The Dissertation Committee for Viktoria Yuryevna Topper Certifies that this is the approved version of the following dissertation:

Integrative analysis of endocrine-disrupting chemical effects in the developing hypothalamus: adult behaviors and neural networks

Committee:

Andrea Gore, Supervisor

David Crews

Adron Harris

Hans Hofmann

Steven Phelps

Integrative analysis of endocrine-disrupting chemical effects in the developing hypothalamus: adult behaviors and neural networks

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Viktoria Yuryevna Topper, B.S.

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For my husband, Dr. Stephen Topper

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Integrative analysis of endocrine-disrupting chemical effects in the developing hypothalamus: adult behaviors and neural networks

Viktoria Yuryevna Topper, Ph.D.

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Supervisor: Andrea C. Gore

Endocrine-disrupting chemicals (EDCs) are environmental pollutants known to perturb hormone systems and interfere with normal endocrine function. Exposure to EDCs during hormone-sensitive developmental periods can result in profound dysfunction in reproductive physiology and behavior. In this dissertation, effects of gestational exposure to a class of EDCs called polychlorinated biphenyls (PCBs) were examined in the developing hypothalamus, which is known to control reproductive physiology and behavior in vertebrates. The specific hypothesis was that PCBs caused changes in sexually dimorphic hypothalamic nuclei, resulting in perturbation of adult sociosexual behaviors and alteration of neural networks with changes in expression of microRNAs and genes during development and in adulthood.

This research focused on two brain areas relevant to understanding the PCB effects on the developing hypothalamus: 1) microRNA and related target gene expression during postnatal development, 2) adult sociosexual behaviors and gene expression. In both sections, molecular changes were examined in two sexually dimorphic hypothalamic

nuclei, medial preoptic nucleus (MPN) and ventromedial nucleus (VMN), known for their role in regulation of sociosexual behavior. In the first section of the dissertation, the effects of PCBs were examined on the expression of microRNAs and target genes at four ages during postnatal [P] development (P15, P30, P45, and P90). Age and sex specific effects were observed in both MPN and VMN, with greater effects in the MPN. The second research section of the dissertation explored whether sociosexual behaviors, namely ultrasonic vocalizations and sociosexual preference behaviors, were altered by gestational PCBs. Expression of forty-eight neuroendocrine candidate genes was also examined in the MPN and VMN of the same animals. Several sociosexual behaviors were affected, including number and acoustic properties of ultrasonic vocalizations, and nose-touching with opposite-sex animals. Gene expression was altered in sex and region-specific manner in the brains of behaviorally affected rats. Taken together, these findings suggest that gestational PCBs have lasting effects on molecular mechanisms during postnatal development and in adulthood, and could result in altered sociosexual behavior. These results have implications for human health and disease, as early life exposures to EDCs have been linked to reproductive decline in humans.

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Chapter 1: General introduction

Background on reproductive physiology

Sex differences in reproductive physiology and behavior are critical to the survival of vertebrate animals. Reproduction is controlled by the hypothalamic-pituitary-gonadal [HPG] axis, and sexual dimorphism is apparent at all levels of the system. The HPG axis includes finely controlled hypothalamic signals to the anterior pituitary, which in turn sends hormonal stimuli to the gonads, ovaries in females, or testes in males (Gore, 2010). The hypothalamus, located at the most rostral region of the brainstem, is a key neural center that regulates homeostatic functions in the body, integrates internal and external stimuli in the environment, and orchestrates the energetically costly process of reproduction. The gonadotropin-releasing hormone [GnRH] neuron bodies are located in hypothalamic preoptic area [POA] and mediobasal hypothalamus, with their axons extending to the median eminence at the base of the hypothalamus (Yin *et al.*, 2010). The GnRH peptide is released from the axonal neuroterminals in the median eminence into the portal capillary system in a pulsatile manner. The release of a neurotransmitter peptide directly into the bloodstream is a defining feature of neuroendocrine cells; thus the GnRH peptide can be also appropriately referred to as a hormone or neurohormone.

In the bloodstream, GnRH is transported to the anterior pituitary, where it acts upon its receptors to cause a similarly pulsatile release of pituitary gonadotropins: luteinizing hormone [LH] and follicle-stimulating hormone [FSH]. These hormones are released into the general circulation, and exert their effects on the gonads to drive

steroidogenesis and gametogenesis. Gonadal steroids [estrogen, testosterone, and progesterone], also known as sex steroid hormones, then act upon their respective steroid hormone receptors that are widely and heterogeneously expressed in reproductive and non-reproductive target tissues, including reproductive tract, genitalia, breast, bone, fat, muscle, kidney, liver, and many other organ systems (Takeda *et al.*, 1990; Kuiper *et al.*, 1997; Uotinen *et al.*, 1999). Steroid hormone receptors are also abundant in the central nervous system and pituitary gland, which enables gonadal hormones to exert feedback on the hypothalamic and pituitary levels of the HPG axis (Chakraborty *et al.*, 2003).

In the male brain, steroid hormone feedback involves negative feedback from testosterone and estradiol (Gooren, 1989). In females, the ovarian hormones estradiol and progesterone exert mostly negative feedback on the hypothalamic and pituitary hormone release. However, during the mid- to late follicular stage in primates, or on the day of proestrus in rats with a four-to-five-day estrous cycle, estradiol stimulates the release of GnRH, with the subsequent surge in LH, which causes ovulation (Gore, 2010). Interestingly, GnRH neurons themselves do not express most of the steroid hormone receptors; instead, negative feedback occurs via indirect effects of hormones on interneurons and glial cells that are abundant in estrogen [ER], progesterone [PR], and androgen receptors [AR] and support the GnRH neuron function (Herbison, 2008).

Hormonal and sex differences in the hypothalamus

During development, the brain is organized by gonadal steroids during the critical period of sexual differentiation from late embryonic to early postnatal development (Phoenix *et al.*, 1959; Barraclough, 1961). The concept of this critical period was first suggested by Phoenix *et al.* in 1959 and has since been extensively studied. Briefly, in male mammals, the developing embryonic testes produce testosterone (Forest, 1975), which is released into fetal general circulation, masculinizing reproductive and non-reproductive tissues. In the brain, this masculinization is achieved through high levels of the aromatase enzyme, which converts fetal testosterone to estradiol (Roselli *et al.*, 1985). Estradiol binds to estrogen receptors and activates the signaling cascades responsible for masculinization and defeminization of the developing male brain (Nordeen *et al.*, 1983; Kudwa *et al.*, 2005). Conversely, in the developing females, the ovary produces relatively low amounts of sex steroid hormones, resulting in brain feminization and demasculinization. These organizational effects of hormones on the male and female developing brains are necessary for the manifestation of sexually dimorphic adult physiology and behavior.

