPA exposure in rats and mice induces social behavior deficits, replicating ASD-like symptoms in humans. While studies with rats and mice have provided valuable insights into the effects of prenatal VPA exposure, few considered both sexes and none have examined the effects of VPA in a relevant specie model that exhibits strong social behaviors, which are deficient in ASD. Behaviors such as social attachment and partner preference are not displayed by rats and mice, and therefore these behaviors have not been previously assessed in response to prenatal VPA exposure. The socially monogamous nature of the prairie vole (*Microtus ochrogaster*) has granted us that previously unexplored advantage to investigate the consequences of prenatal VPA exposure on the ability to form social attachments or social bonding. In Chapter I, along with introducing main ASD pathologies and introducing this valuable animal model, we review several neural mechanisms that have been implicated in VPA-induced behavior, molecular, and anatomical deficits in humans and traditional laboratory rodents.

In Chapter 2, we sought to determine if prenatal VPA exposure can trigger behavioral, molecular, and neuromorphological deficits in the prairie vole, in an effort to better understand the mechanisms underlying ASD-like social impairments. Prairie voles exposed to VPA at 600 mg/kg on embryonic day 12.5 engaged in fewer social affiliative behaviors in a familial context, exhibited

fewer social interactions with novel conspecifics, and showed enhanced anxiety-like behavior. Interestingly, among numerous genes that we examined, and are linked to autism spectrum disorders, expression of *avpr1a*, an essential social behavior-relevant gene, and *mecp2*, an important player in the regulation of synaptic plasticity and social behaviors, were the only two genes whose expression were down-regulated in the medial prefrontal cortex (mPFC), a brain region implicated in complex cognitive and social behaviors. Considering that evidence of atypical structure and function in the mPFC has been linked to individuals with ASD, in VPA-exposed rats and mice, and now in our VPA-exposed prairie voles, we next sought to examine changes in mPFC dendritic spine density and morphology. However, we found no alterations in dendritic spine density and morphology in the mPFC of adult prairie voles, suggesting that although prenatal VPA exposure induces underweight phenotypes throughout development, adolescent social deficits, and down-regulation of genes relevant to social behaviors, it does not seem to alter neuronal morphology and likely communication between neurons in mPFC. However, this finding needs to be further explored. It is important to note that co-parental care to the VPA-exposed offspring remained sustained and similar to vehicle-exposed offspring, implying that VPA treatment effects in the offspring is not the consequence of differences in paternal and/or maternal care. Following VPA-exposed rat and mouse models, we report that both male and female prairie voles prenatally exposed to VPA exhibit affiliative behavior and social interaction impairments, associated with a reduction of social behavior- and ASD-relevant genes in the mPFC, but no alterations in dendritic spines morphology or density in this brain area. Our data suggest that prenatal exposure to VPA in male and female prairie voles could be a useful animal model for investigating how developmental perturbations early in life lead to long-term changes in social behaviors that are perturbed in ASD.

In Chapter 3, to address how prenatal VPA exposure affects opposite-sex social attachments in prairie voles, we followed the same VPA exposure paradigm, allowed subjects to reach sexual maturity, and assessed their ability to form partner preferences and display selective aggression following two weeks of cohabitation with mating. Similarly to the cohort of VPA-exposed prairie voles tested for adolescent social behaviors, this group received normal co-parental care. Remarkably, VPA-exposed subjects displayed a robust preference for their opposite-sex partner, versus an opposite-sex stranger, and correspondingly displayed high levels of aggression against same-sex intruders. These results suggest VPA alters only certain behavioral domains such as sex-naive anxiety and affiliative behaviors, but does not alter other domains such as social bonding with opposite sex individuals

Finally, in Chapter 4, we provide a summary of our findings and a general discussion of their implications, as well as future directions for the study of the consequences of prenatal VPA exposure in prairie vole social monogamous behaviors. Taken together, this work demonstrates that prairie voles are valuable animal models for validating the use of the prenatal VPA exposure model of ASD. Significantly, using the socially monogamous nature of the prairie vole allowed us to highlight an important distinction between same-sex social affiliative behaviors and the underlying processes of enduring sexual attachments affected by prenatal VPA exposure—a peculiarity observed in a subtype of the autism spectrum.

CHAPTER 1

GENERAL INTRODUCTION

"It seems that for success in science or art, a dash of autism is essential. For success the necessary ingredients may be an ability to turn away from the everyday world, from the simple practical, an ability to rethink a subject with originality so as to create in new untrodden ways, with all abilities canalised into the one speciality." – Hans Asperger

1.1 Autism spectrum disorder, prevalence, and economic costs

Autism spectrum disorder (ASD) embodies a spectrum of complex, pervasive, and heterogeneous neurodevelopmental disorders; characterized by core symptoms of (1) social-communication impairments and (2) a display of repetitive, restricted interests and behaviors. To enhance diagnosis, treatment, and research, the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) has classified all subtypes under the umbrella term "autism spectrum disorder," by merging the milder form previously labeled as Asperger's syndrome to the most severe form previously termed as "classic autism" (American Psychiatric Association., 2013). Accordingly, ASD encompasses an array of diverse etiologies and developmental trajectories that ultimately influence the degree of core symptom severity, language skill, cognitive function, and medical comorbidities (Powell & Monteggia, 2013). Although the population-wide prevalence of ASD is approximately 1%, individuals with ASD have average medical expenditures that far exceed those of typically developed individuals by $4,110-6,200 per year, with medical costs for Medicaid-enrolled children with ASD averaging $10,709 per child (six times higher than costs for children without ASD) (Peacock, Amendah, Ouyang, & Grosse, 2012; Shimabukuro, Grosse, &

Rice, 2008). Moreover, the extensive effects of ASD reach past the medical and welfare sectors, due to economic burden extending to the education, social care, housing, and labor markets. The annual combined direct medical, non-medical, and productivity expenditures are forecasted to reach $461 billion by 2025, alarmingly double the estimated cost for 2015 (Leigh & Du, 2015). For the majority of the 20th century, ASD was considered an incredibly rare condition, with an incidence of 1 children per 5,000 in 1975 (Silberman, 2015). But as a consequence of increased public and clinical awareness, and broadened diagnostic criteria, ASD prevalence has skyrocketed in recent years. In the United States, the average ASD prevalence in 2000 was 1 in 150 children, and has subsequently risen to 1 in 59 children in 2014, according to the Center of Disease Control estimates (Baio et al., 2018). Several studies have characterized the contribution of genetic predisposition, gene and environmental interactions, and disruption to epigenetic regulation as factors that contribute to the phenotypic heterogeneity of ASD. The following sections briefly define and outline current research on ASD neuropathology.

1.2 Genes and common genetic pathways in ASD

Disorders caused by a single gene mutation, otherwise known as monogenetic disorders, were the first to be recognized as causes for ASD. Among many, monogenetic disorders associated with ASD consist of chromosomal abnormities, such as fragile x syndrome and Rett syndrome. Accounting for 10-20% of ASD cases, inherited or *de novo* copy number variations (CNVs) and chromosomal rearrangements are far more frequent and affect more genes, compared to typically developed siblings and 1-2% of the general population with non-ASD-related monogenetic disorders (Devlin & Scherer, 2012; Huguet, Ey, & Bourgeron, 2013). Neuronal genes with *de novo* coding-sequence mutations are also associated with 5-10% of individuals with ASD. Moreover,

individuals with ASD who suffer from cognitive development and intellectual disability are observed to carry an enrichment of CNVs in numerous genes associated with regulating neuronal development, cell proliferation, and neuronal plasticity (Pinto et al., 2010). For instance, repeated expansions of the CGG trinucleotide in the *FMR1* gene is a detrimental cause of intellectual disability and physical abnormalities in patients with fragile X syndrome, a neurodevelopmental disorder with 1-2% ASD comorbidity (Budimirovic & Kaufmann, 2011). The *FMR1* gene is located on the X chromosome and encodes the FMRP protein, an RNA-binding protein involved in activity-regulated localization and translation of mRNA in dendrites and synapses, stability and translation in activity-dependent synaptogenesis, and plasticity mechanisms (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004; Gatto & Broadie, 2010). Mice with the *FMR1* gene knocked out have been extensively studied and display altered social interactions, decreased vocalizations, decreased anxiety-like behavior, and impaired learning and memory (Qin et al., 2011; Santos, Kanellopoulos, & Bagni, 2014).

Another X-linked dominant mutation known to cause profound cognitive impairments is Rett syndrome, a neurodevelopmental disorder in which 95% of cases are caused by mutations in the *MeCP2* gene that encodes the methyl-CpG-binding protein (MeCP2) (Amir et al., 1999). MECP2 is involved in transcription regulation, bearing methylation-dependent transcriptional repressor activity, and is involved in regulation of RNA splicing (Pruitt, Tatusova, & Maglott, 2007). Importantly, MECP2 is important for embryonic development, as its own dysfunction during the critical period of synaptic maturation leads to several abnormalities, that range from impairments in brain development, perturbations in neuronal expression of other genes, and changes in synaptic and other extra-cellular signals (Kaufmann, Johnston, & Blue, 2005).

Importantly, the majority of clinical and translational studies have implicated MECP2 co-occurrence with ASD (Vourc'h et al., 2001).

Mutations in ASD-related genes involved in the formation of excitatory and inhibitory synapses include the neurexin (*NRXN*), neuroligin (*NLGN*), and shank (*SHANK*) genes. NRXN and NLGN proteins are trans-synaptically interacting cell adhesion proteins that are important for the maintenance and maturation of functional synapses by mediating signaling across the synapse and shaping neuronal networks (Chih, Engelman, & Scheiffele, 2005; Kleijer et al., 2014; Krueger, Tuffy, Papadopoulos, & Brose, 2012; Kwon et al., 2012; Sudhof, 2008; Varoqueaux et al., 2006). Neurexin isoforms bound to neuroligins can form trans-synaptic complexes that regulate selective extracellular interactions such as synaptic strength and long-term plasticity at glutamatergic and GABAergic synapses (Aoto, Martinelli, Malenka, Tabuchi, & Sudhof, 2013; Boucard, Chubykin, Comoletti, Taylor, & Sudhof, 2005; Chih, Gollan, & Scheiffele, 2006). The first association with the NRXN-NLGN complex and ASD identified a mutation in the X-linked genes *NLGN3* and *NLGN4* in siblings with ASD (Jamain et al., 2003). Subsequent studies discovered *NLGN3* mutations caused defective neuroligin trafficking, leading to accumulation of the protein in the endoplasmic reticulum and decreased *NLGN3* delivery to the cell surface (Comoletti et al., 2004). Mutations of *NRXN1* in mice induces impaired social interactions, increased auto-grooming, and increased inhibitory synaptic transmission (Tabuchi et al., 2007). SHANK proteins are multi-domain postsynaptic density scaffold proteins that serve to attach neurotransmitter receptors, ion channels, and other membrane proteins to the actin cytoskeleton and G-protein coupled signaling pathways, and thus play a role in synapse formation and dendritic spine maturation (Pruitt et al., 2007). Mutations in the *SHANK3* gene are characterized by impairments in social interaction and communication, and restricted behavioral patterns and interests—core symptoms of ASD.

Disrupted *SHANK3* expression in mouse models induces increased brain size, impaired social interactions, altered vocalizations, and increased anxiety-like disorder (Peca et al., 2011). Inheritance of two SHANK2 mutations decreases dendritic spine volume, while SHANK2 deficient mice exhibit hyperactivity, deficits in social interactions, and impaired vocalizations (Berkel et al., 2012; Schmeisser et al., 2012; Won et al., 2012).

The neurotransmission of oxytocin (OXT) and arginine-vasopressin (AVP), and their receptors are central in neural and physiological pathways that underlie social and affiliative behaviors. Notably, polymorphisms in the *oxtr* and *avpr1a* receptors contribute to ASD diagnosis and phenotypic heterogeneity. Oxytocin is involved in regulating female intercourse, triggering contractions during child birth, lactation, maternal attachment, and female pair bonding. Oxytocin enriches an individual's positive social interactions by fostering relaxation, trust, empathy, and altruism. Given the importance of oxytocin's role in modulating social behaviors in humans and animals, it has been proposed that dysregulation of its signaling pathways underlies many social disorders. Genetic variations in oxytocin receptors can affect a person's empathetic behavior by impairing their ability to understand facial expressions and to feel distress at other's suffering (Rodrigues, Saslow, Garcia, John, & Keltner, 2009). Several other human genetic studies have discovered significant associations between common and rare oxytocin receptor variants and ASD in multiple ethnic populations (Egawa et al., 2015; Gregory et al., 2009; Jacob et al., 2007; Wu et al., 2005). Blockade or ablation of oxytocin receptors in animal models results in deficits in social interaction and preference for social novelty, elevated aggressive behaviors, and inhibition of partner preference formation (Pobbe, Pearson, Blanchard, & Blanchard, 2012; Pobbe, Pearson, Defensor, et al., 2012; Sala et al., 2011; Takayanagi et al., 2005; J. R. Williams, Insel, Harbaugh, & Carter, 1994). Alternatively these behavior deficits can be rescued by subsequent administration

of oxytocin or oxytocin receptor agonists (Sala et al., 2011; J. R. Williams, Carter, & Insel, 1992). Although the use of intranasal oxytocin has received focus as a potential ASD treatment, current clinical trials have generated varied results for its clinical efficacy (Anagnostou et al., 2014; Guastella et al., 2015). Acute intranasal, but not chronic intranasal (Anagnostou et al., 2012; Dadds et al., 2014; Guastella et al., 2015), oxytocin administration has reliably yielded positive outcomes for ameliorating ASD symptoms, such as improving comprehension of affective speech (Hollander et al., 2007), enhancing eye contact (Auyeung et al., 2015), strengthening social interactions (Andari et al., 2010), and reducing repetitive behavior (Hollander et al., 2003). Discrepancies in chronic intranasal treatments indicate factors that influence the efficacy of oxytocin administration need to be further examined. For instance, it is important to examine administration schedules, as reports on dose concentration and frequency of administration affecting individuals with ASD are currently unavailable. While no clinical studies have reported severe adverse effects, studies in prairie voles and mice have provided evidence of negative effects in response to chronic oxytocin treatment (Bales et al., 2013; Huang et al., 2014). Additionally, individual variation in drug response to oxytocin treatment is a factor that can contribute to its efficacy, as participant age and ASD symptom severity requires different administration schedules and delivery routes of oxytocin (Okamoto, Ishitobi, Wada, & Kosaka, 2016).

Oxytocin moreover is an indispensable "switch" that alters the function of the main inhibitory neurotransmitter in the adult brain, γ-aminobutyric acid (GABA). During fetal development GABAergic synaptic transmission is in fact excitatory and can exert widespread trophic effects (Ganguly, Schinder, Wong, & Poo, 2001; Kilb, 2012), such as the promotion of rapid fetal brain development (Cancedda, Fiumelli, Chen, & Poo, 2007; Foradori & Handa, 2008). Mediated by high intracellular chloride ion concentrations ($[Cl^-]_i$), a deficiency of the K^+/Cl^- co-

transporter KCC2 (that extrudes Cl^-), and up regulation of the $Na^+/K^+/Cl^-$ co-transporter NKCC1 (that imports Cl^-), GABA exerts robust depolarizing and excitatory actions on immature, but not adult, neurons (Ben-Ari, 2017). Interestingly, the switch of GABA function begins as OXT levels within the womb rise considerably during labor and vaginal delivery. These elevated OXT levels lower the $[Cl^-]_i$ inside fetal neurons, a state that persists after birth and thus shifts the actions of GABA from excitatory to inhibitory. The switch can be delayed by chronic inhibition of $GABA_A$ receptors and conversely accelerated by increased $GABA_A$ receptor activation (Ganguly et al., 2001). Moreover, a recent study using the valproic acid and fragile X rodent models in rats reported that the oxytocin-mediated neuroprotective GABA excitatory→inhibitory shift during delivery is abolished as a result of persistent elevated $[Cl^-]_i$ in hippocampal neurons that demonstrated increased excitatory GABA, enhanced glutamatergic activity, and elevated gamma oscillations during delivery. Likewise, inhibiting oxytocin signaling in intact rat dams produced offspring that exhibited electrophysiological and behavioral ASD-like features. Taken together, these studies emphasize the importance of oxytocin-mediated GABAergic inhibition, and shed light in a connection between defective chloride channels and ASD pathogenesis (Tyzio et al., 2014).

Vasopressin acting through its receptor, V1aR, is involved in regulating male sexual stimulation, maternal responses to stress, male aggression, and male and female pair bonding. Genetic variations in the vasopressin (*avpr1a*) gene have been associated with perceived marital problems and marital status in men, and a lower inclination toward altruistic behavior in children (Avinun et al., 2011; Walum et al., 2008). Several studies have found genetic association and rare variants in the *avpr1a* gene that are identified with ASD in American, Israeli, and Korean populations (Bachner-Melman et al., 2005; S. J. Kim et al., 2002; Yang et al., 2010). Significantly, a*vpr1a* knockout mice have been shown to exhibit impairments in social interactions, social

recognition, and reduction of anxiety-like behavior (Bielsky, Hu, Ren, Terwilliger, & Young, 2005; Bielsky, Hu, Szegda, Westphal, & Young, 2004; Egashira et al., 2007). Vasopressin acting through V1aR can stimulate aggressive behavior. Administration of a vasopressin antagonist, SRX251, to golden hamsters significantly reduced the frequency of offensive behavior in a resident-intruder test (Ferris et al., 2006). In a human fMRI study, intranasal vasopressin antagonist administration lowered neural response to angry faces (Lee et al., 2013). Administration of a V1AR antagonist reduced passive coping behavior and reduced anxiety in rats when tested in the forced swim test and elevated plus maze (Ebner, Wotjak, Landgraf, & Engelmann, 2002; Liebsch, Wotjak, Landgraf, & Engelmann, 1996). These studies suggest that V1AR antagonists may be used to treat interpersonal violence or stressful behaviors co-occurring in bipolar disorder, schizophrenia, and ASD. Disturbances of the oxytocin and vasopressin systems on social interactions have thus led researchers to suggest the involvement of these neurotransmitters in conditions described by social deficits in ASD and ASD-related conditions.

In summary, genes highly implicated in ASD are those that regulate social behaviors through chromatin remodeling, and regulation of mRNA translation and synaptic function. The genetic component to ASD vulnerability is further underlined by recurrence risk within families. Although the sibling recurrence rate of ASD is 18.7% higher than the general population (Ozonoff et al., 2011), the concordance rate for monozygotic twins is substantially higher, reaching 70-90%, than dizygotic twins (0-30%) (Daghsni et al., 2018). Interestingly, the absence of an all-inclusive ASD concordance in monozygotic twin studies indicates that environmental factors and perturbations to epigenetic mechanisms operate on or enhance the contributions of ASD susceptibility genes.

1.3 Environmental factors in ASD

Environmental factors interact with the genome during prenatal and postnatal development and contribute to increased ASD risk. Contrary to popular belief, congenital rubella syndrome (CRS) is not completely eradicated and in fact affects up to 5% of pregnant women globally and has an incidence of 4.12-7.3% with ASD (Hwang & Chen, 2010). CRS is a maternal infection with possible links to ASD, as CRS children display hyperactivity, increased muscle tone or stiffness, and may develop diabetes mellitus type 1 as adults (Hutton, 2016).

Epidemiological studies have linked maternal exposure to the immunomodulatory drug thalidomide to increase ASD risk to the fetus. Used by pregnant women in the 1950s, a marked increased risk for ASD was observed when thalidomide exposure to the embryo occurred 20-24 days post-conception, producing sustained cranial nerve motor nuclei injuries, and shortened brain stem (Rodier, Ingram, Tisdale, & Croog, 1997). Due to the precise time frame of thalidomide's injurious effects to exposed children, and its close association with ASD, it has been suggested that thalidomide interferes with the sequence formation of the rhombomeres, which are transiently separated segments of the developing neural tube that develop into the brainstem and cranial nerve motor nuclei in the course of neural tube closure (Rodier, Ingram, Tisdale, Nelson, & Romano, 1996).

Moreover, maternal use of the anticonvulsant valproic acid (VPA) causes harm to the fetus to the same degree as thalidomide, as VPA perturbs discrete neural processes during development that lead to a constellation of physical deformities, indicative of injury around the time of neural tube closure during the first trimester: neural tube defects, congenital heart disease, dysmorphic craniofacial features, abnormally shaped or posteriorly rotated ears, genital abnormalities, and limb defects (Arndt, Stodgell, & Rodier, 2005). Importantly, prenatal VPA exposure in children is

associated with impaired cognitive function, verbal intellectual abilities (Meador, 2008) , and a seven-fold occurrence risk for the development of ASD (Roullet, Lai, & Foster, 2013). First licensed in the United States in 1978, reports of VPA's potential for causing minor and major malformations to exposed children began to be published in the 1980s (Kennedy & Koren, 1998), such as linking prenatal VPA exposure to fetal neural tube defects (Robert & Guibaud, 1982) and clinical features of fetal valproate syndrome (DiLiberti, Farndon, Dennis, & Curry, 1984). Close to a decade later, clinical studies began to associate VPA and ASD when children with fetal valproate syndrome moreover displayed classic autism symptoms (Christianson, Chesler, & Kromberg, 1994; P. G. Williams & Hersh, 1997). Although VPA is typically administered throughout the entire pregnancy, the teratogenic effect of VPA that increases ASD risk is estimated to occur during the first trimester (Roullet et al., 2013). Based on analogous developmental periods and clinical behavioral outcomes, studies using rodent models have provided valuable insights into VPA's mechanisms of action and its impact on social development.

1.4 Perturbations of epigenetic mechanisms in ASD

Adapted from: Elvir L, Duclot F, Wang Z, Kabbaj M. Epigenetic Regulation of Motivated Behaviors by Histone Deacetylase Inhibitors. Neuroscience and biobehavioral reviews 2017 Oct 8. pii: S0149-7634(17)30069-6.

1.4.1 An introduction to molecular aspects of epigenetics

The idea of an "epigenetic landscape" was first coined by Conrad Waddington in the 1940s to describe the creation of a phenotype as a result of elaborate interplays during development between the environment and the genome (Waddington, 2012). Recent attempts to establish a common definition have introduced molecular concepts to describe epigenetics as "mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA

sequence" (Allis, Caparros, Jenuwein, & Reinberg, 2007). We broadly define epigenetics to indicate the mechanisms influencing gene expression that induce changes in phenotype without changing the primary DNA sequence.

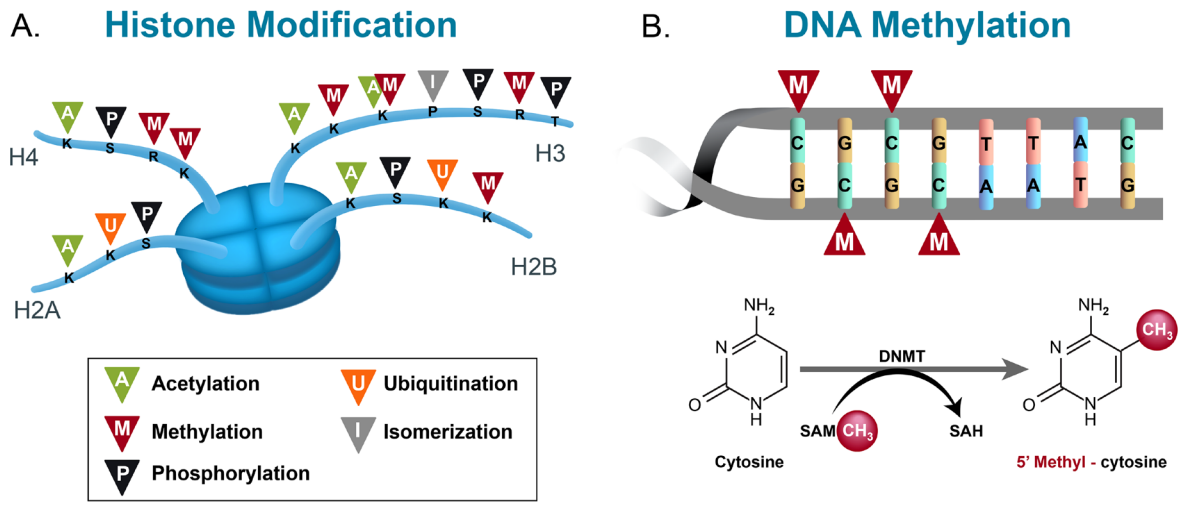

Figure 1. Illustration of major epigenetic modifications: histone modification (***A***) and DNA methylation (***B***). Abbreviations: K, S, R, and P – lysine, serine, arginine, and proline residues, respectively, DNMT – DNA methyltransferase, SAM – S-Adenosyl methionine, CH_3 – methyl group, SAH -- S-Adenosyl homocysteine (Elvir, Duclot, Wang, & Kabbaj, 2017).

While illustrated independently in Figure 1, epigenetic modifications form a multifaceted, collaborative, and cross-regulating system, which is oftentimes reversible, to control gene expression. Changes in epigenetic patterns were initially thought to only be heritable as epigenetic programming can begin while the fetus is developing in the uterus, but this notion is challenged by the fact that phenotype and epigenetic patterns vary as genetically identical twins become older (Fraga et al., 2005). Additionally, maternal environmental exposures during gestation and preconceptual paternal exposure to environmental stimuli can impact the offspring's phenotype via alterations of epigenetic marks (Day, Savani, Krempley, Nguyen, & Kitlinska, 2016; Perera &

Herbstman, 2011). Epigenetic changes can indeed result in enduring neuroadaptations influencing the organism's subsequent response to stress, metabolism, or social behaviors, thereby altering its vulnerability to the development of diseases. Interestingly, in addition to allowing us to comprehend the link between environmental influences and phenotypical changes, understanding how social behaviors are regulated by epigenetic mechanisms can thus offer the opportunity for therapeutic interventions in individuals suffering from neuropsychological disorders, such as addiction or social impairments, and neurodevelopmental disorders, such as ASD. Specifically, epigenetic dysregulation of ASD candidate genes comprise of changes in histone modifications and DNA methylation, which are both important epigenetic markers with regard to abundance and function (Dai & Wang, 2014).

1.4.1.1 Histone acetylation and methylation

Histones are highly basic proteins that associate with negatively-charged DNA in the nucleus with the purpose of packaging the DNA into chromatin. Histones can be modified at specific residues by various post-translational modifications (PTM) that include, among others, acetylation, phosphorylation, methylation, ubiquitination, sumoylation, and isomerization predominantly at their N-terminal tails (Grayson & Guidotti, 2013). These unique chemical signatures can affect global chromatin assembly, transcription factor binding, or recruitment of transcriptional cofactors. Histone PTMs also permit for chromatin relaxation or compression around genetic loci leading to the promotion or repression of gene transcription. Notably, histone modifications can occur on the tails of the 4 core histone proteins H2A, H2B, H3, and H4 (Figure 1A), and these modifications can interact with each other, thereby opening a wide array of histone

PTM combinations and a complex regulation of gene transcription (Nightingale et al., 2007; van Attikum & Gasser, 2009).

Acetylation of histone tail lysine residues is known to occur in different organisms for the regulation of diverse nuclear and cytoplasmic processes. Considered a very common PTM that targets larger macromolecular complexes, acetylation of lysine residues can occur in over 1,750 proteins that together comprise 3,600 lysine acetylation sites (Choudhary et al., 2009). Importantly, this acetylated state is controlled through the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Figure 2). HATs neutralize the positively charged ϵ-amino group of specific lysine residues by transferring an acetate group from acetyl-CoA to the lysine residue, thereby enhancing their acetylation, and thus rendering DNA more accessible by decreasing binding affinity between the histone tail and DNA (Shogren-Knaak et al., 2006), otherwise the positive lysine binds to negatively charged DNA in the absence of the acetyl group. HDACs hydrolyze the acetyl moiety, *i.e.* remove the acetyl groups, and reduce DNA accessibility for transcription to occur. Through the balance between HAT and HDAC activities, the levels of histone acetylation are thus regulated in a highly dynamic manner that represent a unique molecular framework for the integration of environmental stimulations on gene expression. Indeed, although proteins carrying HAT or HDAC activities have non-histone targets, HATs generally favor DNA availability to transcription factors, thereby promoting DNA transcription at a given locus, whereas HDACs generally act as transcriptional corepressors by deacetylating core histone proteins, resulting in chromatin condensation and the inhibition of transcriptional activating complexes (Haery, Thompson, & Gilmore, 2015). In this context, HATs and HDACs represent valuable candidates for pharmacological intervention at the interface of gene and environment interactions. HDAC expression in the central nervous system exhibits a neuronal

subpopulation specificity that further supports an isoform specificity in their involvement in motivated behaviors (Elvir et al., 2017). Notably, this observation is of particular interest as it provides an important layer of specificity to the actions of HDAC inhibitors (HDACi) in the central nervous system.

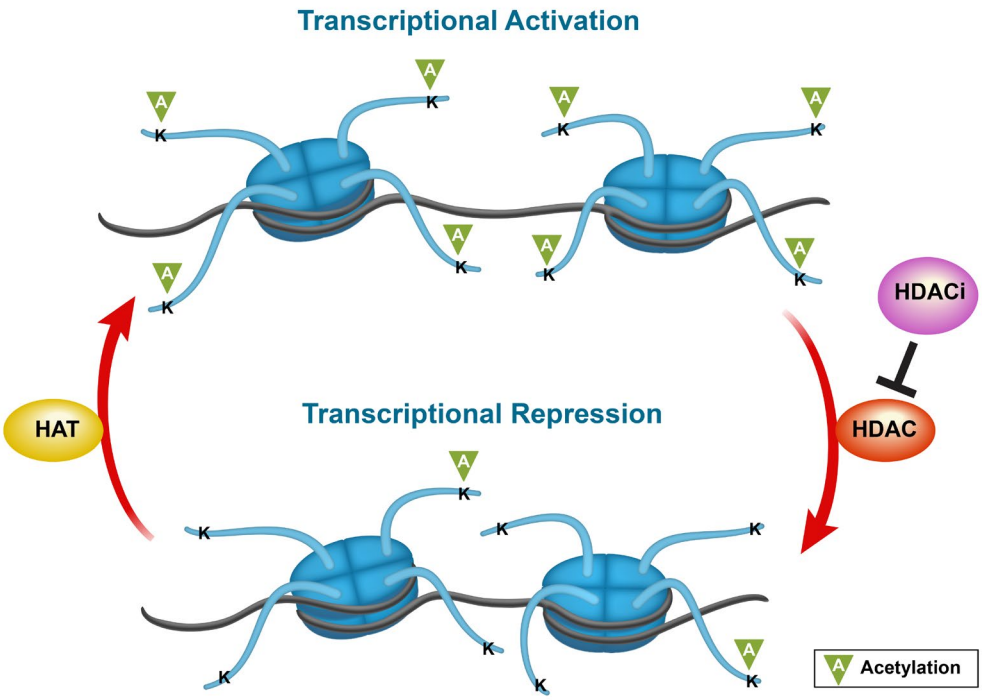

Figure 2. Illustration of chromatin conformation according to HAT/HDAC balance. Abbreviations: K – lysine residue, HAT – histone acetylase, HDAC – histone deacetylase, and HDACi – histone deacetylase inhibitor (Elvir et al., 2017).

HDACi have emerged as promising cancer therapeutic agents as atypical patterns of histone modifications are involved in cancer development. HDACi have the potential to target non-histone proteins to inhibit cell proliferation, stimulate cell cycle arrest, inhibit angiogenesis, and induce senescence of cancer cells (Bolden, Peart, & Johnstone, 2006; Peng & Seto, 2011). Presently, HDACi are being used as stand-alone therapies or in combination with other agents,

chemotherapies, radiation therapies, or immune checkpoint therapies in numerous clinical cancer trials (Mazzone, Zwergel, Mai, & Valente, 2017; Qiu, Xiao, Li, & Li, 2017; Zain & O'Connor, 2010). However, clinical results do not coincide with preclinical work in terms of efficacy against solid tumors and off-target toxicity with negative side-effects (Subramanian, Bates, Wright, Espinoza-Delgado, & Piekarz, 2010). Approaches to surmount these setbacks involve localized administration to sidestep systemic toxicity and the formation of new isoform selective HDACis (Gryder, Sodji, & Oyelere, 2012).

While histone acetylation occurs at lysine residues to induce transcriptional activation, histone methylation is multifaceted as it can ensue at both lysine and arginine residues on histone tails, the number of methyl groups can vary (mono-, di-, and tri-), and the residue modified and its location designates whether transcriptional activation or repression occurs (Table 1). For instance, silenced genes contain regions of di- and tri-methylated histone H3 at lysine 9 (H3K9), whereas transcriptional activation correlates with di- and tri-methylation of histone H3 at lysine 4 (H3K4) (Gupta et al., 2010). Similar to the way HATs and HDACs operate, histone methyltransferases transfer the methyl group(s) from S-adenosylmethionine to lysine or arginine residues, while demethylases act to remove methyl groups from the same histone residues. However, unlike the process of changing the lysine residue charge, and thus impacting the association between DNA and histones that occurs through histone acetylation, histone methylation status allows for recruitment of diverse methylation "reader" proteins that activate or inhibit gene transcription (Gu & Lee, 2013). Additionally, methyl isoforms (such as H3K4me1, H3K4me2, and H3K4me3) are discrete epigenetic marks that can be produced by a solitary methyltransferase advancing to add methyl groups in succession or by numerous methyltransferases operating synchronously.

Table 1. Forms of histone acetylation and methylation modifications and their potential crosstalk in regulation of gene transcription on histones H3 and H4 (Dai & Wang, 2014). K: lysine residue; R: arginine residue; ac: acetylation; me1: mono-methylation; me2: di-methylation; me3: tri-methylation.

Histone	Residue Location	Modification	Indication of Transcription
H3	K79	me1 me2 me3	Active
	K36	ac me1 me2 me3	Active
	K27	ac me1	Active
		me2 me3	Inactive
	K23	ac	Active
	K18	ac	Active
	K14	ac	Active
	K9	ac me1	Active
		me2 me3	Inactive
	K4	ac me1 me2 me3	Active
	R2	me	Inactive
H4	K20	ac me1	Active
	K16	ac	Active
	K12	ac	Active
	K8	ac	Active
	K5	ac	Active

1.4.1.2 DNA methylation

The most studied epigenetic modification is DNA methylation, a process that consists of a methyl moiety transferred from S-adenosylmethionine to the 5' position of cytosines or adenines, catalyzed by DNA methyltransferases (DNMTs) (Jangra et al., 2016). Notably, DNA methylation in the mammalian brain can be particularly dynamic, with demethylation or *de novo* methylation events occurring rapidly following neuronal activity (Guo et al., 2011). Furthermore, the methylation status of cytosine residues at the 5' position of the cytosine rings in the CG sequence can affect gene transcription in complex ways depending mostly on the genomic localization and the transcriptional cofactor environment and has thus been associated with major enduring neuroadaptations underlying higher order brain processes (Meaney & Szyf, 2005).

1.4.2 Epigenetic mechanisms in ASD

Rett syndrome patients, who possess mutations in MeCP2, display increased density of histone H3, and decreased levels of the transcriptional activator H3K4me3. Although levels of H3K9ac and H3K27ac were not affected (Lilja et al., 2013), MeCP2 knockout mice display genome-wide increased histone acetylation, indicating that MeCp2 is associated with decreased global H3 acetylation levels. Besides its involvement with Rett and ASD pathology, MeCP2 acts as a bridge between histone methylation on lysine 9 (H3K9me) and DNA methylation, two chromatin-associated gene silencing mechanisms, to reinforce a repressive chromatin state (Fuks et al., 2003).

In the next sections of this chapter, I focus on ASD-related behavioral and molecular changes known to be associated with prenatal VPA exposure in humans and in traditional

laboratory rodent species. Following this overview, I will highlight the novel advantage of studying prairie vole behavior and neurobiology in response to prenatal VPA exposure.