Although many parts of the nervous system are sexually dimorphic in structure and function, the most well-studied component is the hypothalamus. For example, the anteroventral periventricular nucleus [AVPV], which provides input to GnRH neurons and is important for the female preovulatory GnRH/LH surge, is approximately two times larger in volume, cell number, and density in adult females than in males (Bleier *et al.*, 1982; Sumida *et al.*, 1993). Conversely, the volumes of the sexually dimorphic nucleus of

the preoptic area [POA] and the medial preoptic nucleus [MPN] are two to four times higher in male rats than in the female rats (Gorski *et al.*, 1978; Gorski *et al.*, 1980). These regions are involved in regulating masculine and feminine sexual physiology and behavior [POA, MPN] (Davis *et al.*, 1996). Beyond the hypothalamus, the spinal nucleus of the bulbocavernosis [SNB] and bed nucleus of stria terminalis [BNST] are both larger in males than in females (Nordeen *et al.*, 1985; Chung *et al.*, 2000). In males, the SNB is involved in maintaining the penile erection, while females lack the bulbocavernousus muscle that the SNB innervates, thus this region is virtually absent and plays minimal physiological role in females (Breedlove, 1986; Johansen *et al.*, 2004). The BNST is involved in masculine and feminine reproductive physiology and affective behavioral pathways (Simerly, 2002). Thus, many regions of the rodent nervous system are sexually dimorphic in size and structure, due to the effects of sex hormones on the developing brain.

While morphological differences in brain regions are part of sexual differentiation of the brain, more importantly, the neurochemistry and expression of neuropeptides, neurotransmitters, and receptors exhibit a number of sex differences. This dissertation will discuss a subset of such targets: steroid hormone receptors [*Ar,* androgen receptor; *Esr1,* estrogen receptor α; *Esr2,* estrogen receptor β], kisspeptin [*Kiss1*], and social behavior genes [*Oxt,* oxytocin; *Oxtr,* oxytocin receptor; *Avp,* vasopressin*; Avpr1a,* vasopressin receptor 1a], specifically in rats and mice.

A variety of immunohistochemical studies have shown that there is sexual dimorphism in the distribution pattern of neuronal cell bodies and fibers containing *Ar,*

Esr1, Esr2 and *Kiss1* in the hypothalamus (Herbison *et al.*, 1993a; Herbison *et al.*, 1993b; Dickerson *et al.*, 2011a). Furthermore, *in situ* hybridization histochemical studies have revealed sex differences in mRNA expression of the same signaling receptors in the hypothalamus (Simerly *et al.*, 1990; Chakraborty *et al.*, 2010). In adult females, *Esr1, Esr2,* and *Kiss1* are more abundant in the medial basal hypothalamus and preoptic area than in adult males; whereas *Ar* expression is higher in adult male hypothalamus than in adult females (Lauber *et al.*, 1991; Herbison *et al.*, 1993a; Imamura, 2011; Walker *et al.*, 2012). Additionally, *Kiss1* gene and kisspeptin protein expression levels in the anteroventral periventricular nucleus [AVPV] of the hypothalamus are sexually differentiated, with adult females displaying higher levels than males (Kauffman, 2009).

The neuropeptides and their receptors involved in social and sociosexual behaviors are, not surprisingly, sexually dimorphic in the brain. Within and beyond hypothalamus, *Oxtr, Avp, and Avpr1a* gene expression is higher in males than in females in ventromedial hypothalamus [*Oxtr*], bed nucleus of stria terminalis [*Avp*], and medial amygdala [*Oxtr, Avp, Avpr1a*] (de Vries *et al.*, 1984; Bale *et al.*, 1995; Ferguson *et al.*, 2001). Oxytocin gene expression was examined but found not to be sexually dimorphic in paraventricular nucleus of the hypothalamus, among other regions (Nomura *et al.*, 2002).

Sexually dimorphic behaviors and hormonal influences

Sex differences in brain morphology and steroid hormone levels are necessary for the manifestation of sex-appropriate behaviors, including copulatory and social behaviors. In male rodents, commonly analyzed copulatory behaviors include mounting females, penile intromissions, and ejaculations. Measurements of feminine copulatory behavior include proceptive [hops, darts, ear wiggling] and receptive [lordosis] behaviors. The brain regions controlling these behaviors in both sexes are almost entirely overlapping and include medial amygdala, BNST, POA, and ventromedial nucleus [VMN], as well as other midbrain, hypothalamic, and forebrain regions (Malsbury *et al.*, 1977; Mathews *et al.*, 1977; Clark *et al.*, 1981; Kato *et al.*, 2000; Hull *et al.*, 2006). It is possible that within these regions, the differences in steroid hormone receptors, neurotransmitters, neuron numbers, and phenotypes result in sex differences in behavior. Additionally, sex-dependent levels of estradiol, progesterone, and testosterone may exert sex-typical effects on the HPG axis, leading to the manifestation of male-like and female-like behaviors (Crews, 2012).

Male copulatory behaviors

The importance of testosterone in regulating male-typical sexual behavior is shown by castration studies. Castration caused a decrease in serum testosterone levels and a corresponding decrease of sexual behavior, while administration of exogenous androgen reinstated the mounting behavior to pre-gonadectomy levels (Davidson, 1966; McGinnis *et al.*, 1989). Although testosterone is the most abundant sex steroid hormone

in males, administration of its aromatization product, estradiol, has also been shown to elicit male-typical behaviors in gonadectomized rats and quails (Clancy *et al.*, 2000; Ball *et al.*, 2004). Progesterone has been similarly successful in reversing the effects of castration on the sexual behavior of gonadectomized sexually naïve male rats (Wagner, 2006). Surprisingly, administration of the exogenous non-aromatizable androgen dihydrotestosterone does not reinstate sexual behavior in gonadectomized male rats and quails, but does so in guinea pigs and whiptail lizards (Alsum *et al.*, 1974; Wade *et al.*, 1993).