1.5 Valproic acid: a double edged drug

Valproic acid (2-propylvaleric acid, 2-propylpentanoic acid or n-dipropylacetic acid) is a branched short-chain fatty acid, with a half-life of 9-16 hours, that was initially employed as a "physiologically inert" solvent for dissolving organic compounds targeting various diseases, such as acute tonsillitis and kidney stones (Nogrady, 1985). VPA's anticonvulsant property was coincidently discovered when these structurally unrelated drugs, while using VPA as the vehicle, exhibited antiepileptic qualities and thus leading to the realization that it was indeed VPA's chemical properties that produced these profound antiepileptic effects (Meunier, Carraz, Neunier, Eymard, & Aimard, 1963; Nogrady, 1985). Ensuing controlled clinical trials established its accompanying mood-stabilizing effects for bipolar disorder, depression, anxiety, and migraines. Furthermore, due to its reliable clinical tolerability, low cost, ease in crossing the blood brain barrier, and its diverse mechanisms of action, VPA has become a desirable candidate drug for treating cancer, HIV, and Alzheimer's disease. However, the noteworthy therapeutic potential of VPA must be scrutinized, as it likewise possesses adverse effects and significant teratogenicity risk.

1.5.1 VPA's mechanisms of action

As an anticonvulsant agent, VPA acts to increase synaptic availability of GABA and decrease neuronal excitation. Specifically, VPA inhibits GABA degradation, increases GABA synthesis, and decreases turnover, all of which ultimately work to potentiate the inhibitory activity

of GABA (Mesdjian et al., 1982). Conversely, VPA weakens the Na^+ and Ca^{2+} ion channels, the voltage-gated K^+ channel, and reduces NMDA-mediated excitation (VanDongen, VanErp, & Voskuyl, 1986; Zeise, Kasparow, & Zieglgänsberger, 1991). Recent work has proven VPA as an epigenetic regulator as well. VPA is an HDACi that targets class I and II HDACs, which play central roles in cellular activity, cell cycle, cell differentiation, DNA repair, and apoptosis (Elvir et al., 2017). Interestingly, VPA induces indirectly a concomitant increase of methylation at H3K4, and wherein the abundance and degree of methyl isoforms at H3K4 (me1, me2, and me3) are associated with H3 acetylation status (Nightingale et al., 2007). The exact mechanism is not yet known, but it likely that VPA does not directly enhance the enzymatic activity of DNA demethylases. However, through HDACi activity, VPA enables methylated DNA to be more accessible, which is confirmed by the observation that inhibition of HAT diminishes the demethylation effect triggered by VPA. In addition, VPA decreases expression of chromatin maintenance proteins: SMCs 1-6, DNMT1, and HP2 (Chateauvieux, Morceau, Dicato, & Diederich, 2010).

1.5.2 Teratogenicity of VPA in rats and mice

The most environmentally triggered model of ASD results from embryonic VPA exposure in rodents (Favre et al., 2013). The model was first proposed in 1996 by Rodier *et al*., who were the first group to link prenatal VPA exposure to disruptions in cranial nerve motor nuclei development during neural tube closure (Rodier et al., 1996). Prenatal VPA exposed rats on embryonic day 12.5 (E12.5) recapitulated the core cellular malformations of the neuropathological disorder by exhibiting significant reduction of cranial nerve nuclei, and altered numbers of cerebellar Purkinje cells and deep nuclei (Rodier et al., 1996). Additionally, VPA-induced HDACi

has been shown to play a key role in cortical pathology and impaired social behaviors in mice, as Nissl-positive cells in the middle and lower layers of the mPFC and in the lower layers of the somatosensory cortex at PND56 were reduced by VPA exposure (on E12.5). Moreover, VPA exposure on E12.5 seems to produce only transient hyperacetylation levels of H3 and H4 for up to 6 hours in the embryonic brains, but does not affect total histone levels (Kataoka et al., 2013). After 12-24 hours, H3 and H4 acetylated levels return to normal, followed by an elevation of apoptotic cell death in the neocortex and a decline of cell proliferation in the ganglionic eminence. These molecular and cellular changes were accompanied by social interaction deficits, anxiety-like behavior, and memory deficits at PND28-56, but only true in VPA-exposed mice at E12.5, but not at E9 and E14.5 (Kataoka et al., 2013). Cell density in other regions, such as the striatum, amygdala, and hippocampus, were not affected by VPA exposure and thus indicating that specific brain regions, like the mPFC, may be more vulnerable to injury during development following VPA exposure (Kataoka et al., 2013). Inhibition of HDACs can result in hyperacetylation of histones, consequently elevating transcription of many genes important for development and genes related to neural tube formation (Massa, Cabrera, Menegola, Giavini, & Finnell, 2005; Okada, Endo, Singh, & Bhalla, 2005; Stodgell et al., 2006; Wlodarczyk, Craig, Bennett, Calvin, & Finnell, 1996). Besides inducing changes in histone acetylation, VPA can also cause genome wide methylation status modifications (Detich, Bovenzi, & Szyf, 2003; Fukuchi et al., 2009; Milutinovic, D'Alessio, Detich, & Szyf, 2007). Specifically, prenatal VPA exposure on E12.5 can induce demethylation in the promoter regions of *wnt1* and *wnt2* in the mPFC and hippocampus, accordingly increasing both mRNA and protein expression of *wnt1* and *wnt2* (Wang et al., 2010). With β-catenin expression levels likewise increased, it was not surprising to find the activity of the Wnt/β-catenin pathway to also be upregulated in the VPA-exposed animals (Wang et al., 2010).

The Wnt/β-catenin pathway plays a role in normal embryonic development by its involvement in proliferation, differentiation, apoptosis, and process outgrowth (Ciani & Salinas, 2005; Endo & Rubin, 2007; Ille & Sommer, 2005). Likewise, disruption of the Wnt/β-catenin pathway components lead to morphological and functional impairments similar to those found in individuals with ASD.

A recent study in rats investigated the effect of *in utero* VPA exposure at E12.5 on neural morphological rearrangement in the prefrontal cortex (PFC), the nucleus accumbens (NAcc), ventral and dorsal hippocampus (VH and DH), and basolateral amygdala (BLA) immediately after weaning (PND21), during prepubescence (PND35), and adulthood (PND70) (Bringas, Carvajal-Flores, Lopez-Ramirez, Atzori, & Flores, 2013). Exposure to VPA increased dendritic spine density in the NAcc and VH, with a reduction in the PFC, DH, and BLA at PND70. Increases in neuronal arborization were observed in the NAcc, layer III of the PFC and BLA, with reduced neuronal arborization in the VH and DH at PND70. The morphological arrangements in these regions accompanied VPA behavioral impairments in which hyper-locomotion and exploratory behavior, with an increase of head-dipping duration in the hole-board test were displayed by VPA-exposed rats compared to controls (Bringas et al., 2013). Likewise, several functional and structural connectivity studies have revealed patients with ASD to have long-distance under-connectivity between functional brain regions and simultaneous local over-connectivity within neighboring neural ensembles (Palau-Baduell, Salvado-Salvado, Clofent-Torrento, & Valls-Santasusana, 2012; Wass, 2011). This pattern of connectivity could justly describe the connectivity altered in the VH-PFC-NAcc and BLA-PFC-NAcc pathways in VPA-exposed rats: the PFC pyramidal neurons receiving diminished excitatory input from the hippocampus and BLA, while NAcc medium spiny neurons receive enhanced excitatory input from the hippocampus, PFC,

and BLA. Bringas *et al*., hypothesize that the enhanced excitatory inputs to the NAcc result in reduced GABAergic drive from the ventral pallidum to the medial-dorsal thalamus while changes in dendritic arborization may result in local over-connectivity in the BLA, PFC, and NAcc circuitry and local under-connectivity at the level of the VH and DH at PND70 (Bringas et al., 2013).

Prenatal VPA exposure can cause alterations of neurotransmitter systems at excitatory and inhibitory synapses in the basal forebrain and dorsal forebrain in rodents. The endogenous opioid peptide systems play a key role in the regulation and facilitation of stress responses affect states, nociception, locomotion, learning, memory, and fear (Good & Westbrook, 1995; McGaugh, Cahill, & Roozendaal, 1996; Olson, Olson, & Kastin, 1996; Valverde, Tzavara, Hanoune, Roques, & Maldonado, 1996), all of which are disrupted in autistic individuals (Frith & Happe, 2005; Ozonoff, Goodlin-Jones, & Solomon, 2005). Rats exposed to VPA on E12.5 display decreased proenkephalin mRNA expression in the dorsal striatum and the NAcc (core and shell), correspondingly inducing increased anxiety-like behavior, diminished basal hedonic tone, without impacting learning and memory (Schneider, Ziolkowska, Gieryk, Tyminska, & Przewlocki, 2007). Prenatal VPA exposure significantly increases NMDA receptor-mediated transmission by selectively increasing subunits NR2A and NR2B and thus increases plasticity in the neocortex via enhancement of postsynaptic long-term potentiation (Rinaldi, Kulangara, Antoniello, & Markram, 2007). Suppression of NMDA receptor hyperfunction caused by prenatal VPA exposure rescues social deficits and repetitive behaviors (Kang & Kim, 2015). Further work is however necessary to investigate the antagonist's effects on synaptic transmission. Prenatal VPA exposure also upregulates expression of the α4 subunit of $GABA_AR$ and hippocampal BDNF mRNA, which are involved in epileptogenesis. Expression of the γ2 subunit of $GABA_AR$, GAD65, GAD67, and the

ion co-transporter KCC2 was downregulated; these components are involved in the development of GABAergic inhibitory neurons (Fukuchi et al., 2009).

It is important to note that VPA pharmacokinetics and its embryotoxicity is gestational stage-dependent, as VPA concentrations in pregnant mice injected with 500 mg/kg VPA on gestation day 13 (G13) exhibit slow VPA clearance from plasma and blood when compared to pregnant mice injected on G7, who rapidly clear out VPA within 6 hours. Overall on G13 in pregnant mice, VPA concentrations remain significantly high in the plasma (~600-800 ug/ml) and brain (~300 ug/g) after 6 hours of injection (Ohdo, Watanabe, Ogawa, Yoshiyama, & Sugiyama, 1996). On embryonic day (E13), this delay of VPA clearance from the plasma and brain of the dam closely reflects the VPA concentration in the offspring, as VPA concentrations remain significantly higher after 6 hours post injection in E13 embryos (800 ug/g) versus E7 embryos (100 ug/g) (Ohdo et al., 1996). Intriguingly, ASD-like behavioral deficits induced by prenatal VPA exposure are very specific to the developmental period E12.5 in mice (Kataoka et al., 2013) and rats (K. C. Kim et al., 2011). Indeed, mice exposed to VPA on E12.5, but not E9 and E14.5, display social interaction impairments and enhanced anxiety-like behavior on PND28-64, accompanied by cell density changes in the mPFC and transient histones H3 and H4 hyperacetylation (Kataoka et al., 2013). Similarly, VPA-exposed rats exhibit reduced adolescent (PND28) social affiliation with familiar conspecifics and decreased social interactions with novel conspecifics when exposed to VPA on E12.5, but not on E7, E9, and E15, in rats (K. C. Kim et al., 2011).

1.5.3 Limitations of VPA-exposed rat and mouse models

Non-human animal research has remained heavily male-biased (Beery & Zucker, 2011), and consequently it is expected that limited studies on rats and mice have reported the effects of

prenatal VPA exposure in both sexes (Kataoka et al., 2013; K. C. Kim et al., 2016; K. C. Kim et al., 2013). This is a significant limitation, while a 4:1 male to female ratio is observed in the human ASD population (Baio et al., 2018), the male: female ratio in children prenatally exposed to VPA that develop ASD is 1:1 (Rasalam et al., 2005). Of the few studies reporting the effects of VPA exposure, male and female rodents equally display certain ASD-like deficits, such as increased repetitive/stereotypic-like activity (Schneider et al., 2008). Therefore studies on males cannot be generalized to females, as sex differences at all levels of biological organization, brain development, neuroanatomy, and neurochemistry exist; and may accordingly contribute to the susceptibility to disease.

Most laboratory rat and mouse models (Silverman, Yang, Lord, & Crawley, 2010) of ASD are inbred and provide valuable evidence for genetic susceptibility in ASD, particularly when considering that phenotypic variability is reduced by a homogenous genetic background. However, the use of inbred strains and transgenic lines can restrict our interpretation of the clinical behavioral and biological heterogeneity in ASD. Importantly, genetic susceptibility accounts for only 20-25% of ASD cases, with each rare gene mutation or variant accounting no more than 1-2%. Therefore, while the genetically diverse background of outbred strains results in unpredictable genotypes and phenotypes, they might be more suitable for modeling the heterogeneous human ASD population.

Studying the effects of prenatal VPA exposure in a relevant species that exhibits complex social behaviors, such as monogamous opposite-sex social attachments, has yet to be reported. This is due, perhaps in part because traditional laboratory rats and mice are promiscuous breeders, do not exhibit bi-parental behaviors, and typically prefer social novelty over familiar conspecifics. This is a significant drawback given that, like the general population, high-functioning individuals with ASD are interested in initiating and maintaining romantic relationships, and express a

spectrum of gender identities and sexual orientations (Dewinter, De Graaf, & Begeer, 2017; Strunz et al., 2017).

1.6 The prairie vole model

The prairie vole is an excellent animal model for understanding the neurobiology of prosocial behaviors and social cognitive deficits exhibited in psychiatric and neurodevelopmental disorders. It is one of the rare animal species that exhibits social behaviors that recapitulate the complexity of some human social behaviors. As part of the 3% of mammalian species that are socially monogamous, female and male prairie voles form long-lasting social attachments with their mating partners and display selective aggression toward intruders (J. R. Williams, Catania, & Carter, 1992), while providing sustained bi-parental care for their offspring (Ahern, Hammock, & Young, 2011). These unique social bonding behaviors are modulated by underlying networks of neuropeptides, neurotransmitters and their receptors, such as oxytocin, vasopressin and dopamine, and offer a distinct advantage toward understanding the neurobiology of complex social attachments. Additionally, prairie voles are continuously outbred in laboratory settings and display a high degree of genetic diversity, relative to rat and mice strains, therefore presenting an advantage for investigating individual variation and molecular mechanisms that underlie social behaviors affected in neurodevelopmental diseases.

1.6.1 Oxytocin, vasopressin, and social attachment in prairie voles

Synthesized in the hypothalamus and released from the posterior pituitary, oxytocin (OXT) and vasopressin (AVP) are 9-amino peptides that are released into specific brain areas where they bind to their receptors to affect social information processing and social behaviors. Compared to

non-monogamous vole species, the monogamous prairie vole has by far higher oxytocin receptor (OXTR) density in the prefrontal cortex and nucleus accumbens (Insel & Shapiro, 1992), which is indeed crucial for the formation of pair bonds in female prairie voles (L. J. Young, Lim, Gingrich, & Insel, 2001). The importance of OXT-mediated pair bonding is exemplified when sex-naïve female prairie voles that receive intra-NAcc OXT infusions form a partner preference, while sex-naïve females that receive intra-NAcc vehicle infusions or intra-NAcc OXT with concurrent administration of an OXT antagonist did not form a pair bond (Liu & Wang, 2003). Vasopressin likewise is important in paternal and pair bonding in male as well as female prairie voles. Vasopressin receptor 1a (AVPR1a) density in the ventral pallidum of male prairie voles is significantly higher than non-monogamous meadow voles (Insel, Wang, & Ferris, 1994). Moreover, AVP infusions into the lateral septum of sex naïve male prairie voles reliably induces a pair bond (Wang, Ferris, & De Vries, 1994), while infusion of a AVPR1a antagonist into the LS or VP prevents pair bonding in males that have mated with their partner (Lim et al., 2004; Wang et al., 1994). OXT and AVP work simultaneously to facilitate formation of pair bonds by enhancing the saliency of social information, increasing social motivation and investigation, and converge the olfactory signature of a mate upon dopaminergic pathways (L. J. Young, 2008).

1.6.2 Neural circuit of pair bonding

Behaviors cannot arise from the activity of a single neuron, but instead rely on neuronal communication to orchestrate behaviors, giving rise to our perceptions, and dictating how we experience the world. Indeed, given the diversity and complexity of social behavior, we still observe conserved mechanisms and general principles operating to control behavior at the neural and genetic level. Although specific behavioral outcomes vary widely from species to species, the

biological needs that drive these behaviors are deeply shared. For instance, the hedonic brain circuit in humans and circuit of social attachment in prairie voles (Figure 3) share numerous resemblances. For instance, both circuits (1) are densely innervated with dopamine, oxytocin and vasopressin receptors, (2) act through the same brain regions that process social information, and (3) have the OXT and AVP neurons projection to important areas of the mesolimbic dopaminergic reward pathway. The evidently strong overlap between hedonic brain circuits and social attachment suggests that both may draw on the same psychological constructs, and perhaps the same biological substrates (Insel, 2003).

Stimulating dopamine neurons in the ventral tegmental area (VTA) enhances reinforcing behaviors and its afferents are highly linked to reward and pair bonding (Curtis & Wang, 2005; L. J. Young, 2008). For example, mating in prairie voles activates VTA, medial amygdala (MeA) and medial preoptic area (POA) of the hypothalamus, resulting in increased dopamine release from the VTA to the nucleus accumbens (NAcc), caudate putamen (CP), and prefrontal cortex (PFC). Moreover, the PFC has strong glutamatergic projections to the NAcc, whereas the NAcc sends GABAergic projections to the ventral pallidum (VP). Social recognition is important for pair bonding and is initiated when olfactory signals from the partner are processed in the olfactory bulbs (OB), which project to the MeA and lateral septum (LS). Olfactory learning and memory is further facilitated when OXT is released from the PVN after mating, and hypothalamic OXT fibers project and act on the PFC, NAcc, and MeA. Moreover, the MeA itself is a major source of AVP fibers projecting to the LS and VP. As a result, mating induces activation of NAcc dopamine receptors, OXT receptors in the PFC and NAcc of females, and AVP receptors in the VP of males. The reinforcing, hedonic properties of mating become coupled with olfactory cues, developing into a conditioned partner preference for the mate (L. J. Young & Wang, 2004). Significantly,

abnormalities in these circuits, brain regions, and neurotransmitter systems influence cognitive function and social behavior in the form that we ascribe to psychological and neurodevelopmental disorders, like depression and ASD.