Sexual behavior in male rodents is not merely a simple effect of hormones but requires integration of sensory, integrative, and motor neural pathways. During a sociosexual encounter, olfactory information about an opposite-sex partner is carried to the olfactory bulb for interpretation. It gets further processed in the medial amygdala, the BNST, and the hippocampus, which in turn send projections to the POA, the central site of the sensory input integration in males (Kato *et al.*, 2000; Hull *et al.*, 2006). Efferent projections from the POA coordinate the CNS response by sending projections to the medial prefrontal cortex [mPFC], nucleus accumbens [NAc], as well as the VMN that are known to regulate the performance of motivated behaviors and help orchestrate the act of copulation (Malsbury *et al.*, 1977; Mathews *et al.*, 1977; Clark *et al.*, 1981; Balfour *et al.*, 2004; Bell *et al.*, 2009). The CNS input from the POA and the sensory input from the male genitalia is integrated in the brainstem nuclei, which send efferent projection to the SNB region, effecting a sensory-motor response pattern that results in penile erection and male-typical mounting behavior.

The importance of sex hormone signaling in the POA is highlighted by lesion studies, which inhibit male-typical sexual behavior in all vertebrate males (Paredes, 2003). Furthermore, c-fos studies have shown that POA is activated in response to sociosexual stimuli, such as presence of a female conspecific or following exposure to an odor from an estrous female (Heeb *et al.*, 1996; Neal *et al.*, 2007). Expression of steroid hormone receptors [*Ar* and *Er*] in the POA mediates effects of these hormones on male-typical behaviors. Additionally, intracranial implantation of androgens into the POA elicits mounting behavior in gonadectomized males (Fisher, 1956; Morgantaler *et al.*, 1978).

Female copulatory behaviors

In females, estrogen and progesterone are fundamental to the expression of copulatory behaviors. Increases in estradiol and progesterone during the follicular phase of the estrous cycle cause positive feedback on the HPG axis, resulting in ovulation and period of maximum receptivity, called behavioral estrus in rodents (Feder, 1981). In ovariectomized females, this positive feedback is abolished, as is ovulation and estrus. Administration of exogenous estradiol and progesterone to ovariectomized females in a pattern that mimics the natural production of hormones, reverses the effects of ovariectomy on sexual behavior and expression of estrogen and progesterone receptors in the relevant behavioral brain regions (Pfaff *et al.*, 1994).

The neural circuitry underlying female copulatory behaviors involve sensory, integrative, and motor neural pathways, similar to males. Sensory input from the body

flanks and genital region in females is carried to the motor nuclei in the brainstem and is then transmitted to hypothalamic, midbrain, and forebrain regions (Pfaff *et al.*, 1994). Integration of this input occurs in the ventromedial nucleus of the hypothalamus [VMN], which controls lordosis behavior (Malsbury *et al.*, 1977; Mathews *et al.*, 1977; Clark *et al.*, 1981). The afferent output from the VMN relays the information to the mPFC, NAc, and POA, as well as the motor control centers in the brainstem and spinal cord to coordinate the spinal response of dorsiflexion (Balfour *et al.*, 2004; Bell *et al.*, 2009).

Mate choice behaviors

Beyond the mechanics of copulation, another critical aspect of sociosexual interactions is the process of finding and evaluating a mate. Females must both perceive and evaluate male cues prior to reaching a mating decision. It is easy to envision that mate choice behaviors rest on species-specific exchange of communication signals, used to enable an individual to evaluate another as a potential mate. In some species, males are known to "advertise" their reproductive fitness through extravagant mating rituals, to influence the choice of receptive females (Andersson, 1994). Other cues, including auditory and even environmental cues have been shown to influence female mate choice (Westneat *et al.*, 2000). The organizational and activational effects of hormones influence female mate preference and selectivity (Wilczynski *et al.*, 2010). Developmental studies have shown that prenatal treatment with androgens and estrogens alters the mate preference for an opposite-sex partner, and results in abnormal mate choices (Matochik *et al.*, 1992). Similarly, gonadectomy studies have established the dependence of mate

preference behaviors on proper hormone levels during puberty and in adulthoo. Studies using neural c-fos activation in a sociosexual context to determine the neural circuitry underlying female mate choice behaviors have identified forebrain, midbrain, and hypothalamic nuclei, similar to the brain regions regulating masculine and feminine copulatory behaviors (Gentner *et al.*, 2001; Hoke *et al.*, 2004; Woolley *et al.*, 2008).

Production of ultrasonic vocalizations

Acoustic modalities are central in female choice and male mating displays for many species (Emerson, 2001; Collins, 2004). Rats vocalize in the ultrasonic range [22kHz to about 80kHz], which is above the human hearing capacity [20Hz to 20kHz], thus they remain mostly undetected by human observers. Two types of vocalizations are emitted by adult Sprague-Dawley rats, categorized by the general frequency of the calls as low [22-kHz] and high frequency [50+-kHz] ultrasonic vocalizations [USVs] (Brudzynski, 2001; Brudzynski *et al.*, 2002). Evidence from behavioral and pharmacological studies suggests that USVs vary depending on social situations and experience, and may represent distinct affective states of the vocalizing subject. Adult rats emit low-frequency 22-kHz USV when exposed to predators (Blanchard *et al.*, 1991), or other aversive stimuli, such as unescapable foot-shocks (Vivian *et al.*, 1993; Antoniadis *et al.*, 1999; Wohr *et al.*, 2005; Borta *et al.*, 2006). While 22kHz USVs are important in certain social situations as described above, I will focus my review on 50kHZ USVs, which are relevant to my project.

50kHZ USVs may serve a communicative function in rodents. They are emitted by rodents after removal of their partner from the home cage, or after a rat is separated and placed into a new clean cage (Wohr *et al.*, 2008). Thus, the USV calls are thought to establish, re-establish, or maintain social contact. 50kHz USVs also occur in affective social contexts, including but not limited to juvenile play (Knutson *et al.*, 1998), tickling (Burgdorf *et al.*, 2001), social exploratory activity (Brudzynski *et al.*, 2002), and mating behavior (McGinnis *et al.*, 2003). Since 50-kHz USVs are also expressed during anticipation of copulation (Bialy *et al.*, 2000), play (Knutson *et al.*, 1998), and food (Panksepp *et al.*, 2000), it has been suggested that these calls are a sensitive marker for reward states and could reflect a positive affective state (Knutson *et al.*, 1999; Panksepp *et al.*).

Sex steroid hormones are known to influence the production of 50kHz USVs (Ball *et al.*, 2003; Moore *et al.*, 2005; Bass *et al.*, 2008). For example, testosterone affects vocalizations through actions on behaviorally relevant brain areas [motivational brain regions and central vocal motor nuclei] (Yamaguchi, 2002) or through actions on peripheral organs involved in USV production [larynx and syrinx] (Taylor, 2010; Pasch *et al.*, 2011). Thus, it is not surprising that numerous sex differences in USV production exist. In mongolian gerbils and prairie voles, adult males vocalize more than females (Brown *et al.*, 1988; Ma *et al.*, 2014). In another study, male golden hamsters were found to produce a higher number of complex, frequency-modulated USV calls compared to the females. Entropy of the calls [measure of how energy is dispersed over the frequency] was lower in males than in female golden hamsters (Fernandez-Vargas *et al.*, 2015). In

the same animals, interquartile bandwidth similarly showed lower measures in males than in females (Fernandez-Vargas *et al.*, 2015).

MicroRNAs and sexually dimorphic brain development

MicroRNAs are small non-coding RNA molecules that exert their effects on gene expression by binding to messenger RNA [mRNA], leading to degradation of the mRNA molecule or inhibition of protein translation (Kim *et al.*, 2009). The identification of the first microRNA was done using forward genetics assays in *C. elegans*, which uncovered a novel, non-coding RNA molecule that mediated the temporal pattern of body formation in the worm (Lee *et al.*, 1993; Wightman *et al.*, 1993). Initially believed to be a finding confined to worms, the identification of phylogenetically conserved *let-7* microRNA in rodents spurred an interest in the field of small regulatory RNAs (Reinhart *et al.*, 2000). Interestingly, the sexually dimorphic nature of microRNAs was obvious from the initial studies. In *C. elegans*, *let-7* is required for worm viability during development, and its absence was noted to be lethal [LEThal-7], with females dying at L3 larval stage [early developmental stage] and males dying at L4 [later developmental stage]. In the 20 years following the identification of the first microRNA, more than 2000 microRNAs have been identified in mammalian species, regulating nearly all essential cellular processes in development and disease (Olive *et al.*, 2015).

In the brain, microRNAs are involved in numerous processes, including immune responses, apoptosis, and cancer (Kim *et al.*, 2008). They were found to be important in neurodevelopment (Conaco *et al.*, 2006; Tan *et al.*, 2012), circadian signaling [e.g. *mir-*

132, mir-219] (Cheng *et al.*, 2007), and neurotransmitter and growth factor regulation [e.g. *mir-7*] (Kocerha *et al.*, 2009; Miller *et al.*, 2012; Sakai *et al.*, 2013). The expression of some microRNAs is hormone-sensitive, and microRNAs in turn influence the expression of genes involved in mediating hormone responses (Klinge, 2009; Rao *et al.*, 2013). For example, estradiol treatment altered the microRNA profiles in breast cancer cell lines (Klinge, 2009) and in select brain areas in aging rat females (Rao *et al.*, 2013).

Many microRNAs display sexually dimorphic expression. Studies in fish (Bizuayehu *et al.*, 2012), neonatal and adult rodent brains (Olsen *et al.*, 2009; Morgan *et al.*, 2012; Murphy *et al.*, 2014), and post-mortem examinations of human brains (Mellios *et al.*, 2012) have revealed sex-dependent differences in expression of individual microRNAs. For example, in the brains of the teleost fish *Hippoglossus hippoglossus* [Atlantic halibut], *mir-451* and *mir-9* were higher and lower, respectively in 3-year-old females compared to the males (Bizuayehu *et al.*, 2012). Morgan and Bale (Morgan *et al.*, 2012) demonstrated that seven microRNAs were sexually dimorphic in the whole brains of neonatal rats on postnatal [P] day 0 [day of birth]. Importantly, a single prenatal injection of aromatase inhibitor, which inhibits the conversion of testosterone to estradiol, feminized microRNA expression in the newborn males on P0. This suggests that some microRNAs in the brain affect or are affected by genes regulating the process of sexual differentiation. Furthermore, a number of hypothalamic microRNAs are expressed in a sexually dimorphic manner during development and are involved in mechanisms permitting or leading to puberty onset [e.g., *lin28/let-7* family] (Sangiao-Alvarellos *et al.*, 2013). Finally, studies of post-mortem brains have identified *mir-30b*, which is estrogen

responsive, at significantly lower levels in females compared to males (Mellios *et al.*, 2012).

Endocrine-disrupting chemicals (EDCs)

Endocrine-disrupting chemicals (EDCs) are defined as "exogenous chemicals, or mixture of chemicals, that interferes with any aspect of hormone action" (Zoeller *et al.*, 2012). Many effects of EDCs are mediated by hormone receptors such as estrogen receptors, androgen receptors, thyroid receptors, and others, which are widely distributed in the brain, and thereby perturb endocrine and neurobiological systems. While the majority of EDCs tested have estrogenic properties, EDCs can also be anti-estrogenic, androgenic, anti-androgenic, or anti-thyroidogenic (Kelce *et al.*, 1997; Schantz *et al.*, 2001; Zoeller *et al.*, 2005). EDCs may also act via membrane steroid hormone receptors (Tokumoto *et al.*, 2007) and thereby act via non-genomic mechanisms. EDCs have been also known to affect more global changes in steroid synthesis, metabolism, and degradation by influencing the expression of steroidogenic enzymes (Colciago *et al.*, 2009). Furthermore, EDCs can have neurotoxic effects on neuroendocrine physiology via modulation of neurotransmitter systems (Corey *et al.*, 1996; Morse *et al.*, 1996; Seegal *et al.*, 2005). Beyond their effects on the reproductive and thyroid axis (Zoeller *et al.*, 2005; Dickerson *et al.*, 2007), EDCs can also affect hormone signaling in stress [hypothalamic-pituitary-adrenal axis], growth [somatotropic axis], lactotrophic axis, and neurotransmitter signaling of neural and metabolic peptides (Schantz *et al.*, 2001; Scarth, 2006).