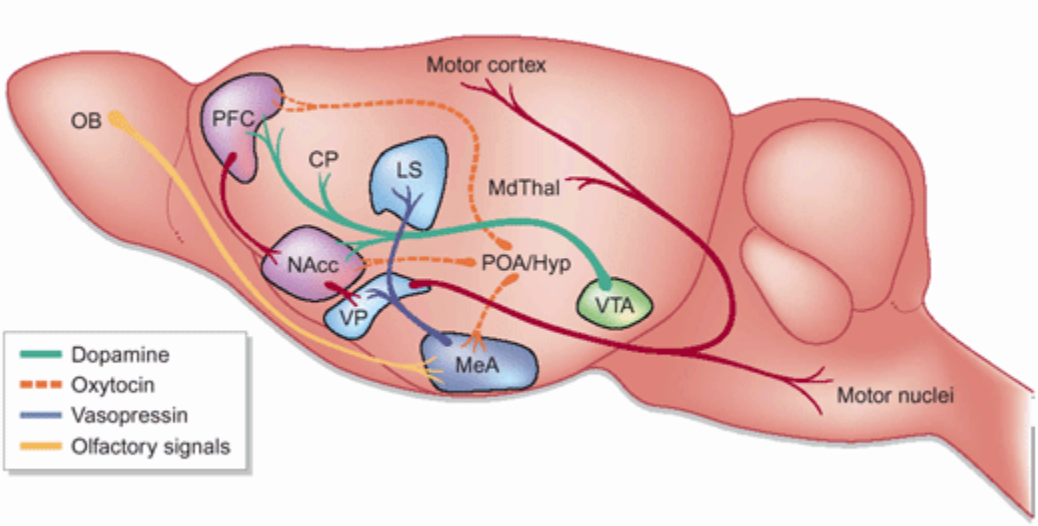

Figure 3. Neural circuit model of pair bonding (L. J. Young & Wang, 2004).

1.7 Research aims

In this work, we took advantage of the social monogamous nature of the prairie vole to examine the effects of *in utero* valproic acid exposure on male and female progeny. We use the same VPA exposure paradigm that has reliably induced ASD-like behavioral and molecular phenotypes in rats and mice: inject pregnant prairie voles with 600 mg/kg VPA on E12.5, examine affiliative and social behaviors of the VPA-exposed offspring, and social behavior, and ASD-relevant molecular and neuroanatomical targets in an effort to better understand the mechanisms underlying social deficits in ASD. Accordingly, chapter 2 focuses on validating the use of the prairie vole in the VPA model of ASD by assessing the effects of prenatal VPA exposure on same-

sex adolescent social and anxiety-like behaviors. Subsequently, we measure changes in expression of socially- and ASD-relevant genes and alterations in dendritic spine density and morphology in the medial prefrontal cortex in an effort to understand VPA-induced social behavior deficits. While most studies in VPA-exposed rats and mice fail to report effects in both sexes, this research includes both male and female prairie voles. In Chapter 3, we assess the effects of prenatal VPA exposure on the ability of prairie voles to form opposite-sex social attachments: partner preference formation and selective aggression, behaviors impossible to study in rats and mice. In this way, we were able to characterize same-sex and opposite-sex social behaviors in response to VPA exposure.

CHAPTER 2

EFFECTS OF PRENATAL VPA EXPOSURE ON ADOLESCENT SOCIAL BEHAVIOR, CELLULAR, AND MOLECULAR ALTERATIONS

Adapted from: Sailer L, Duclot F, Wang Z, Kabbaj M. Consequences of prenatal exposure to valproic acid in the socially monogamous prairie voles. Submitted to Scientific Reports Aug 2018.

2.1 Introduction

Neurodevelopmental disorders, such as schizophrenia and ASD, are likely the result of a dynamic interaction between genetics, environmental factors, and epigenetic mechanisms (Bale et al., 2010). For instance, prenatal exposure to the histone deacetylase inhibitor (HDACi) sodium valproate (or valproic acid, VPA) has been linked to greater occurrence of ASD in humans (Alsdorf & Wyszynski, 2005; Bromley et al., 2008; Gentile, 2014; Mawer et al., 2010; Meador et al., 2012). Histone deacetylases (HDACs) can regulate the activity state of chromatin and repress gene expression through the removal of acetyl groups from the tail of core histones (Elvir et al., 2017). Akhtar et al (2009) demonstrated that class I HDACis, such as VPA, form a developmental switch that modulates synapse maturation and functions by inhibiting HDAC1 and HDAC2 in immature and mature neurons. Inhibition of class I HDACs during early synaptic development causes a robust facilitation of excitatory synapse maturation and a modest increase in synapse numbers which may be associated with behavioral deficits observed in ASD (Akhtar et al., 2009).

Supporting evidence in rats and mice reveals that *in utero* exposure to VPA induces ASD-like social behaviors, such as social interaction impairments, altered ultrasonic vocalizations, and repetitive auto-grooming (Cohen, Varlinskaya, Wilson, Glatt, & Mooney, 2013; Moldrich et al., 2013), as well as cellular, and molecular deficits including alterations in glutamatergic neuronal differentiation (K. C. Kim et al., 2013) and dendritic spine number in limbic regions (Bringas et

al., 2013). Notably, the diverse effects of VPA are contingent upon the developmental period of administration. For instance, the negative effects following prenatal VPA exposure do not appear during postnatal chronic treatment with HDAC inhibitors VPA and SAHA (Foley, Cassidy, & Regan, 2014). While studies with rats and mice have provided valuable insights into the effects of prenatal VPA exposure, few considered both sexes (Kataoka et al., 2013; K. C. Kim et al., 2016; K. C. Kim et al., 2013) and none have examined the effects of VPA in a relevant species that exhibits strong social behaviors, which are deficient in ASD.

The prairie vole is an excellent animal model for understanding the neurobiology of prosocial behaviors and social cognitive deficits exhibited in psychiatric disorders. It is one of the rare animal species that exhibit social behaviors that recapitulate the complexity of some human social behaviors. The prairie vole is among the 3% of mammalian species that are socially monogamous—forming long-lasting social attachments with their mating partners and displaying selective aggression toward intruders (J. R. Williams, Catania, et al., 1992)—and provide sustained co-parental care for their offspring (Ahern et al., 2011). These behaviors involve neuropeptides and their receptors, such as oxytocin and vasopressin (K. Gobrogge & Wang, 2016; Tabbaa, Paedae, Liu, & Wang, 2016). For example, mating triggers vasopressin release and activation of vasopressin V1aR receptors, while inhibition of vasopressin receptors prevents the formation of partner preference in male prairie voles (Cho, DeVries, Williams, & Carter, 1999; Donaldson, Spiegel, & Young, 2010). Overexpression of V1aR, through genetic manipulation, in a promiscuous vole species results in the ability to form an exclusive partner preference (Lim et al., 2004).

Therefore, in this study, we aimed to validate the VPA model in male and female prairie voles to elucidate the molecular mechanisms by which social behaviors are perturbed, such as

affiliation with cage-mates and interaction with novel conspecifics of the same sex. We assessed how expression of genes important in the modulation of social behaviors are altered, and assessed how cortical spine density and morphology during adulthood is affected.

2.2 Materials and methods

In this experiment, we examined the effects of prenatal VPA exposure on social behaviors, prefrontal cortex gene expression of molecules previously implicated in ASD, and prefrontal cortex dendritic spine density (Bringas et al., 2013; Mahmood et al., 2017). To this end, male and female prairie voles prenatally-exposed to VPA or saline were weighed on PND21, 28, 35, and 90, and subjected to a series of behavioral tests on days PND38-47 to determine the effects of VPA on social affiliation, social interaction, anxiety-like behaviors, and locomotion during adolescence (Figure 4A).

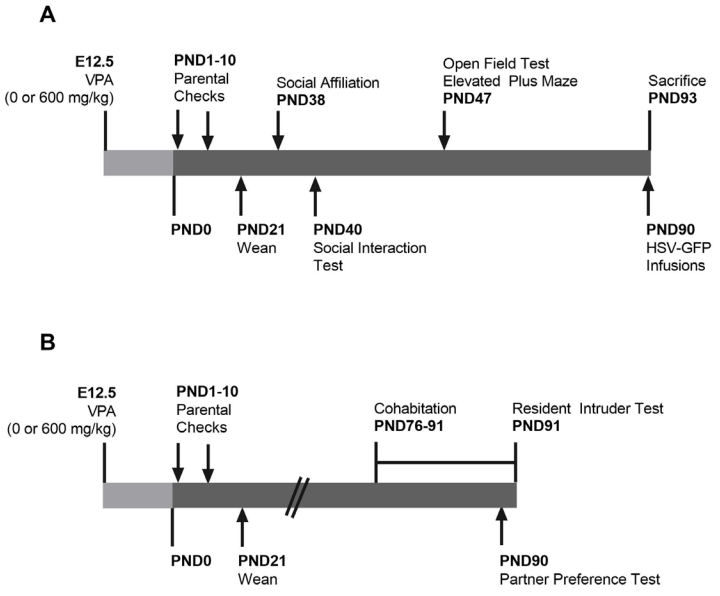

Figure 4. Experimental time lines of experiment 1 (***A***) and experiment 2 (***B***). E12.5, embryonic day 12.5; VPA, valproic acid; PND, postnatal day.

2.2.1 Animal subjects

Male and female prairie voles (*M. ochrogaster*) were produced from laboratory-bred colonies at Florida State University, weaned on postnatal day (PND) 21 and housed with same-sex littermates. All animals received food and water *ad libitum* and were maintained at 20 °C on a 14:10 light-dark cycle. Experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Florida State University.

2.2.2 Prenatal exposure to valproic acid

Adult (90 days old) sexually naïve female and male prairie voles were pair-housed and visually recorded for the first 3 days of cohabitation to confirm the day of mating—then considered as embryonic day 0 (E0). On gestation day 12.5, timed-pregnant female prairie voles received a single intraperitoneal (*i.p.*) dose of 600 mg/kg VPA (Sigma–Aldrich, St. Louis, MO, USA) or an equivalent volume of 0.9% saline (vehicle) and subsequently left undisturbed with their male partner and offspring until the time of pup weaning on PND21. The day and dose (600 mg/kg) of VPA was chosen as it is established to cause substantial social impairments in rats and mice (Bambini-Junior et al., 2011; Moldrich et al., 2013). Finally, the offspring of the time-mated dams were pair-housed so that treatment and sex matched between the two cagemates.

2.2.3 Parental behavior spot checks

In order to assess parental behaviors, spot checks were conducted twice daily from PND1-10 of the time-mated dams and sires, as described previously (Tabbaa, Lei, Liu, & Wang, 2017). In the morning (0900) and late afternoon (1700), parental behaviors (*i.e.* nest occupancy, passive

nursing, and active nursing) were thus scored by five spot checks conducted with 5-min interval for a total of 10 measures per day.

2.2.4 Social affiliation

Adolescent affiliative behavior with a cage-mate was measured on PND38. Subjects were placed in a novel cage (12 × 28 × 16 cm) with their cage-mate for 30 minutes, during which the mean duration and frequency of affiliative behaviors (side-to-side contact), social interaction (anogenital sniffing), and non-social cage exploration were quantified by an observer blind to treatment condition using a computer data acquisition system (JWatcher_V1.0 Windows version with JRE 1.5.0, http://www.jwatcher.ucla.edu).

2.2.5 Social interaction

A modification of the social interaction test (Nadler et al., 2004), was performed on PND40. The three-chamber apparatus consisted of a neutral cage (12 × 28 × 16 cm) joined to two identical side cages by plastic tubes (7.5 × 16 cm) connected with sensors detecting chamber transition frequency and duration. Smaller, cubical wire cages were used to contain either the unfamiliar animal (UA) or toy in the side cages (Figure 3A). The test subject was placed in the middle chamber with the tubes closed off and habituated for 10 minutes. At the end of habituation, the dividers were raised, allowing the test subject to move freely throughout all three chambers of the apparatus for a 10-minute test session. Location of the social side was alternated on consecutive sessions and the apparatus was cleaned with 70% ethanol (EtOH) between sessions. The duration and frequency of time spent investigating either the UA or toy was quantified by an observer blind to treatment condition.

2.2.6 Elevated plus maze test

The testing apparatus for the elevated plus maze (EPM) consisted of two open arms (35 cm x 6.5 cm) and two closed arms (35 cm x 6.5 cm x 15 cm) that cross in their middle as previously described (Sun, Smith, Lei, Liu, & Wang, 2014). On PND47, subjects were placed in the center, facing a closed arm and behavior was videotaped for 5 minutes to assess anxiety-like behaviors. The latency to enter an open arm, as well as time spent and frequency of entries into each arm were scored by an experimenter blind to treatment conditions. The apparatus was cleaned with 70% EtOH between sessions.

2.2.7 Open field test

The open field test was performed as previously described (Pan, Liu, Young, Zhang, & Wang, 2009), to assess locomotor activity on PND47. Test subjects were placed in the center of a squared open field arena (56cm L x 56cm W x 20 cm H) and allowed to explore freely for 10 minutes. Their behavior was videotaped and EthoVision XT 8.5 (Noldus, Leesburg, VA, USA) was used post-testing to measure locomotion, scored as distance traveled (cm) during the 10 min. test. Visual cues were kept constant and the arena was cleaned with 70% EtOH in between sessions.

2.2.8 Virus delivery, immunohistochemistry (IHC), spine density, and morphology measurements

At PND90, four males per group received bilateral stereotactic infusions of the HSV-GFP viral vector (p1005+ HSV plasmid expressing GFP under the control of cytomegalovirus promoter, prepared by Dr. Rachael Neve, MIT Viral Core Facility) into the medial prefrontal

cortex (mPFC) as previously described (Sarkar & Kabbaj, 2016). Coordinates were as follows: +1.9 mm anterior, ±0.5 mm lateral, and -2.5 mm ventral relative to bregma. The viral vector was delivered at a rate of 0.1 µl/min using a 27-gauge syringe (Hamilton Laboratory Products, Reno, NV) for a total volume of 500 nL per side. Subjects were sacrificed and perfused 3 days after viral vector injection, and 50 µm thick coronal sections containing HSV-injection within the mPFC were processed for IHC as described previously (Sarkar & Kabbaj, 2016). Briefly, after blocking in 5% normal goat serum and 0.3% Triton X-100 for 1 hour, sections were incubated overnight with a chicken anti-GFP antibody (1:500; Abcam ab13970, Cambridge, MA) at 4°C, and incubated with an Alexa Fluor 488–conjugated goat anti-chicken (1:1000; Life Technologies A11039, Grand Island, NY) for 3 hours. Labeled pyramidal neurons of layers III and V within the prelimbic region of the mPFC were imaged under 63× oil objective (Zeiss Plan Apochromat, numeric aperture = 1.40) of a Zeiss LSM880 confocal laser scanning microscope (Carl Zeiss, Germany) and the GFP tag was excited using an argon/krypton 488-nm laser line. For spine density and morphology analysis, Z-stacks of images were collected at 3x optical zoom and 0.39-µm step sizes using the Zen software (black edition, Carl Zeiss, Germany). Spine density and morphology were quantified using Neurolucida 360 and analyzed using Neurolucida Explorer and expressed as the number of spines per 10-µm dendritic segments, as previously described (Sarkar & Kabbaj, 2016).

2.2.9 Tissue processing and RNA extractions

Subjects that did not receive intra-mPFC HSV-GFP infusions were terminated by rapid decapitation on PND93, their brains rapidly extracted and frozen on dry ice before being stored at -80 °C. Tissue punches (1-mm diameter) were then taken bilaterally from the entire mPFC from 200 µm thick coronal sections and stored at -80 °C until further processing for total RNA extraction

using TRI-reagent according to the manufacturer's protocol (Molecular Research Center) and as previously described (Duclot et al., 2016). Notably, one half of the animals per group was used for RNA extraction, whereas tissue from the other half of the animals was used for chromatin immunoprecipitation.

2.2.10 Semi-quantitative real-time PCR

Total RNA (350-500 ng) was reverse-transcribed with the cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific) to examine mRNA expression for *avpr1a*, methyl CpG-binding protein 2 (*mecp2*), *oxtr*, *nlgn1*, *bdnf*, *psd95*, *shank1*, *shank2*, and *shank3* by semi-quantitative real-time PCR in triplicates (See Table 2 for primer sequences). Primer specificity was verified by melt curve analysis. For each primer pair, amplified cDNA was normalized to nicotinamide adenine dinucleotide dehydrogenase (NADH), as described previously (Duclot et al., 2016), and presented as percentage of saline-exposed controls.

Table 2. Primer sequences (5'-3') used for amplification of target mRNA after Trizol extraction.

Target	Primer Sequence	
avpr1a	Forward	GAGGTGAACAATGGCACTAAAACC
	Reverse	CCAGATGTGGTAGCAGATGAAGC
mecp2	Forward	GAGGGAGAGCGCAAAGACAT
	Reverse	GCTAACTCTCTCGGTCACGG
oxtr	Forward	TCCAAGGCCAAAATCCGCACGG
	Reverse	GGCAGAAGCTTCCTTGGGCGC
nlgn1	Forward	CTTTCCAGCTGGGCTGTTAG
	Reverse	ATCGATCACAGGTCCAAAGG
bdnf	Forward	CCATAAGGACGCGGACTTGTAC
	Reverse	TTGGAGATGTGGTGGAGAGG
psd95	Forward	GGATATGAGTTGCAGGTGAACGG
	Reverse	TGAAGCCCAGACCTGAGTTACC
shank1	Forward	GCACAGACAGCCACCACGGA
	Reverse	GTCTTCAGAGAGCCTCTGCCGCT

Table 2 – continued

Target	Primer Sequence	
shank2	Forward	CTGCAGAGAACGTGGCCATAGAATC
	Reverse	TCGTACACGGAGTTCACATCAGAGG
shank3	Forward	CAAGTCGTCCAGCCTCTCCATC
	Reverse	CCCACATCGAACTTGCTCCAAA
nadh	Forward	CTATTAATCCCCGCCTGACC
	Reverse	GGAGCTCGATTTGTTTCTGC

2.2.11 Chromatin immunoprecipitation

Phosphorylated cAMP response element-binding protein (pCREB) and MeCP2 binding at target genes promoters in the mPFC were analyzed by chromatin immunoprecipitation using the ChromaFlash High-Sensitivity ChIP Kit (Epigentek, Farmingdale, NY, USA) following the manufacturer's instructions. Chromatin was sheared using a Bioruptor sonicator (Diagenode, Denville, NJ, USA) for 20 cycles (30 sec on, 30 sec off) with continuous cooling to fragments of 200-600 bp. Successful DNA immunoprecipitation was verified *a priori* with positive (RNA polymerase II) and negative (non-immune IgG) control antibodies. Immunoprecipitations for pCREB and MeCP2 were carried out with 0.10 µg of anti-pCREB (Millipore 06-519, Billerica, MA, USA) and 0.10 µg of anti-MeCP2 (Diagenode C15410052, Denville, NJ, USA). Immunoprecipitated DNA was analyzed in triplicates by real-time PCR with standard curves made from pooled DNA input samples to determine binding to the *avpr1a* and *mecp2* promoters. The primers were designed to amplify a 75-bp-long region located 216-bp upstream of the first exon coding for the prairie vole *avpr1a* gene (Genbank accession #AF069304) and an 87 bp-long region located 3,118 bp upstream of the first exon encoding the prairie vole *mecp2* gene (Genbank accession #NW004949275.1). As a positive control, pCREB binding to *bdnf* (Koo et al., 2015; Rube et al., 2016) was analyzed (see Table 3 for primers sequences). Primer specificity was

verified by melt curve analysis and each sample was normalized to the respective DNA input value and expressed as a percentage of Saline-treated voles.