Polychlorinated biphenyls (PCBs) and their effects in the brain

PCBs are a class of industrial chemicals and known EDCs that were mass-produced in the United States for 40 years but eventually banned in the late-1970s for their toxic effects. Initially these chemicals were employed mostly in encapsulation of closed-system apparatuses such as transformers and capacitors (Carpenter, 1998). However, their stable chemical compositions and the ability of chemists to alter the structure-function relationship by varying their degree of chlorination led to the wide use of PCBs as industrial lubricants, adhesives, and sealants (Davila, 1993). Structurally, PCBs can be divided into two groups: coplanar and noncoplanar. The structural differences dictate functional effects of the PCBs, with the coplanar PCBs acting mostly at the steroid hormone receptors, and noncoplanar PCBs affecting neurotransmitter regulation (Corey *et al.*, 1996; Morse *et al.*, 1996; Schantz *et al.*, 2001; Seegal *et al.*, 2005); however the distinction is not absolute. Some coplanar PCBs can also bind aryl hydrocarbon receptor, inducing dioxin-like effects (Denison *et al.*, 2003). The degree of chlorination of the PCB compounds also dictates their metabolism rate and exposure routes. Heavily chlorinated PCBs have a longer half-life both in the environment and within the living organisms, while lightly chlorinated PCBs are readily metabolized and do not bioaccumulate (Platonow *et al.*, 1975; Hermanson, 1989). Humans and wildlife are exposed to heavily chlorinated PCBs via ingestion of contaminated water and foodstuffs, primarily fish and meats, while lightly chlorinated PCBs are frequently aerosolized and thus exposures occur through inhalation or ingestion after concentration in plants.

This dissertation will focus on the PCB mixture Aroclor 1221 [A1221], a reconstituted mixture of lightly chlorinated PCB congeners that has a relatively short half-life, and is weakly estrogenic (Dickerson *et al.*, 2007). This compound has been previously shown to have effects on rodent reproductive physiology and behavior following prenatal exposure. There are many other EDCs, such as organochlorine compounds, bisphenol A, and pesticides, which share some mechanistic features with PCBs, but are beyond the scope of this dissertation.

PCBs and the developing hypothalamus

Several studies have investigated the effects of gestational PCB exposure on brain sexual differentiation, highlighting the importance of the critical period of brain development. Gestational exposures to PCBs disrupted expression of steroid hormone receptors, aromatase activity, and neurotransmitter systems in age-, sex-, and PCB-specific manners. Estrogen and progesterone receptors are sensitive targets of gestational PCB exposures and may contribute to their effects on brain development. For example, gestational exposure to Aroclor 1254 [A1254] increased ERα [estrogen receptor α] and decreased PR [progesterone receptor] gene expression in the VMN of embryonic [E20] female rats (Lichtensteiger, 2003). Aromatase activity has also been investigated in many of the developmental PCB studies. Gestational exposure has been shown to downregulate aromatase protein expression and activity in the POA of neonatal [P0] male rats. That same study also demonstrated an effect of PCBs at the estrogen receptors, which contributed to a hormonal imbalance and resulted in altered brain development (Hany *et*

al., 1999). However, a separate study demonstrated an increase in aromatase gene expression at P21 in males exposed to PCBs during prenatal and postnatal development [from embryonic day 15 to postnatal day 21] (Colciago *et al.*, 2009). Thus, when considering effects of gestational PCBs on gene and protein expression, age at analysis is important. The sex of the examined individual is equally important. Short-term gestational exposure to A1254 decreased AR protein expression in the hypothalamus of embryonic female but not male rats at E20 (Colciago *et al.*, 2006). Other heavily chlorinated PCB congeners have also been shown to affect neurotransmitter regulation, including serotonergic, dopaminergic, and cholinergic systems at the gene and protein expression levels in a sex-specific manner (Corey *et al.*, 1996; Morse *et al.*, 1996; Seegal *et al.*, 2005).

Studies of prenatal exposure to A1221 demonstrated decreased numbers of ERα-positive cells in anteroventral perioventricular nucleus [AVPV] in adult female rats at postnatal [P] day 60. In the POA of the same females, gestational exposure to A1221 decreased expression of androgen receptor, insulin-like growth factor 1, NMDA receptor subunit NR2b, and transforming growth factor β1, among others (Dickerson *et al.*, 2011b). In another study, A1221-treated adult females had increased expression of kisspeptin, ERα, and prodynorphin genes in the AVPV at P90 (Walker *et al.*, 2014). As a whole, the studies in the Gore lab and others suggest that gestational exposures to PCBs disrupt the normal pattern of gene and protein expression in the developing brain.

PCB effects on adult behaviors

PCB exposures during development have been associated with changes in adult behaviors related to social and sexual interactions. Gestational exposure to A1221 disrupted sexual and social behavior in adult rats. This was manifested as an increase in time to mate in paced mating experiments in exposed females (Steinberg *et al.*, 2007), and by decreased time spent nose-touching with same-sex gonadecomized males in sociability paradigm in exposed males (Reilly, under review). Exposure to other PCBs has shown similar effects on reproductive and social behaviors in rats. For instance, female rats that were gestationally exposed to PCBs showed depressed sexual receptivity (Wang *et al.*, 2002) and altered timing of mating events (Chung *et al.*, 2001). In another study, juvenile as well as adult social behaviors were altered in gestational PCB-exposed rats (Jolous-Jamshidi *et al.*, 2010). Cummings *et al* demonstrated that females exposed to PCBs shortly after birth had decreased interest in males in a partner preference test (Cummings *et al.*, 2008). Furthermore, maternal behaviors in rodents and play behaviors in humans and rodents were disrupted by gestational PCB exposures (Cummings *et al.*, 2005; Bell, communication). Additionally, PCBs affected cognitive development (Stewart *et al.*, 2003), hyperactivity-associated behaviors (Neugebauer *et al.*, 2015), and taste preference (Kaya *et al.*, 2002). In summary, there is a robust literature for developmental effects of PCB exposures on a variety of behaviors, which may be due to PCB effects on developing neural networks.

PCBs and other EDC effects on microRNA expression

The effects of EDCs on microRNAs is a burgeoning field that includes studies on several tissue and cell types, but there has been little research on the nervous system and the developing brain. Some work has been conducted in cell lines on the EDC bisphenol A [BPA]. In *in vitro* immortalized cytotrophoblast placental cell lines, BPA strongly induced *mir-146a* expression (Avissar-Whiting *et al.*, 2010), and in human MCF-7 breast cancer cell line, BPA treatment altered the expression of multiple microRNAs, including *mir-21* (Tilghman *et al.*, 2012). Effects of another estrogenic EDC, nonylphenol, were investigated in mouse Sertoli cell lines in which a number of microRNAs were up- or down-regulated (Choi *et al.*, 2011). In animal studies, oral BPA given to adult male rats for two months caused alterations in the transcriptional profiles of mRNA and microRNA expression within the penile shafts and resulted in a moderate corporal veno-occlusive erectile dysfunction (Kovanecz *et al.*, 2014).

Relatively little work has been published on EDCs and microRNAs in the brain, either *in vitro* or *in vivo*. Lesiak *et al* investigated the effects of PCB 95 on microRNA expression in primary dissociated rat hippocampal cultures and found that neurotoxic effects of PCB 95 were mediated via *mir-132* upregulation, which in turn suppressed the translation of p250GAP, a negative regulator of synaptogenesis (Lesiak *et al.*, 2014). Oral RDX, a common environmental contaminant, fed to adult mice for 28 days altered microRNA expression in the liver and brain (Zhang *et al.*, 2009). These studies point to the susceptibility of microRNA signaling to endocrine disruption.

Summary and goals of this dissertation

Taken together, these data emphasize the importance of sexual dimorphisms in reproductive physiology and outline their sensitivity to endocrine disruption. A few global conclusions can be made. First, low-dose EDC exposures during the sensitive perinatal developmental periods underlie the field the developmental [fetal] basis of adult disease and will be the focus of this dissertation. This concept includes a potentially long latency from exposure to disease or dysfunction. Second, the examination of sexually dimorphic endpoints provides a sensitive framework for discerning the organizational effects of hormones and EDCs during development on microRNAs, genes, and adult behaviors in both sexes. Finally, and of particular relevance to humans, due to the reality of world-wide contamination, humans are primarily exposed to non-occupational low-level mixtures of various chemicals. Thus, this dissertation will focus on gestational exposure to low-levels PCBs, and analyze sexually dimorphic endpoints that are related to brain sexual differentiation.

In my dissertation, the effects of A1221 are investigated during the perinatal period when levels of endogenous hormones vary precipitously and are sex-specific, and the developing fetus exhibits heightened sensitivity to even slight changes in the hormonal milieu. Therefore, introduction of exogenous hormone mimics such as A1221 allows for the investigation of perturbation of hormone-dependent brain sexual differentiation and its influence on permanent organizational effects on the developing neuroendocrine system, affecting physiology and behavior in adulthood.

Chapter 2 examines the effects of sex and age on the expression of hormone-sensitive hypothalamic microRNAs during postnatal development and investigates the effects of endocrine disruption on microRNA expression. This work is the first developmental profile of hormone-sensitive microRNAs in two hypothalamic nuclei, the medial preoptic nucleus and ventromedial nucleus of the hypothalamus. Additionally, to my knowledge, there are no published studies that have examined the effects of gestational EDCs on microRNA expression.

Chapter 3 investigates the effects of gestational PCB exposure on sexually-dimorphic adult behaviors and gene expression relevant brain nuclei. Two sexually dimorphic adult behaviors examined are the production of ultrasonic vocalizations and sociosexual preference behaviors. The focus of the molecular work in Chapter II is the examination of gene expression changes in two sexually dimorphic hypothalamic brain regions, the MPN and VMN, known for their involvement in reproductive physiology and behavior. Relationships between behaviors and gene expression are also determined.

In summary, the effects of PCBs on the developing HPG axis range from disruptions of neural differentiation of sexually dimorphic brain regions, to alterations in gene and protein expression, steroidogenic enzymatic activity, serum hormone levels, to interference with adult reproductive behaviors. From the studies described herein, it can be concluded that the developing neuroendocrine system constitutes a sensitive target of endocrine disruption. This dissertation will provide novel insight into the mechanisms by which PCBs cause neuroendocrine dysfunction.

Chapter 2: Sexually dimorphic effects of gestational endocrine-disrupting chemicals on microRNA expression in the developing rat hypothalamus

Abstract

MicroRNAs are small non-coding RNAs involved in post-transcriptional regulation of gene expression. This study had two goals: first, to examine expression of eight microRNAs in two hypothalamic regions for developmental change and possible sexual dimorphisms, and second, to investigate whether low-level gestational exposures to environmental endocrine-disrupting chemicals [EDCs] altered these expression patterns. Pregnant Sprague-Dawley rats were injected on gestational days 16 and 18 with vehicle [DMSO], estradiol benzoate [EB, 50 μg/kg], or a weakly estrogenic mixture of polychlorinated biphenyls [A1221, 1 mg/kg]. Pups were born and littermates were euthanized on postnatal days [P] 15, 30, 45, or 90. The medial preoptic nucleus [MPN] and ventromedial nucleus [VMN] of the hypothalamus were assayed for expression of selected microRNAs [*let-7a, let-7b, mir-124a, mir-132, mir-145, mir-219, mir-7, mir-9*]. MicroRNAs showed robust developmental changes in both regions, and most were sexually dimorphic in the MPN, but not the VMN. In the MPN, EDC effects on microRNA expression were sex and age-specific. In females, most microRNAs had higher expression levels in PCB- and EB-exposed animals compared to vehicle during the pubertal transition [P30]. In males, microRNA levels were lower in PCB- than vehicle-exposed groups in adulthood [P90]. There were fewer effects of treatment in the

VMN. *In silico* target prediction analysis was used to select mRNA targets of the affected microRNAs for gene expression analysis by qPCR [*Ar, Pgr, Ppara, Igf1r, Grin2a*, *Clock*, *Lin28b, Lepr*]. Modest effects of treatment were found but no clear relationship with microRNAs was identified. In summary, hypothalamic microRNAs are sexually dimorphic and regulated by EDCs in a sex-, developmental age-, and brain region-specific manner.

Introduction

During the period of brain sexual differentiation in gestation and early postnatal life, gonadal hormones organize the development of brain structures that govern sex-typical physiology and behavior (Phoenix *et al*., 1959; Wallen, 2009). Exposure to exogenous hormones or endocrine-disrupting chemicals [EDCs] during this life stage results in structural and functional neurobiological changes (Diamanti-Kandarakis *et al*., 2009). The underlying molecular pathways for these effects are varied, and can involve gene and protein expression, apoptosis, neurogenesis, and molecular epigenetic mechanisms such as DNA methylation and histone modifications (Dolinoy *et al*., 2007; Bredfeldt *et al*., 2010; Dickerson *et al*., 2011a; Dickerson *et al*., 2011b; Komada *et al*., 2012).

A recently identified player in brain sexual differentiation are microRNAs, a family of small regulatory noncoding RNAs that bind to the 3'-untranslated region of a target mRNA, causing mRNA translational repression and/or degradation (Filipowicz *et al*., 2005). The expression of some microRNAs is hormone-sensitive, and microRNAs, in

turn, influence the expression of genes involved in mediating hormone responses (Klinge, 2009; Rao *et al*., 2013). Individual microRNAs are expressed in the nervous system in a region- and development-specific manner (Olsen *et al*., 2009; Ziats *et al*., 2014). Some, such as *mir-124a* and *mir-9* are important in neurodevelopment (Conaco *et al*., 2006; Tan *et al*., 2012), and are highly expressed in the hypothalamus (e.g. *mir-7, mir-132, mir-219*) (Kocerha *et al*., 2009; Miller *et al*., 2012; Sakai *et al*., 2013). Furthermore, a number of hypothalamic microRNAs are expressed in a sexually-dimorphic manner during development [e.g., *lin28/let-7* family] (Sangiao-Alvarellos *et al*., 2013). Although research on links between prenatal hormones on microRNA expression on the brain is limited, work on prenatal or maternal stress demonstrates effects on expression of a subset of these and other microRNAs (Morgan *et al*., 2011; Zucchi *et al*., 2013).