Table 3. Primer sequences (5'-3') used for amplification of target genomic DNA.

Target	Primer Sequence		Origin
avpr1a	Forward Reverse	CAGTCAGCGGCAGTAGACG TGCTCCAGCCCCCTTTACAA	Own design
*mecp2*_1	Forward Reverse	GGCTTTTACCACAGCCCTCT CTCAAGCTGGCCGGGAG	Own design
*mecp2*_2	Forward Reverse	AGATGTCACGCTCTTAGGGC CAGTGCTAACCATCCGATCCA	Own design
bdnf	Forward Reverse	CAGGTATTCTTTTCCTCGCTGT CGCTCCAAAATCTGACTCTCTC	(Rube et al., 2016)

2.2.12 Statistical analyses

All data are presented as the means ± SEM. Parental behaviors were analyzed by using a 3-way mixed ANOVA to compare the effects of sex, treatment, and postnatal day using SPSS Version 22 software (IBM Corp, Armonk, NY, USA). Body weight and weight gain were analyzed by using a 2-way mixed ANOVA to compare the effects of treatment and postnatal day. For comparisons of sex and treatment, data were analyzed by 2-way mixed ANOVA. For comparisons of sex, treatment, and stimulus preference, data was analyzed by using a 3-way mixed ANOVA. If any of these parent ANOVAs resulted in main effects or significant interactions (alpha set at 5%), Tukey's post-hoc tests were performed to determine source of significance. All molecular data and experiment 2 data were analyzed using an unpaired two-tailed *t*-test after verification of normality. Additionally, partial Eta-squared (ηp^2) and Cohen's *d* are reported as measures of effect size for ANOVA and *t*-tests, respectively.

2.3 Results

2.3.1 VPA treatment to dams did not affect bi-parental behavior

Maternal licking and grooming are vital for the development of social behaviors in rodents (Francis, Diorio, Liu, & Meaney, 1999; Nguyen, Bagot, Diorio, Wong, & Meaney, 2015; Zhang, Labonte, Wen, Turecki, & Meaney, 2013). Furthermore, bi-parental care, a unique social behavior in prairie voles, is an important aspect for the development of social attachment (Ahern et al., 2011; Tabbaa et al., 2017). In this context, we first assessed the effects of VPA treatment on bi-parental behaviors by conducting home-cage spot checks during the first ten postnatal days (PND1-10). Paternal presence in the nest was low when compared to maternal presence (Figure 5A, Females vs Males, 3-way mixed ANOVA: $F_{1,44} = 106.62$, $p < 0.0001$, $\eta p^2 = 0.71$), but did not differ whether the partner dam was treated with saline or VPA (2-way mixed ANOVA, $F_{1,22} = 0.73$, $p = 0.4005$, $\eta p^2 = 0.03$). Interestingly, however, maternal nest occupancy was higher in VPA-treated dams on PND1 than in saline-injected dams ($p < 0.0001$), resulting in a different evolution of maternal presence in the nest over PND by VPA treatment (2-way mixed ANOVA, $F_{1,22} = 7.35$, $p = 0.013$, $\eta p^2 = 0.251$). Nevertheless, although passive and active nursing increased and decreased over PND, respectively (passive nursing: $F_{9,198} = 2.56$, $p = 0.0084$, $\eta p^2 = 0.104$ for PND; active nursing: $F_{9,198} = 2.9$, $p = 0.003$, $\eta p^2 = 0.116$ for PND; Fig. 5B), saline- and VPA-treated dams were undistinguishable (passive nursing: $F_{1,22} = 0.44$, $p = 0.5123$, $\eta p^2 = 0.020$ for treatment, $F_{9,198} = 0.26$, $p = 0.9838$, $\eta p^2 = 0.012$ for interaction; active nursing: $F_{1,22} = 0.02$, $p = 0.880$, $\eta p^2 = 0.001$ for treatment, $F_{9,198} = 0.62$, $p = 0.7808$, $\eta p^2 = 0.027$ for interaction), demonstrating that despite a lower maternal presence observed during the first PND, VPA injections do not affect bi-parental care in prairie voles.

It is important to note, however, that despite similar bi-parental care, VPA-exposed offspring exhibited lower body weight than saline-exposed controls from weaning (PND21) to PND90 (2-way mixed ANOVA, $F_{1,118} = 16.32$, $p < 0.0001$, $\eta p^2 = 0.122$ for treatment, $F_{3,354} = 1112.65$, $p < 0.0001$, $\eta p^2 = 0.904$ for time, and $F_{3,354} = 1.479$, $p = 0.2199$, $\eta p^2 = 0.012$ for the interaction, Fig. 6A). Nevertheless, VPA- and saline-exposed prairie voles exhibited a similar body weight gain from PND21 throughout the experiment (2-way mixed ANOVA, main effect of treatment: $F_{1,118} = 1.28$, $p = 0.261$, $\eta p^2 = 0.011$, main effect of time: $F_{1,118} = 7.53$, $p = 0.007$, $\eta p^2 = 0.060$, and for interaction: $F_{1,118} = 0.14$, $p = 0.713$, $\eta p^2 = 0.001$, Fig. 6B), suggesting an unaltered post-weaning development in VPA-exposed offspring.

2.3.2 Exposure to VPA in utero alters adolescent social affiliation and social interaction

Impairments in social interaction are characteristic phenotypes observed in ASD and in other neurodevelopmental disorders, as well as in rats and mice prenatally exposed to VPA (Kataoka et al., 2013; Markram, Rinaldi, La Mendola, Sandi, & Markram, 2008). Therefore, we analyzed in VPA-exposed prairie voles the levels of social affiliation and social interaction with same-sex siblings and novel same-sex conspecifics during adolescence (PND38 and PND40, respectively, Fig. 7 and 8).

In line with the known impairments in social behaviors following prenatal VPA exposure in rats and mice (Kataoka et al., 2013), both male and female VPA-exposed offspring spent less time in side-to-side contact with a same-sex sibling than saline-exposed controls ($F_{1,29} = 33.37$, $p < 0.0001$, $\eta p^2 = 0.535$ for treatment, $F_{1,29} = 0.29$, $p = 0.595$, $\eta p^2 = 0.010$ for sex, and $F_{1,29} = 0.065$, $p = 0.801$, $\eta p^2 = 0.002$ for the interaction; Fig. 7A). Contrary to side-by-side contact, however, VPA-exposed prairie voles presented with more non-affiliative behaviors than their saline-exposed

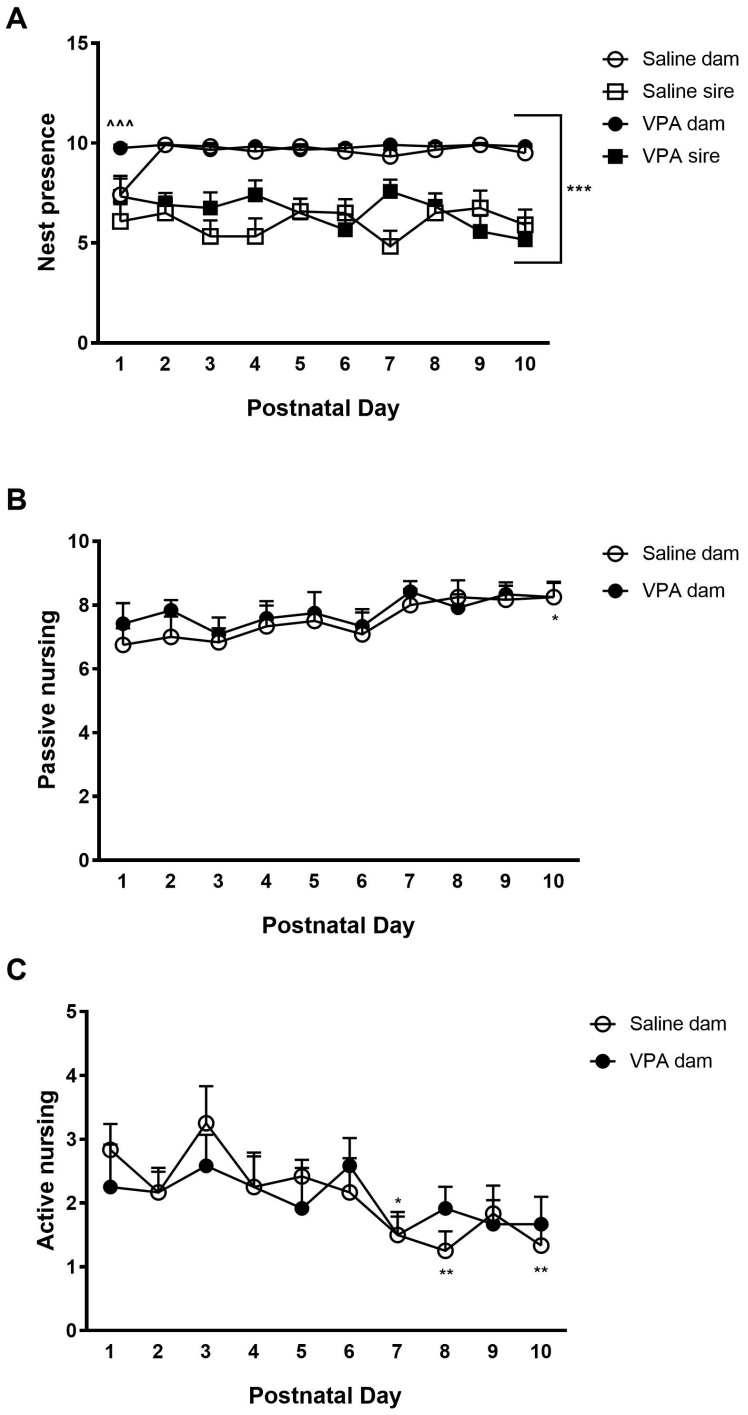

Figure 5. Evolution of bi-parental behaviors throughout postnatal days (PND) 1-10. Sires spent less time in the nest than dams (***A***). Dams engage in passive nursing (***B***) and active nursing (***C***) equivalently, regardless of treatment. Data are presented as mean ± SEM; n = 10, 2-way mixed ANOVA with Tukey's post hoc test: ***$p < 0.0001$ dams vs sires; ^^^$p < 0.001$ VPA dam vs saline dam on PND1; *$p < 0.05$, **$p < 0.01$ vs saline dam on PND1.

counterparts in a sex-dependent manner. Indeed, while prenatal VPA exposure led to higher levels of anogenital sniffing than saline-exposed controls overall ($F_{1,29} = 30.63$, $p < 0.0001$, $\eta p^2 = 0.514$ for treatment, Fig. 7B), this effect was more pronounced in females than males ($F_{1,29} = 8.37$, $p = 0.0072$, $\eta p^2 = 0.224$ for sex, and $F_{1,29} = 7.98$, $p = 0.01$, $\eta p^2 = 0.216$ for the interaction, Fig. 7B) and was reflected by higher levels of anogenital sniffing in VPA-exposed females than males ($p = 0.002$). Although similar effects on non-social exploration were observed, as VPA-exposed prairie voles exhibited higher levels than their saline-exposed counterparts overall ($F_{1,29} = 19.99$, $p < 0.0001$, $\eta p^2 = 0.408$ for treatment, Fig. 7C), sex differences were less pronounced. Indeed, despite a significant interaction with treatment ($F_{1,29} = 4.32$, $p = 0.0467$, $\eta p^2 = 0.130$ for sex:treatment interaction, and $F_{1,29} = 1.14$, $p = 0.295$, $\eta p^2 = 0.038$ for sex), VPA-exposed males and females spent a similar amount of time in non-social exploration ($p = 0.149$). Altogether, these observations indicate that prenatal VPA exposure reduces the levels of affiliative behaviors in both male and female prairie voles, along with a coherent increase in non-affiliative and non-social behaviors that, interestingly, is more pronounced in females than males.

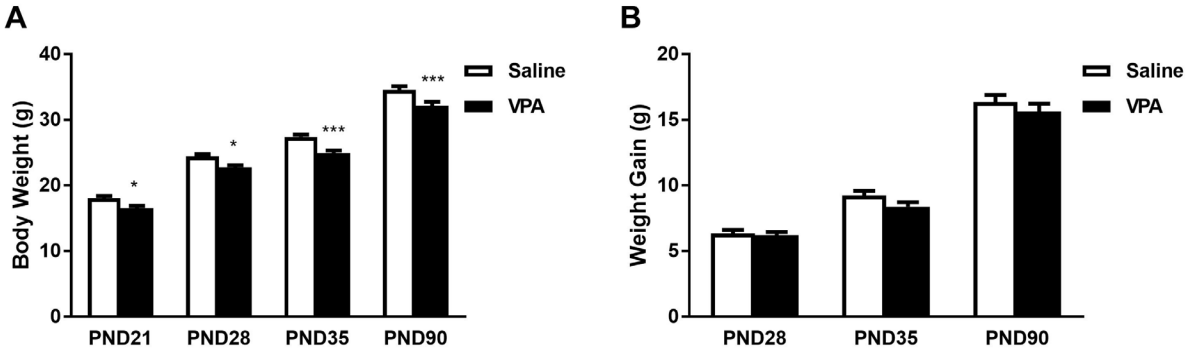

Figure 6. Body weight (***A***), but not weight gain (***B***), is decreased by prenatal VPA exposure. Body weights were measured upon weaning (PND21), during adolescence (PND28 & PND35), and sexual maturity (PND90). Data pooled from males and females, presented as mean ± SEM; n = 25-35; *$p < 0.05$, 2-way mixed ANOVA with Tukey's post hoc test: *$p < 0.05$, ***$p < 0.001$.

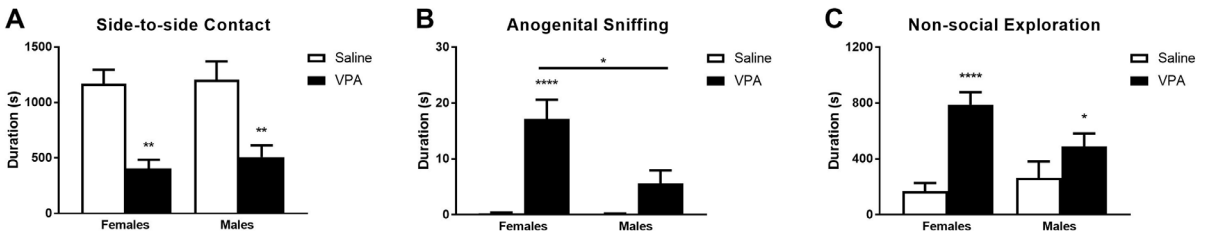

Figure 7. Adolescent social affiliation in response to exposure to VPA *in utero*. Duration of side-to-side contact with cage-mate (***A***), anogenital sniffing (***B***), and non-social cage exploration (***C***) behaviors in prairie voles prenatally exposed to saline (white bars) or VPA (black bars). Data are presented as mean ± SEM; n = 8-9, 2-way ANOVA with Tukey's post hoc test.

Following examination of affiliative behaviors, social interaction with a novel same-sex conspecific and a toy was assessed on PND40 (Fig. 8A). Regardless of sex and treatment, all animals preferred to investigate an unfamiliar animal (UA) versus a toy during the social interaction test (mixed model 3-way ANOVA, stimulus main effect: $F_{1,93} = 72.22$, $p < 0.001$, $\eta p^2 = 0.437$, Fig. 8B). Yet VPA-exposed females and males spent significantly less time with the UA when compared to saline-treated females and males (mixed model 3-way ANOVA, stimulus:treatment interaction $F_{1,93} = 5.64$, $p = 0.02$, $\eta p^2 = 0.057$; stimulus:sex interaction: $F_{1,93} = 0.98$, $p = 0.324$, $\eta p^2 = 0.010$). VPA did not affect global investigative and exploratory behaviors as indicated by total cage transitions (Fig. 8C, 2-way ANOVA, $F_{1,116} = 1.76$, $p = 0.187$, $\eta p^2 = 1.487$ for treatment, $F_{1,116} = 0.99$, $p = 0.321$, $\eta p^2 = 0.837$ for sex, and $F_{1,116} = 0.12$, $p = 0.734$, $\eta p^2 = 0.097$ for the interaction). These data thus demonstrate that prenatal VPA exposure decreases social interaction with novel conspecifics during adolescence in both females and males.

As increased anxiety is often observed in individuals with ASD (Vasa & Mazurek, 2015) and in VPA-exposed rats and mice (Kataoka et al., 2013; Markram et al., 2008), we tested whether VPA-exposed subjects at PND47 exhibit anxiety-like behaviors on the EPM (Figure 9A). A main effect of treatment (2-way ANOVA, $F_{1,117} = 5.21$, $p = 0.0243$, $\eta p^2 = 0.043$), without interaction

with sex ($F_{1,117} = 0.99$, $p = 0.3206$, $\eta p^2 = 0.008$) was observed in the percentage of time spent in the open arms. As locomotion in an open field arena remained unaffected by sex or VPA (Figure 9B, 2-way ANOVA, $F_{1,117} = 2.08$, $p = 0.152$ for treatment, $F_{1,117} = 1.19$, $p = 0.277$ for sex, and $F_{1,117} = 0.70$, $p = 0.1036$ for the interaction), these data suggest that prenatal VPA exposure increases anxiety levels in both the male and female offspring.