The effects of prenatal EDCs on developmental expression of microRNAs have not been studied in the hypothalamus, but their effects have been shown in other tissues including mouse Sertoli cells (Choi *et al*., 2011), whole brains, and livers (Zhang *et al*., 2009); rat penile shafts (Kovanecz *et al*., 2014) and hippocampal cultures (Lesiak *et al*., 2014); and human breast carcinoma (Tilghman *et al*., 2012; Teng *et al*., 2013) and placental cell lines (Avissar-Whiting *et al*., 2010). In the current study, we addressed whether a class of prenatal EDCs affect the expression of microRNAs during brain sexual differentiation following prenatal exposure of rats to A1221, a mixture of polychlorinated biphenyls [PCBs] that has previously been shown to perturb expression of sexually dimorphic genes in the brain and cause reproductive and behavioral phenotypic changes in adulthood (Steinberg *et al*., 2007; Steinberg *et al*., 2008; Dickerson *et al*., 2011a;

Dickerson *et al*., 2011b; Walker *et al*., 2013; Walker *et al*., 2014). We also assayed several mRNA targets of the microRNAs. Work was conducted on two sexually-dimorphic, hormone-sensitive hypothalamic regions involved in reproductive physiology and behavior (Malsbury *et al*., 1977; Mathews *et al*., 1977; Clark *et al*., 1981; Hoshina *et al*., 1994; Kato *et al*., 2000; Hull *et al*., 2006), the medial preoptic nucleus [MPN] and the ventromedial nucleus [VMN].

Materials and methods

Animals and treatments

All protocols were performed in accordance with the guidelines from the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Brain regions were collected from rats used for a published study on effects of EDCs on gene expression in other hypothalamic regions, and detailed husbandry is provided in that report (Walker *et al*., 2014). In brief, Sprague-Dawley dams and sires [Harlan, Houston, TX] were purchased, and provided low phytoestrogen Harlan Teklad 2019 Global Diet *ad libidum* for at least 2 weeks prior to mating. The first day of successful pregnancy was termed embryonic day [E] 0. At the beginning of the third trimester, corresponding to the onset of the period of hypothalamic sexual differentiation, on E16 and E18, the dams were injected intraperitoneally with one of three treatments: vehicle [100% dimethylsulfoxide [DMSO] Sigma number D4540; Sigma, St Louis, Missouri]; 50 μg/kg estradiol benzoate [EB; Sigma number E8515; or 1 mg/kg A1221 [AccuStandard, New

Haven, Connecticut, number C221N], all in 0.1 ml volume. The dosage, route, and timing of exposure were based on published work showing effects on reproductive function and gene expression in exposed rats (Steinberg *et al*., 2007; Steinberg *et al*., 2008; Dickerson *et al*., 2011a; Dickerson *et al*., 2011b; Walker *et al*., 2013; Walker *et al*., 2014).

Brain tissue collection and storage

On P15, P30, P45, and P90, one male and one female littermate were euthanized two - three hours before lights out by rapid decapitation. Trunk blood was collected, allowed to clot, and centrifuged to collect the serum for hormone assays. Body and organ weights were also measured at euthanasia. The brains were dissected and cut into one mm coronal sections using a rat brain matrix, and bilateral micropunches of MPN and VMN were collected using a Palkovits punch [0.98 mm in diameter] (Walker *et al*., 2013). Post-pubertal females were monitored daily by vaginal smears and euthanized on proestrus. Ten animals per group were used for microRNA and mRNA gene expression studies.

RNA isolation, preparation, and real-time PCR

Total RNA was isolated from frozen MPN and VMN punches of individual male and female rats using a mirVana microRNA isolation kit according to the manufacturer's protocols [catalog no. AM1560, Life Technologies, Carlsbad, CA]. All RNA samples were analyzed for quantity by Nanodrop spectrophotometry and run on the Bioanalyzer

2100 [catalog no. 5067-1511, Agilent Technologies, Santa Clara, CA] to assess RNA purity and integrity. Only RNA samples with the RIN of 8 or higher were used.

Total RNA [200 ng total for mRNA, 10 ng for microRNA] was used to generate cDNA. Taqman MicroRNA Reverse Transcription kit [catalog no.4366596] with Taqman RT primers [catalog no. 4440886, Life Technologies] and high-capacity cDNA reverse transcription kit with RNase inhibitor [catalog no. 4374966, Life Technologies] were used for microRNA and mRNA, respectively, according to the manufacturer's recommended protocols.

For microRNA analysis, we selected eight specific microRNA assays based on evidence for their expression in the hypothalamus [*let-7a, let-7b, mir-124a, mir-132, mir-145, mir-219, mir-7, mir-9*] (Cheng *et al*., 2007; Davis *et al*., 2012; Sakai *et al*., 2013). Due to the small amount of starting material, only biological replicates were used for analysis. Technical replicates were run prior to the experiment to validate each assay and intra-assay CV was determined to be <2%. For these and other assays, a no-reverse transcription control was run to confirm the absence of genomic DNA contamination, and a positive control was run on each plate to control for inter-plate variability. Inter-plate variability was <4%.

Messenger RNA targets were selected based on *in silico* predictions of gene targets for the microRNAs [see below] (Dickerson *et al*., 2011a; Dickerson *et al*., 2011b; Casati *et al*., 2013; Walker *et al*., 2013; Walker *et al*., 2014). We conducted real-time PCR analysis using Taqman primer and probe sets for subsets of 8 genes [*Ar, Clock, Lepr, Lin28b, Ppara, Grin2a, Igf1r, Ar, Pgr*] based on microRNA results by sex and

brain region. Samples were run in triplicate and any of the triplicates with a value of 1.5 SD above the mean of that animal was removed, with up to 2 samples per gene removed. None of the triplicates had a coefficient of variation greater than 2%.