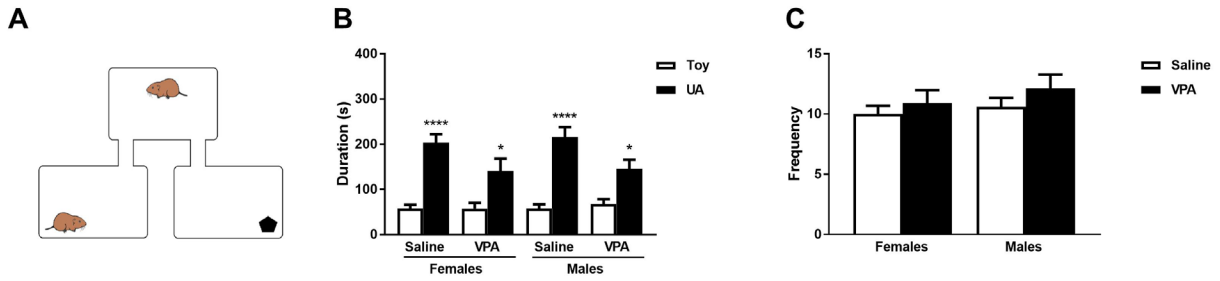

Figure 8. Prenatal VPA exposure reduces social interaction in females and males on PND40. ***A***, Representative image of social interaction test. ***B***, Time spent in contact with a novel toy or a same-sex unfamiliar animal (UA). ***C***, Total number of cage transitions (entry into toy cage + entry into UA cage), does not differ between VPA- and saline-exposed females (p = 0.881) and between VPA- and saline-exposed males (p = 0.676). Data are presented as mean ± SEM; n = 25-35; mixed model 3-way ANOVA with Tukey's post hoc test: * $p < 0.05$ and **** $p < 0.0001$ *vs.* toy; VPA: valproic acid.

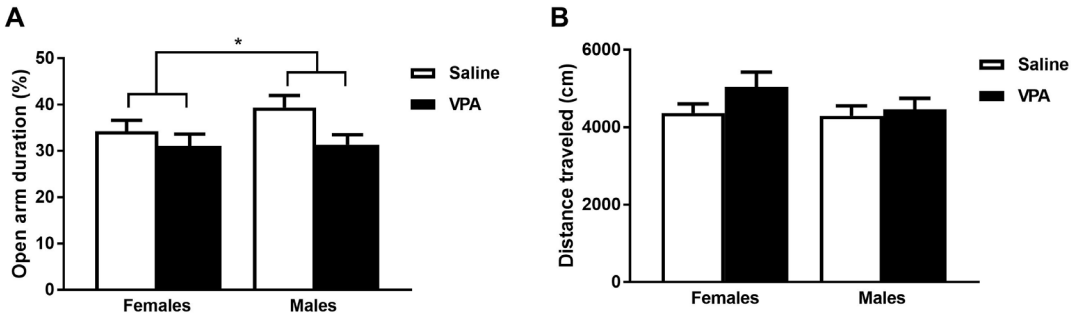

Figure 9. Adolescent anxiety-like behavior on PND47, but not locomotion, is altered by prenatal VPA exposure. Percentage of total time spent in the open arm in the EPM during a period of 5 min (***A***). Distance moved (cm/s) in an open field arena during a period of 10 min (***B***). Data are presented as mean ± SEM; n = 25-35; 2-way ANOVA: * $p < 0.05$ main effect of treatment.

2.3.3 Adult molecular- and plasticity-related alterations in response to prenatal VPA exposure

Evidence of irregular structure and function in the mPFC has been steadily reported in individuals with ASD (Martinez-Sanchis, 2014) as well as in VPA-exposed rodents (Bringas et al., 2013; Lauber, Filice, & Schwaller, 2016). For instance, prenatal exposure to VPA enhances short- and long-term synaptic plasticity (*i.e.* paired-pulse facilitation and long-term potentiation, respectively) in the mPFC of rats (Sui & Chen, 2012). We therefore chose to probe, within the mPFC, for factors known to regulate social bonding—the oxytocin receptor (OXTR) and V1aR—or synaptic plasticity genes involved in ASD pathology. Prenatal exposure to VPA reduced *avpr1a* (two-tailed unpaired *t*-test, $t_{24} = 2.481$, $p = 0.0205$, $d = 0.99$) and *mecp2* ($t_{23} = 2.704$, $p = 0.0127$, $d = 1.14$) mRNA expression, but not *oxtr*, *nlgn1*, *bdnf*, *psd95*, *shank1*, *shank2*, or *shank3* mRNA levels (Figure 10A). Interestingly, a positive correlation between V1aR and MeCP2 mRNA in saline- and VPA-treated voles was observed in both experimental groups (Figure 10B).

Given that MeCP2 is a transcription factor, this positive association might suggest that MeCP2 mediates the down-regulation of *avpr1a*, as recruitment of CREB by MeCP2 at gene promoters results in transcriptional activation (Chahrour et al., 2008). Alternatively, MeCP2 down-regulation could result from signaling events downstream of V1aR, involving for instance the transcription factor CREB and its active phosphorylated form. In order to test these two hypotheses, we next examined the binding of phospho-CREB and MeCP2 to the *avpr1a* promoter in the mPFC and whether this binding was altered following prenatal VPA exposure (Figure 11A). In contrast to this hypothesis, exposure to VPA does not alter the binding of either phospho-CREB (two-tailed unpaired *t*-test, $t_9 = 1.655$ $p = 0.1323$, $d = 1.05$) or MeCP2 ($t_8 = 1.259$, $p = 0.2435$, $d = 0.77$) to the *avpr1a* promoter in the mPFC (Figure 11B and 11C). Interestingly, even though primer

specificity at the *mecp2* promoter was successfully verified at two different loci using input DNA (Figure 12A-12H), no specific amplification of phospho-CREB immunoprecipitated DNA could be achieved at the *mecp2* promoter (Figure 12D & 12H). In light of the successful amplification of the same samples at a positive control locus (*bdnf*, Figure 12I & 12J), this suggests that phospho-CREB does not bind the *mecp2* promoter in our samples. In this context, the positive association between V1aR and MeCP2 mRNA levels is likely indirect, maybe through another common regulator of V1aR and MeCP2.

In addition to alterations in gene expression, individuals with ASD (Courchesne et al., 2011; Zikopoulos & Barbas, 2013) and VPA-exposed rats and mice (Bringas et al., 2013; Mahmood et al., 2017; Rinaldi, Perrodin, & Markram, 2008) exhibit hyperconnectivity in local circuits and hypoconnectivity between brain regions, which, along with alterations in spine density and morphology, are likely to underlie socio-cognitive impairments characteristic of this disorder. For instance, adolescent VPA-treated mice present with alterations in cortical neuron spine morphology concomitant with social interaction impairments (Mahmood et al., 2017). Moreover, prenatal VPA exposure in rats induces cortical spine density loss, with a shift of dendritic refraction to dendritic hypertrophy, throughout development (Bringas et al., 2013). In this context, we analyzed dendritic spine density and morphology in pyramidal layers III and V neurons within the prelimbic region of the mPFC of adult prairie voles prenatally exposed to VPA (Figure 13A and 13B). In all measurements, saline and VPA-exposed prairie voles were undistinguishable (Figure 13C and 13D), suggesting that behavioral alterations subsequent to prenatal VPA exposure are not associated with altered synaptic plasticity in the mPFC of adult prairie voles.

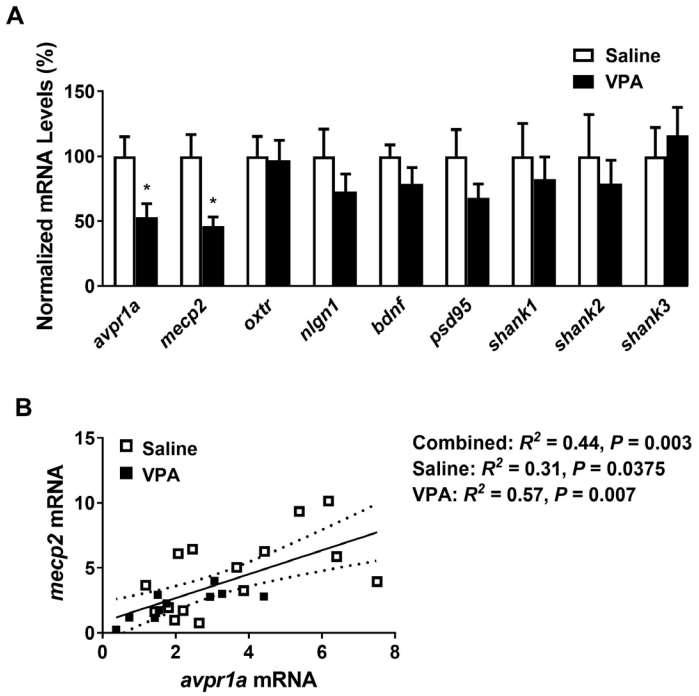

Figure 10. Expression of *avpr1a* and *mecp2* mRNA in the medial prefrontal cortex (mPFC) was downregulated by prenatal VPA treatment, but not *oxtr, nlgn1, bdnf, psd95, shank1, shank2,* and *shank3* (***A***). *Avpr1a* and *mecp2* mRNA in saline- and VPA-treated voles were positively correlated (***B***). Data are presented as mean ± SEM; n = 6-8; two-tailed unpaired *t*-test: **p* < 0.05 vs Saline. Dotted lines: SEM for a 95% confidence interval.

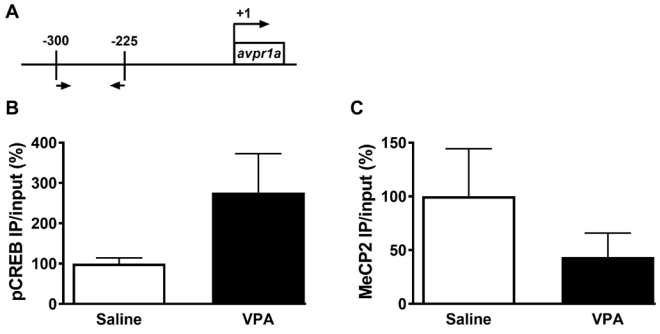

Figure 11. Chromatin immunoprecipitation (ChIP) was used to confirm phospho-CREB (pCREB) and MeCP2 predicted occupancy at the avpr1a promoter region. (***A***) Schematic representation the avpr1a promoter region where the genomic primers were designed (arrows) and their position relative to the transcription start site (+1 site). Prenatal VPA treatment does not alter pCREB (***B***) or MeCP2 (***C***) binding to the *avpr1a* promoter during adulthood. Data was normalized by the respective INPUT value, expressed as percentage of Saline-treated voles and presented as mean ± SEM; n = 5-6.

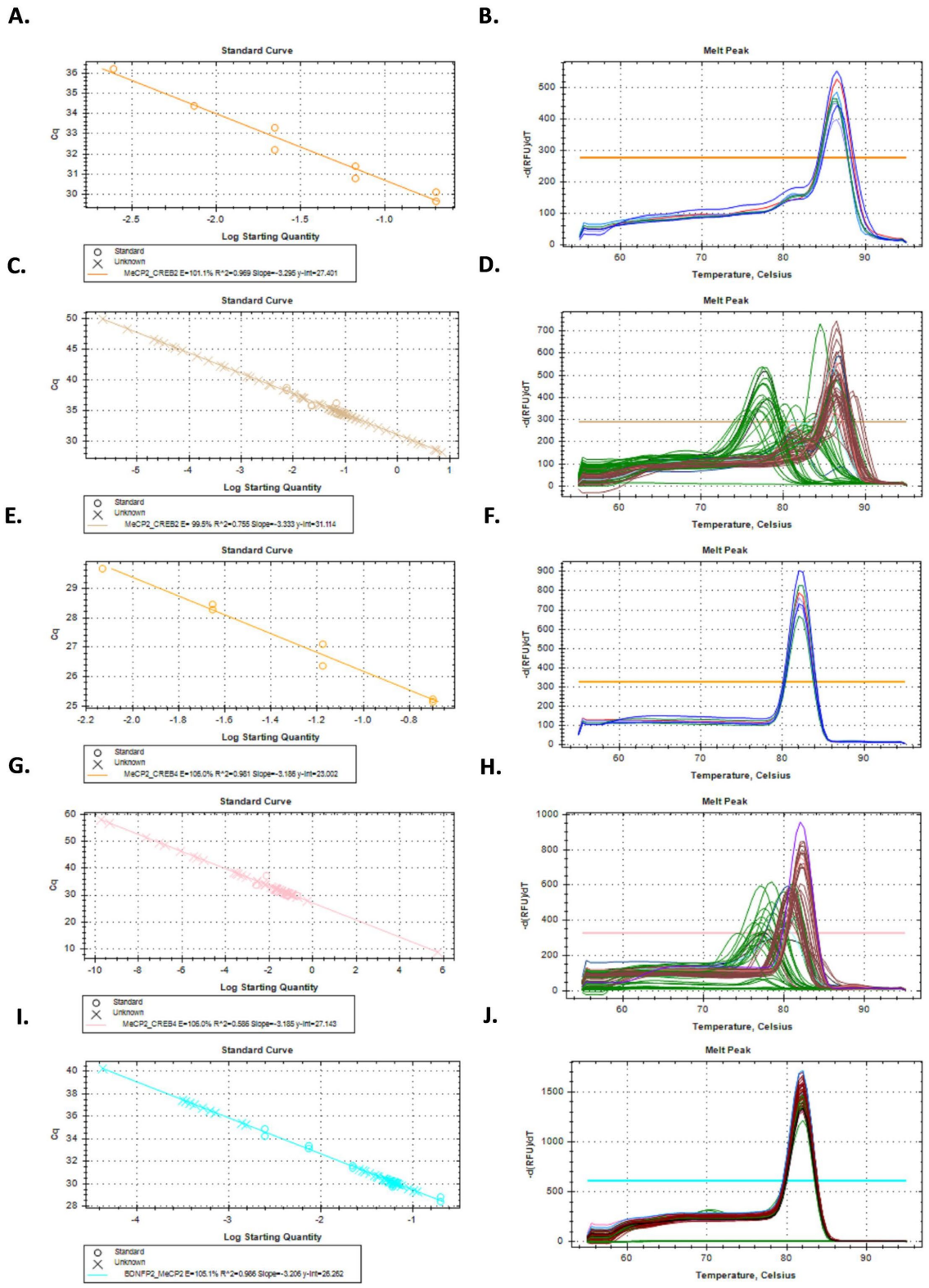

Figure 12. Standard curves and melt peaks for specificity of mecp2 and bdnf qRT-PCRs against immunoprecipitated pCREB. The amplification efficiency (Eff%) for sequentially diluted input

Figure 12 – continued. DNA at two mecp2 promoter loci was 101.1 and 106.0 ,respectively, with coefficients of determination (R^2) as 0.969 and 0.981, respectively (***A*** and ***E***). The uniform melt peaks indicate the same amplification products were formed with varying amounts of template (***B*** and ***F***). While the standard curves for detection of mecp2 from pCREB pull-down produces high primer set efficiencies (Eff% = 99.5, R^2 = 0.755 for ***C***, Eff% = 106.0, R^2 = 0.586 for ***G***), multiple melt peaks (***D*** and ***H***) are produced, indicating non-specific genomic DNA amplification. Amplified bdnf genomic DNA from pCREB pull-down produces high efficiency qPCR primer sets (Eff% = 105.1, R^2 = 0.986 for ***I***) and single melt peaks using varying amounts of DNA input (***J***), indicating specific amplification.

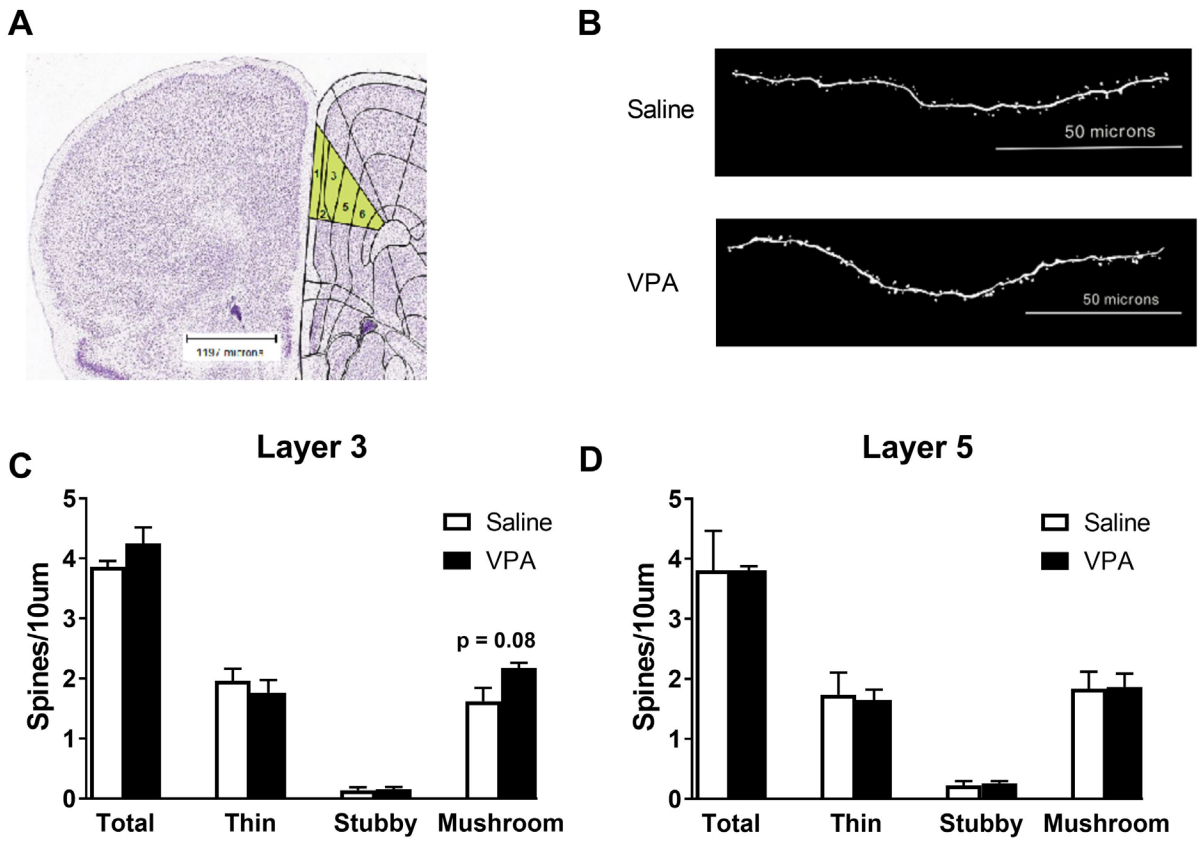

Figure 13. Influence of prenatal VPA exposure in spine density and spine morphology. Representative coronal section through the prefrontal cortex, the prelimbic cortex is highlighted in yellow with delineated pyramidal neuron layers, Image Credit: Allen Institute (***A***). Representative dendritic segments of pyramidal neurons from layer III using a 63x oil immersed objective (***B***). Total, thin, stubby, and mushroom spine density of pyramidal neurons of the layer III (***C***) and layer V (***D***) in the prelimbic cortex. Prenatal VPA treatment did not affect total spine density or spine morphology. Data are presented as mean ± SEM; 8 neurons/animal and n = 3.

2.4 Discussion

In the socially monogamous prairie voles, prenatal VPA exposure leads to behavioral and molecular alterations. Indeed, although VPA-treatment to the dam did not affect parental behaviors, the VPA-exposed adolescent offspring showed reduced social affiliation and higher levels of non-affiliative behaviors with a same-sex sibling. Furthermore, VPA-exposed young adults exhibit lower interaction with novel same-sex conspecifics and elevated anxiety levels than saline-exposed controls. These behavioral alterations are accompanied by a down-regulation of cortical V1aR and MeCP2 mRNA levels. Other synaptic and neuronal plasticity-related genes remained unaffected, as well as dendritic spine density and morphology in the mPFC of VPA-exposed prairie voles. Surprisingly, despite deficits in social affiliations and interactions with a same-sex conspecifics, VPA-exposed prairie voles are still able to display partner preference and selective aggression. Altogether, these data thus indicate that prenatal VPA exposure in prairie voles induces a complex pattern of ASD-like molecular alterations and social deficits.

The attenuation of same-sex social affiliations and interactions observed following prenatal VPA exposure are, interestingly, accompanied by an increase in non-affiliative behaviors and occur in the absence of general locomotion impairments. It is important to note, however, that VPA-exposed animals also exhibited elevated anxiety levels, and thus conceivably explain reduced social interactions with unfamiliar targets. Nevertheless, the observation in the same animals of substantial social deficits in a familiar context during affiliation with a same-sex sibling suggests that the effects of prenatal VPA exposure on social behaviors are not the sole result of an enhanced anxiety towards novel stimuli, but rather reflect more general impairments in social abilities.

Lastly, the origin of the complex profile of social behavior impairments triggered by prenatal VPA exposure remains unclear (Hara et al., 2017; Iijima et al., 2016). Interestingly, one contributing factor could be of environmental nature, through a direct effect of VPA on the dam's maternal care towards the pups. This is especially intriguing in light of the positive association between parental care and the offspring's social behaviors during adolescence (Perkeybile, Griffin, & Bales, 2013). Following VPA treatment, however, we observed indistinct levels of parental care from PND1-10. We can thus rule out the possibility that social impairments in adolescent prairie voles result from different levels of parental care. Yet it is interesting to note that, compared to VPA-exposed rats and *Mecp2*-null mice (Guy, Hendrich, Holmes, Martin, & Bird, 2001; Schneider & Przewlocki, 2005), VPA-exposed offspring exhibited a marked lower body weight throughout postnatal development. In line with the timing of VPA exposure (E12.5), these findings point towards neurodevelopmental alterations.

Associated with Rett syndrome, MeCP2 expression is reduced in VPA-exposed mice (K. C. Kim et al., 2016). Interestingly, evidence of MeCP2's duality as a transcriptional repressor and activator (Horvath & Monteggia, 2018) opens the possibility of a direct transcriptional control of *avpr1a* by MeCP2 in our study, as supported by the down-regulation of both mPFC V1aR and MeCP2 mRNA expression in VPA-exposed prairie voles. Nevertheless, pCREB and MeCP2 binding to the *avpr1a* promoter did not differ between VPA-exposed and saline-exposed groups, whereas pCREB binding at the *mecp2* promoter was undetectable. As a result, the positive association between V1aR and MeCP2 mRNA levels is likely indirect or CREB-independent. Despite the identification of *oxtr* as an ASD genetic risk factor (Baribeau et al., 2017), mPFC OXTR mRNA levels did not differ between saline- and VPA-exposed prairie voles. In agreement with our study, 20 mg/kg, but not 100 mg/kg, chronically VPA-exposed rats display reduced

OXTR binding in the amygdala but not in other limbic regions (Bertelsen et al., 2017). Thus our data supports this dose- and structure-specificity in the regulation of OXTR expression following prenatal VPA exposure. Moreover, prenatal VPA exposure did not alter the expression of neuroplasticity genes *nlgn1*, *bdnf*, *psd95*, *shank1-3*, suggesting an unaltered neuronal plasticity in the mPFC of adult VPA-exposed prairie voles when compared to saline-exposed controls. Intriguingly, mutations of these synaptic molecules are associated with ASD pathogenesis in humans and ASD rodent models alike (Blundell et al., 2010; Hung et al., 2008; Sadakata et al., 2007). For example, MeCP2 down-regulation, with concurrent up-regulation of cortical PSD95 and Shanks 1-3, is linked to glutamatergic synapse development impairments in VPA-exposed rats during early postnatal life (PND14) (K. C. Kim et al., 2016). Notably, this mPFC hyper-synaptic function is normalized during adolescence (PND48) and then diminished in adult (PND120) VPA-exposed rats compared to saline-exposed controls (Martin & Manzoni, 2014), suggesting that VPA-exposed neurons transition through a normal period between a hyper- to hypo-synaptic function. Conceivably, VPA-exposed prairie voles likewise undergo a homeostatic compensatory mechanism (Walcott, Higgins, & Desai, 2011) on PND90, explaining the evidence of unaltered mPFC neuroplasticity genes.

Accordingly, dendritic spine density and morphology in the adult mPFC were similar between VPA- and saline-exposed prairie voles. This finding can appear surprising in light of the spine pruning deficits and increased spine density observed in ASD patients (Tang et al., 2014), or the reduced brain weight, cortical thickness, dendritic branching, spine density, and Nissl-positive cells in the PFC of mice and rats prenatally exposed to VPA (Bringas et al., 2013; Hara et al., 2012; Mychasiuk, Richards, Nakahashi, Kolb, & Gibb, 2012). Similarly, total and mushroom-type spine density in cortical neurons are reduced in Rett syndrome and related rodent models

(Belichenko & Dahlstrom, 1995; Bittolo et al., 2016). In MeCP2-null mice, however, while spine density is lower during early postnatal development, its levels are comparable to wildtypes during adolescence and adulthood when social deficits are well established, suggesting the action of a secondary compensatory mechanism (Chapleau et al., 2012). Given the MeCP2 down-regulation observed in the mPFC without alterations in spines density at adulthood (PND90), we can thus speculate that prenatal VPA exposure in prairie voles may resemble Rett syndrome-like pattern of alterations in neuronal morphology. Nevertheless, future studies aimed at examining dendritic spine-subtype and total spine density throughout development are warranted.

CHAPTER 3

EFFECTS OF PRENATAL VPA EXPOSURE ON PAIR BONDING

Adapted from: Sailer L, Duclot F, Wang Z, Kabbaj M. Consequences of prenatal exposure to valproic acid in the socially monogamous prairie voles. Submitted to Scientific Reports Aug 2018.

3.1 Introduction

Our findings from Chapter 2 demonstrate that prenatal administration of the histone deacetylase inhibitor, valproic acid (VPA), decreases the preference for same-sex familiar and novel conspecifics during adolescence. This reduced social preference was accompanied by a downregulation of *avpr1a* in the medial prefrontal cortex during adulthood in the same animals. Here we examined the effects of VPA on the ability to form mating-induced social attachments in prairie voles, socially complex behaviors not exhibited by traditional laboratory rats and mice.

3.2 Materials and methods

In order to examine the effects of prenatal VPA exposure on partner preference formation and mating-induced aggression in adult male prairie voles, subjects were exposed to VPA at E12.5 and allowed to age to adulthood. At PND76, male prairie voles were pair-housed with ovariectomized females for 2 weeks—a paradigm known to reliably induce partner preference and selective aggression in prairie voles (K. L. Gobrogge & Wang, 2011; K. A. Young, Gobrogge, Liu, & Wang, 2011)—before a partner preference test on PND90 and resident-intruder testing at PND91 (Fig. 1B).

3.2.1 Animal subjects

Male and female prairie voles (*M. ochrogaster*) were produced from laboratory-bred colonies at Florida State University, weaned on postnatal day (PND) 21 and housed with same-sex littermates. All animals received food and water *ad libitum* and were maintained at 20 °C on a 14:10 light-dark cycle. Experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Florida State University.

3.2.2 Prenatal exposure to valproic acid

Adult (90 days old) sexually naïve female and male prairie voles were pair-housed and visually recorded for the first 3 days of cohabitation to confirm the day of mating—then considered as embryonic day 0 (E0). On gestation day 12.5, timed-pregnant female prairie voles received a single intraperitoneal (*i.p.*) dose of 600 mg/kg VPA (Sigma–Aldrich, St. Louis, MO, USA) or an equivalent volume of 0.9% saline (vehicle) and subsequently left undisturbed with their male partner and offspring until the time of pup weaning on PND21. The day and dose (600 mg/kg) of VPA was chosen as it is established to cause substantial social impairments in rats and mice (Bambini-Junior et al., 2011; Moldrich et al., 2013). Finally, the offspring of the time-mated dams were pair-housed so that treatment and sex matched between the two cagemates.

3.2.3 Parental behavior spot checks

In order to assess parental behaviors, spot checks were conducted twice daily from PND1-10 of the time-mated dams and sires, as described previously (Tabbaa et al., 2017). In the morning (0900) and late afternoon (1700), parental behaviors (*i.e.* nest occupancy, passive nursing, and

active nursing) were thus scored by five spot checks conducted with 5-min interval for a total of 10 measures per day.

3.2.4 Cohabitation and partner preference test

Immediately after cohabitation for 2 weeks (Figure 4B), the partner preference test was conducted on PND90 as previously described in a three-chamber apparatus (Duclot et al., 2016). Briefly, the male subject was placed in the central cage connected at either side to two identical cages—one containing the female used for cohabitation (termed "partner"), and the other containing a stranger female—and allowed to freely explore for 3 hours. A trained experimenter blind to the treatment groups quantified the subject's side-to-side contact duration with either the partner or stranger. Partner preference is defined as an experimental group reaching statistical significance by spending more time in side-by-side contact with the partner over the stranger.

3.2.5 Resident intruder test

On PND91, levels of selective aggression exhibited by VPA- and saline-exposed males was examined using the resident intruder test (RIT) for a period of 10 min as previously described (K. L. Gobrogge, Jia, Liu, & Wang, 2017). After removing the female partner from the home cage of the male subject, a sex-naïve male intruder was introduced and the duration and frequency of aggressive behaviors (classified as lunges, bites, and chases) as well as latency to attack were scored (K. L. Gobrogge et al., 2017).

3.2.6 Statistical analyses

All data are presented as the means ± SEM. Parental behaviors were analyzed by using a 3-way mixed ANOVA to compare the effects of sex, treatment, and postnatal day using SPSS Version 22 software (IBM Corp, Armonk, NY, USA). Body weight and weight gain were analyzed by using a 2-way mixed ANOVA to compare the effects of treatment and postnatal day. For comparisons of sex and treatment, data were analyzed by 2-way mixed ANOVA. For comparisons of sex, treatment, and stimulus preference, data was analyzed by using a 3-way mixed ANOVA. If any of these parent ANOVAs resulted in main effects or significant interactions (alpha set at 5%), Tukey's post-hoc tests were performed to determine source of significance. All molecular data and experiment 2 data were analyzed using an unpaired two-tailed *t*-test after verification of normality. Additionally, partial Eta-squared (ηp^2) and Cohen's *d* are reported as measures of effect size for ANOVA and *t*-tests, respectively.

3.3 Results

3.3.1 Exposure to VPA in utero does not alter partner preference formation or selective aggression

In light of the deficits in social affiliation and social novelty observed in response to prenatal VPA-exposure, we next investigated whether VPA-exposed prairie voles would retain their ability to form lasting social attachment, a pair-bond. To this end male prairie voles, prenatally exposed to either saline or VPA, were placed in cohabitation with a female in the presence of mating for two weeks and then tested for partner preference and selective aggression (Fig. 4B). Surprisingly, while both saline- and VPA-exposed males spent more time in side-to-side contact with the partner than with the stranger (2-way mixed ANOVA, $F_{1,19} = 165.42$, $p <$

0.0001, $\eta p^2 = 0.897$ for stimulus, $F_{1,19} = 4.28$, $p = 0.052$, $\eta p^2 = 0.184$ for treatment), VPA-exposed males spent more time huddling (or side-by-side contact) with their partner—but not their stranger—than saline-exposed males did ($F_{1,19} = 6.64$, $p = 0.018$, $\eta p^2 = 0.259$, Figure 14A). Furthermore, the total time spent in side-to-side contact with either stimulus (partner + stranger) was similar in both groups (two-tailed unpaired t-test, $t_{19} = 2.069$, $p = 0.052$, $d = 0.92$, Figure 14B), which rules out a non-specific effect of VPA on global social interaction. Altogether, these observations suggest that VPA-exposed males showed higher partner preference than saline-exposed controls. After 24 hours of respite, however, saline- and VPA-exposed males showed similar levels of selective aggression in the resident intruder test as measured by the frequency (two-tailed unpaired *t*-test, $t_{19} = 0.335$, $p = 0.7411$, $d = 0.15$) and duration ($t_{19} = 0.357$, $p = 0.7249$, $d = 0.16$) of aggressive behaviors, as well as the latency to attack ($t_{19} = 0.008$, $p = 0.9934$, $d = 0.003$) (Fig. 14C-14E).

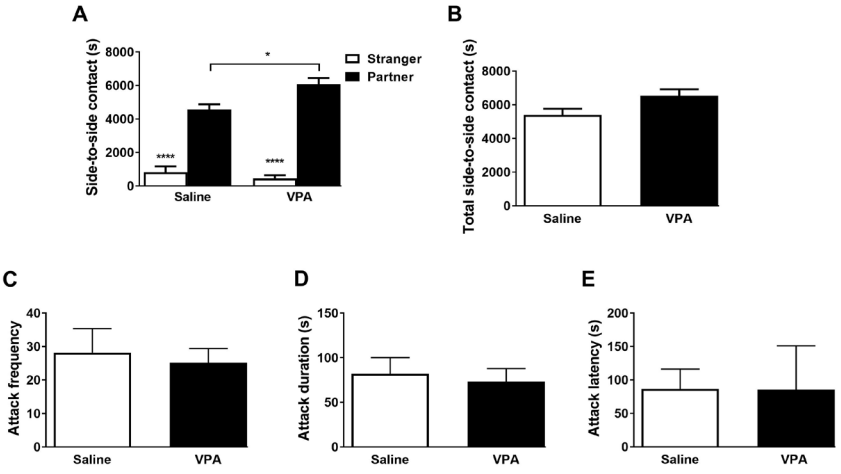

Figure 14. Prenatal VPA exposure does not prevent formation of social bond. ***A***, Saline- and VPA-treated males display a partner preference on PND90. ***B***, Total time spent interacting (stranger + partner).***C***, Saline- and VPA-treated males display similar attack frequency towards unfamiliar male conspecific during the resident intruder test on PND91, as well as attack duration (***D)*** and attack latency (***E)***. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ *vs.* saline group. n = 9-12

3.4 Discussion

Using the validated model of embryonic exposure to VPA (E12.5) (Mabunga, Gonzales, Kim, Kim, & Shin, 2015) in prairie voles, we observed a complex profile of impairments in same-sex social behaviors similar to those seen in rats and mice, thus confirming the validity of the prenatal exposure to VPA model in prairie voles. The socially monogamous nature of this species, however, allows for between-sexes investigations, and especially, the formation of an enduring social attachment referred to as a pair-bond. Interestingly, despite impaired social affiliation with a same-sex sibling, VPA-exposed male prairie voles were capable of forming a pair-bond with a female, as reflected by the presence of partner preference and selective aggression. Moreover, VPA-exposed males huddled more with their partner than saline-exposed males, which would indicate a greater partner preference than controls. Nevertheless, prenatal VPA exposure reduces social interaction with novel conspecifics and increases anxiety levels, which could translate into a reduced motivation to interact with a stranger. Although no significant reduction in side-to-side contact with the stranger was detected, this could explain why VPA-exposed males show higher levels of partner preference but not selective aggression than saline-exposed controls. It is important to note, however, that this preserved ability to form an enduring pair-bond could also suggest that the effects of prenatal VPA exposure on social abilities in prairie voles are age-dependent, and thus no longer detected in adulthood, or can be reversed by social buffering through prolonged exposure to a partner (Donovan, Liu, & Wang, 2018), a hypothesis worth examining in future studies. Our results nonetheless indicate that prenatal VPA exposure does not alter the ability of adult prairie voles to form and maintain a pair-bond, denoting a disconnection between social affiliative behaviors and the underlying processes of an enduring social attachment between sexes. Intriguingly, this is reminiscent of “high-functioning” ASD individuals, many of whom

continue to desire, initiate, and maintain sexual and romantic relationships despite experiencing difficulties with social communications and interactions (Hellemans, Colson, Verbraeken, Vermeiren, & Deboutte, 2007; Strunz et al., 2017).

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The unique social system of the monogamous prairie vole (*Microtus ochrogaster)* makes it an excellent model for investigating the neurobiological mechanisms underlying social bonding in humans. This animal model moreover highlights the importance of normal brain development as conveyed by the impact of environmental factors, such as prenatal exposure to VPA, on an individual's well-being and social development. This work has allowed us to address some major questions about prenatal VPA exposure in prairie voles: Does it alter sex-naïve social and anxiety-like behaviors? Are males and females differentially affected? How is expression of socially- and ASD-relevant genes affected? Does prenatal VPA exposure alter dendritic spine density and morphology? Does prenatal VPA alter mating-induced partner preference and selective aggression? In this work, we argue for the importance of investigating how prenatal VPA exposure alters the neurobiology underlying social behaviors in prairie voles, in an effort to further understand how environmental insults intersect normal neural and social development in humans.

Prenatal VPA exposure is associated with changes in histone methylation, with an increase in histone methylation at H3K4 and a decrease in histone methylation at H3K9 (Tung & Winn, 2010). Therefore a follow-up study is necessary to measure histone methylation at H3K9 and H3K4 at the *avpr1a* promoter within the mPFC. To determine whether prenatal VPA exposure disrupts normal development of sex-naïve social behaviors through changes in epigenetic mechanisms implicated in gene expression of vasopressin and its receptor in the mPFC, it would be important to also conduct studies that enhance V1aR in the mPFC of VPA-exposed prairie voles. Genetic variations in the vasopressin gene in men have been negatively associated to

perceived marital problems and marital status (Walum et al., 2008). Interestingly, mating in male prairie voles triggers the release of vasopressin and activation of vasopressin receptors, and consequently facilitates pair bonding with their female partners. On the other hand, inhibition of vasopressin receptors prevents the formation of a partner preference in male prairie voles (Cho et al., 1999; Donaldson et al., 2010). Furthermore, overexpression of vasopressin receptors results in the ability to form an exclusive partner preference in typically socially promiscuous meadow voles (Lim et al., 2004). Potential disturbances of the vasopressin system on social interactions have led researchers to suggest the involvement of this neurotransmitter in conditions described by social deficits like ASD.

Since we have shown that HDACi TSA facilitates partner preference formation when administered during adulthood in both female and male prairie voles (Duclot et al., 2016), we propose that social deficits in VPA-exposed subjects can be rescued by treatment with postnatal HDACi administration. In an *in utero* VPA exposure study in rats, chronic administration of the HDAC inhibitors VPA and suberoylanilide hydroxamic acid (SAHA) increased acetylation of H3K8 in the cerebellar cortex while only VPA increased acetylation at H3K14. Moreover, acetylation at these lysine residues was correlated with amelioration of social cognition and interaction deficits in the VPA-exposed rats (Foley et al., 2014). Enhancing endogenous vasopressin neurotransmission and its receptor via alterations of histone modifications may prove to be effective for improving social cognition and to facilitate social attachments in prenatally VPA-exposed prairie voles.

It was surprising to find comparable dendritic spine density and morphology in the adult mPFC of VPA- and saline-exposed prairie voles. ASD patients display dendritic spine pruning deficits and increased spine density (Tang et al., 2014), while mice and rats prenatally exposed to

VPA exhibit reduced brain weight, cortical thickness, dendritic branching, spine density, and Nissl-positive cells in the mPFC (Bringas et al., 2013; Hara et al., 2012; Mychasiuk et al., 2012). Remarkably, our VPA-exposed prairie vole model more closely resembles the social behavior and neuroanatomical deficits displayed by MeCP2-null mice. Although dendritic spine density is lower during early postnatal development in MeCP2-null mice, levels are similar to wild types during adolescence and adulthood when social deficits are well established, suggesting the involvement of a secondary compensatory mechanism (Chapleau et al., 2012). Given the MeCP2 down-regulation observed in the mPFC without alterations in spine density at adulthood, we can thus speculate that prenatal VPA exposure in prairie voles may resemble a Rett syndrome-like pattern of neuroanatomical modifications. Nevertheless, it is necessary to examine spine density and spine-subtype density at the same developmental time points during which we observed social deficits in VPA-exposed prairie voles, since MeCP2-null mice indeed possess a reduction of spine density during perinatal development but not during adulthood.

Prenatal VPA exposure in rodents alters neuronal excitability and recapitulates electrophysiological malfunctions found in ASD patients. VPA-exposed rats exhibit enhanced synaptic plasticity in the mPFC, as high frequency stimulation using field excitatory postsynaptic potential (fEPSP) recordings and contralateral stimulation produce enhanced LTP and paired pulse facilitation (Sui & Chen, 2012). However, enhanced neuronal excitability and NMDA currents may distinctly occur soon after birth as a consequence to VPA exposure and gradually restore to normal levels by early adolescence. By measuring miniature excitatory postsynaptic current (mEPSCs) from all inputs to the neuron, Walcott *et al* (2011) observed decreased spike frequency and increased NMDA/AMPA ratios in the mPFC of VPA-exposed rats during pre-adolescent development (<PND22), with differences between control and VPA neurons disappearing by

PND30 (Walcott et al., 2011). Since we did not observe differences in mPFC spine density and spine morphology on PND90, we speculate to observe normal synaptic plasticity in adolescent and adult VPA-exposed prairie voles.

Social bonding between parents and their children, peers, and romantic partners is essential for human physical and psychological health, such as facilitating our ability to cope with internal and external stressors. Pair bonds across different species are formed when an individual develops a preference for a particular conspecific over other potential mates, those pair bonded partners then continue to seek and maintain proximity to one another, experience separation stress, and act as buffers to one another against stressful events (Bales et al., 2017). Particularly, pair bonded prairie voles maintain the stability of their life-time enduring attachments by continually cohabitating and mating with their partners, while being selectively aggressive against potential mating competitors. Granted that prairie voles do not recapitulate every complex human social behavior, past studies have revealed the existence of commonly conserved mechanisms that regulate social bonding in both prairie voles and humans, mainly through the oxytocin, vasopressin, and dopamine systems. This animal model has hence allowed us to understand the molecular mechanisms that contribute to the formation and maintenance of pair bonding in humans.

Disruption of social bonding, through environmental triggers or stress-related events, can manifest as symptoms of mental disorders, such as ASD. This work supports the use of prairie voles as a model organism for dissecting how the molecular mechanisms involved in the development of social behaviors are altered in the VPA exposure model of ASD. We highlight the importance of investigating both sexes, as inclusion of males and females in rat and mouse VPA model studies is insufficient. Correspondingly to the 1:1 male to female ratio in children prenatally exposed to VPA that develop ASD (Rasalam et al., 2005), both male and female prairie voles

develop adolescent social impairments and anxiety-like behaviors in response to prenatal VPA exposure. Yet despite exhibiting sex-naïve social impairments during adolescence, adult VPA-exposed prairie voles are indeed capable of forming and maintaining pair bonds, recapitulating the interests of high-functioning individuals with ASD for initiating and maintaining romantic relationships (Strunz et al., 2017). In addition to displaying similar impairments in social interactions previously described in rats and mice, the prenatal VPA exposure in prairie voles thus allows for the specific investigation of affiliative behaviors within sexes and their disconnection with the formation of enduring social attachment between sexes.

APPENDIX A

IACUC APPROVAL

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August 27, 2018

The Graduate School
Florida State University

To Whom It May Concern:

Concerning the thesis/dissertation submitted to the Graduate School by:

Graduate Student:	**Lindsay Sailer**
Thesis/Dissertation Title:	**Consequences of prenatal exposure to valproic acid in the socially monogamous prairie voles**
Department:	**Biomedical Sciences**
Major Professor:	**Mohamed Kabbaj**

The above named graduate student has provided assurance to the FSU Animal Care and Use Committee that all animal procedures utilized in the work resulting in this thesis/dissertation are described in FSU ACUC Protocol(s):

Protocol Number	Title	Date ACUC Approval
1619	Neurochemical Regulation of Social Attachment	May 25, 2016
1620	Breeding Colony and Holding Protocol for Vole Research Protocols	May 25, 2016
1322	Neurochemical Regulation of Social Attachment	June 22, 2013
1323	Vole Breeding Colony and Holding Protocol for Vole Research Protocols	June 26, 2013

The Animal Care and Use Committee has confirmed that this student was included as a project member during the period covering their thesis/dissertation work. This institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance Number is A3854-01.

Sincerely,

Dr. Kathleen Harper
University Veterinarian
FSU Animal Care and Use Committee

KMH/kjj

APPENDIX B

LIST OF ABBREVIATIONS

ac	acetylation
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	autism spectrum disorder
AVP	vasopressin
avpr1a (or V1aR)	vasopressin 1a receptor
bdnf (or BDNF)	brain-derived neurotrophic factor
BLA	basolateral amygdala
CH3	methyl group
ChIP	chromatin immunoprecipitation
$[Cl^-]_i$	intracellular chloride ion concentration
CRS	congenital rubella syndrome
pCREB	phosphorylated cAMP response element binding protein
CNV	copy number variation
CP	caudate putamen
d	Cohen's *d*
DNMT	DNA methyltransferase
DH	dorsal hippocampus
E12.5	embryonic day 12.5
Eff%	amplification efficiency
EPM	elevated plus maze
EtOH	ethanol

fEPSP	field excitatory postsynaptic potential
FMR1	fragile X mental retardation 1 gene
G13	gestational day 13
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
H	histone
HAT	histone acetylase
HDAC	histone deacetylase
HDACi(s)	histone deacetylase inhibitor(s)
HSV-GFP	herpes simplex virus
i.p.	intraperitoneal
K	lysine residue
KCC2	K^+/Cl^- ion co-transporter
LS	lateral septum
me1	mono-methylation
me2	di-methylation
me3	tri-methylation
MeA	medial amygdala
mecp2 (or MeCP2)	methyl-CpG-binding protein 2
mEPSC	miniature excitatory postsynaptic current
mPFC	medial prefrontal cortex
NAcc	nucleus accumbens
nadh (or NADH)	nicotinamide adenine dinucleotide dehydrogenase

NKCC1	$Na^+/K^+/Cl^-$ ion co-transporter
NLGN	neuroligin
NMDA	N-methyl-D-aspartate
ηp^2	partial Eta-squared
NXN	neurexin
OB	olfactory bulb
OXT	oxytocin
oxtr (or OXTR)	oxytocin receptor
P	proline residue
PND	postnatal day
POA	preoptic area
psd95	post-synaptic density 95
PTM	post-translational modification
qPCR	quantitative polymerase chain reaction
R	arginine residue
R^2	square of Pearson correlation coefficient
S	serine residue
SAH	S-Adenosyl homocysteine
SAHA	suberoylanilide hydroxamic acid
SAM	S-Adenosyl methionine
SEM	standard error of the mean
TSA	trichostatin A
UA	unfamiliar animal

VH ventral hippocampus

VP ventral pallidum

VPA valproic acid

VTA ventral tegmental area

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Zhang, T. Y., Labonte, B., Wen, X. L., Turecki, G., & Meaney, M. J. (2013). Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. *Neuropsychopharmacology, 38*(1), 111-123. doi:10.1038/npp.2012.149

Zikopoulos, B., & Barbas, H. (2013). Altered neural connectivity in excitatory and inhibitory cortical circuits in autism. *Front Hum Neurosci, 7*, 609. doi:10.3389/fnhum.2013.00609

BIOGRAPHICAL SKETCH

EDUCATION

2006-2010 BS, Science, awarded December 2010
University of North Florida, Jacksonville, FL
Major: Biology
DIS Advisor: Radha Pyati

2013- 2018 PhD, Program in Neuroscience, expected award December 2018
Florida State University, Tallahassee, FL
Dissertation: Consequences of prenatal exposure to valproic acid in the socially monogamous prairie vole
Advisor: Dr. Mohamed Kabbaj

SPECIALIZED TRAINING PROGRAMS

2015-2016 National Enhancement of Underrepresented Academic Leaders (NEURAL) Conference; University of Alabama at Birmingham, AL
Program Directors: Dr. Farah Lubin and Dr. Lori McMahon

2017 Summer Program in Neuroscience, Excellence and Success (SPINES); Marine Biological Laboratory, Woods Hole, MA
Program Directors: Dr. Carmen Maldonado-Vlaar and Dr. Gina Poe

TEACHING AND MENTORING

2015-2017 Lecturer, Frontiers in Medicine (IHS4120), two classes every spring semester.
Supervisor: Dr. Michael Blaber
Topic: Molecular mechanisms and potential treatments for Autism Spectrum Disorder

2015-2018 Mentoring high school and undergraduate researchers in the lab: Ashlyn Molinaro, Joyce Wang, Stacey Pierre, Amber Balkcom, Rayyan Darji

2016-2017 Teaching assistant, Research Techniques (BMS5186C), one week every Fall semester.
Supervisor: Dr. Mohamed Kabbaj
Topic: Assisted in providing hands-on training on the *in situ* hybridization technique

FELLOWSHIPS, AWARDS, AND HONORS

2015-2016 NEURAL Conference Travel Award; University of Alabama at Birmingham, Birmingham, AL

2015-2017 Neuroscience Scholars Program Associate; Society for Neuroscience (SfN)

2015-2017 Biomedical Sciences Department Presentation Support Grant; Florida State University, Tallahassee, FL

2015-2018 College of Graduate Students (COGS) Conference Presentation Support Grant; Florida State University, Tallahassee, FL

2016 McKnight Mid-Year Research & Writing Conference invited speaker; Safety Harbor Resort & Spa, Safety Harbor, FL.

2017 SPINES Scholarship for tuition, travel, room and board; Marine Biological Laboratory, Woods Hole, MA

PUBLICATIONS AND PRESENTATIONS

Peer-reviewed journal publications

1. Pyati R, **Elvir LL**, Charles CE, Seenath U, Wolkow TD (2011) Imaging flow cytometry analysis of Schizosaccharomyces pombe morphology. Journal of Yeast and Fungal Research, 2: 106 – 112.

2. Heisler J, **Elvir L**, Barnouti F, Charles E, Wolkow TD, Pyati R (2014) Morphological Effects of Natural Products on Schizosaccharomyces pombe measured by imaging flow cytometry. Natural Products and Bioprospecting, 4: 27-35.

3. **Elvir L**, Duclot F, Wang Z, Kabbaj M (2017) Epigenetic Regulation of Motivated Behaviors by Histone Deacetylase Inhibitors Neuroscience & Biobehavioral Reviews, DOI: 10.1016/j.neubiorev.2017.09.030.

4. **Sailer L**, Duclot F, Wang Z, Kabbaj M. (*Submitted Scientific Reports Aug 2018*) Consequences of prenatal exposure to valproic acid in the socially monogamous prairie voles.

5. **Sailer L**, Duclot F, Wang Z, Kabbaj M. (*In prep*) Individual differences in male prairie vole sexual monogamy.

6. Duclot F, **Sailer L**, Wang Z, Kabbaj M. (*In prep*) Epigenetics of pair bonding.

Selected First Author Accepted Abstracts

1. **Elvir L**, Wang H, Duclot F, Liu Y, Wang Z, Kabbaj M (June 2015). Acute prenatal exposure to valproic acid alters social and anxiety-like behaviors in prairie voles. *NEURAL Conference, Birmingham, AL.*

2. **Elvir L**, Wang H, Duclot F, Liu Y, Wang Z, Kabbaj M (June 2016). Prenatal valproic acid exposure induces autism-related behavioral phenotypes and prefrontal cortex gene expression changes in prairie voles. *NEURAL Conference, Birmingham, AL.*

3. **Elvir L**, Wang H, Duclot F, Liu Y, Wang Z, Kabbaj M (November 2016). Physiological consequences of prenatal exposure to valproic acid in prairie voles. *Society for Neuroscience, San Diego, CA.*

4. **Elvir L**, Wang H, Duclot F, Liu Y, Wang Z, Kabbaj M (November 2017). Consequences of prenatal exposure to valproic acid in prairie vole social behaviors. *Society for Neuroscience, Washington, D.C.*

5. **Sailer L**, Wang H, Duclot F, Liu Y, Wang Z, Kabbaj M (July 2018). Consequences of prenatal exposure to valproic acid in the socially monogamous prairie vole. *International Congress of Neuroendocrinology, Toronto, ON.*

Oral presentations

1. McKnight Mid-Year Research & Writing Conference, "Acute prenatal exposure to valproic acid alters social and anxiety-like behaviors in prairie voles." February 2016, Safety Harbor Resort & Spa, Safety Harbor, FL.

2. FSU Biomedical Sciences Annual Retreat, "Physiological consequences of prenatal exposure to valproic acid in prairie voles." October 2016, Florida State University, Tallahassee, FL.

3. SPINES symposium, "Consequences of prenatal exposure to valproic acid in prairie voles." July 2017, Marine Biological Laboratory, Woods Hole, MA.

4. Biomedical Sciences Department Seminar, "Consequences of prenatal exposure to valproic acid in the socially monogamous prairie vole." June 2018, Florida State University, Tallahassee, FL.

PROFESSIONAL AFFILIATIONS

2013-Present FSU Neuroscience Graduate Student Association (NGSA)

2013-Present FSU Biomedical Sciences Graduate Student Association (BSGSA)

2014-2015 FSU NGSA Committee: Recruitment Officer

2017-2018 North Florida SfN Chapter Secretary

2017-2018 FSU College of Medicine Council on Diversity and Inclusion

OUTREACH

2014 Volunteered for the NGSA Brain Fair, for educating local and statewide K-12 students about the importance of neuroscience research

2015 NGSA Brain Fair Organizing Committee member

2016 Volunteered for the Tallahassee Science Festival, a city-wide event that involves demonstrations and hands-on wet-lab activities in a family-friendly setting

2017 NGSA Friday Neuroscience Lectures Coordinator

2018 NGSA Tallahassee Science Festival Organizing Committee member

TECHNICAL EXPERIENCE

- *Rodent handling:* injections, transcardial perfusion, gross brain dissection, rodent colony management, pup weaning, timed breeding

- *Surgical procedures:* stereotactic surgery for site-specific microinjections, vasectomy, ovariectomy, estradiol pellet priming

- *Behavioral assays:* social interaction test, partner preference test, open field, elevated plus maze, resident intruder test, parental behavior monitoring

- *Wet lab techniques:* sectioning frozen and perfused brain samples; RNA, DNA, and protein isolation with Tri-Reagent, genomic DNA isolation, rodent genotyping, western blot, RT-qPCR, chromatin immunoprecipitation, cell culture, *in situ* hybridization, confocal microscopy, immunohistochemistry, ELISA, radioimmunoassay; competent with designing shRNA oligos and RNAscope

- *Software:* proficient with GraphPad Prism, Neurolucida 360, Neurolucida Explorer, ImageJ, Bio-Rad CFX Manager, LiCor Odyssey, JWatcher, ZEISS Zen Blue, Mouse J, EndNote X8; competent with Noldus EthoVision, SPSS, R, MATLAB