Real-time PCR for microRNA and mRNA analysis was carried out on an ABI ViiA7 using Applied Biosystems TaqMan Universal PCR Master Mix [catalog no. 4324018, Life Technologies] and using the following run parameters: 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

In silico analysis of combinatorial microRNA activity

To determine the potential mRNA targets of the microRNAs that were affected by sex, treatment, or age, we used the prediction program ComiR, chosen for its combinatorial approach in gene target identification, to generate a list of mRNA targets for each region and sex (Coronnello *et al*., 2012; Coronnello *et al*., 2013). The selected genes were verified for the number of binding sites of the predicted microRNAs using Targetscan. The bioinformatic analysis was done across age in four groups: female MPN, male MPN, female VMN, and male VMN. The microRNA groups used for ComiR analysis were *mir-145* and *mir-7* in female MPN; *mir-132, mir-219, mir-9, mir-145, let-7a,* and *mir-124a* in male MPN; *let-7a, mir-124a,* and *mir-219* in female VMN; *mir-124a* in male VMN. DAVID was used to provide biological interpretation of the large gene lists generated by ComiR and annotate them by gene families and function (Huang da *et al*., 2009a; Huang da *et al*., 2009b). Putative targets were chosen for further analysis based on their known role in neuronal function and their algorithm score, as determined

by the computer modeling programs (Huang da *et al*., 2009a; Huang da *et al*., 2009b; Coronnello *et al*., 2012; Coronnello *et al*., 2013).

Hormone assays

Serum testosterone and estradiol levels in the same rats used herein have previously been measured, and assay characteristics and results were published previously (Walker *et al*., 2014). For the current study, hormone concentrations were used for correlation analysis with microRNA and mRNA levels in the MPN and VMN in bionetwork analyses.

Statistics

All microRNA and mRNA data [raw CT values] were normalized within sex to the median of the DMSO P15 group in an R statistical package for qPCR analysis which utilized a generalized linear mixed model with Poisson-lognormal errors and a Bayesian Marco Chain Monte Carlo sampling scheme (Matz *et al*., 2013). All data were normally distributed and homoscedastic. The statistical analysis was done using the multiple comparisons analysis of variance [MANOVA], which addresses the false discovery rates of multiple comparisons, to compare each endpoint [genes and microRNAs] using sex, age, and treatment as independent variables. The R package was run in naïve form, without specifying any control genes, and the statistical significance was set at $P < 0.05$. Post hoc analyses included t-test for sex effect and Tukey HSD for treatment effect and interactions. MicroRNA expression data were graphed as mean fold-change ± standard

error of the mean using DMSO female as a reference group. mRNA expression data were graphed as mean fold-change ± standard error of the mean using DMSO group within sex as a reference group.

Bionetwork analysis

To examine possible relationships among microRNAs, genes, and serum estradiol and testosterone levels, the latter from a companion paper already published on these rats (Walker *et al*. 2014) throughout development, the data were analyzed using a bootstrap technique, as previously reported (Walker *et al*., 2013; Walker *et al*., 2014).

Results

Effects of sex, age, and EDC treatment on microRNA expression in the MPN

Effects of sex, age, treatment, and their interactions, on microRNA expression in the MPN were analyzed (**Figure 2.1**). Detailed statistics for *mir-132, mir-219, mir-7,* and *mir*-9 are presented in **Table 2.1** and statistics for *mir-145, let-7a, let-7b,* and *mir*-124a are shown in **Table 2.2**. In the MPN, five microRNAs showed significant sex effects, with three higher in females than males [*mir-219, mir-7 (both,* $p<0.05$*), mir-145 (*$p<0.005$)], and two higher in males than females [*mir-9 (*$p<0.001$) and *let-7b (*$p<0.05$)]. Significant age effects were found for six microRNAs, all of which increased with advancing age [*mir-132, mir-219, mir-7, mir-145, let-7a, let-7b* (all $p<0.005$)]. *Mir-9* expression decreased significantly with age ($p<0.005$). Only *mir-124a* showed no age-related changes in the MPN.

The effects of prenatal treatment in the MPN were age- and sex-specific. A1221 and EB females had significantly increased expression of six microRNAs and this was specific to one age, P30 [*mir-219*, *mir-132, mir-7, mir-145, let-7a,* and *mir-124a* (all $p<0.005$)]. Expression of *mir-132* and *mir-9* was increased by EB at P15 (both $p<0.005$), and *mir-7* was decreased at P45 in the EB females. In the males, A1221 animals had decreased expression of 6 microRNAs, specifically at P90 [*mir-132, mir-219, mir-9, mir-145, let-7a,* and *mir-124a* (all $p<0.005$)]. *Mir-145* was decreased in A1221 males ($p<0.005$) and *let-7b* was increased ($p<0.005$) at P45. EB males had decreased expression of *mir-145* at P90 ($p<0.005$). EB males had increased expression of *mir-219*, *mir-7*, *mir-9,* and *let-7b* (all $p<0.005$) at P45.

Effects of sex, age, and EDC treatment on microRNA expression in the VMN

In the VMN, main effects of sex, age, treatment, and their interactions on microRNA expression were examined (**Figure 2.2**; detailed statistics for *mir-132, mir-219, mir-7,* and *mir*-9 are presented in **Table 2.3** and statistics for *mir-145, let-7a, let-7b,* and *mir*-124a are shown in **Table 2.4**). Only one sex difference was found, for *mir-219* ($p<0.005$), with expression higher in the females compared to males. All 8 microRNAs increased expression with advancing age in the VMN ($p<0.005$).

The effects of prenatal treatment in the VMN were age- and sex-specific. In the A1221 females, *mir-124a* expression was increased at P15 ($p<0.005$), and *let-7a* expression was decreased at P90 ($p<0.005$). EB females had increased expression at P15 of *mir-219* ($p<0.0005$) and *mir-124a* ($p<0.005$). In the males, A1221 and EB rats had

increased expression at P30 of *mir-9* and *let-7b* (both $p<0.005$), and decreased expression of *mir-219* at P45 ($p<0.0005$). A1221 males also had decreased expression of *mir-124a* at P90 ($p<0.005$).

In silico analysis of combinatorial microRNA activity

In silico analysis was conducted separately for the female MPN, female VMN, male MPN, and male VMN, to identify mRNA targets. The microRNA groupings were: *mir-132, mir-219, mir-9, mir-145, let-7a,* and *mir-124a* in male MPN; *mir-145* and *mir-7* in female MPN; *let-7a*, *mir-124a,* and *mir-219* in female VMN; *mir-124a* in male VMN. To provide an example, the DAVID results in the males in the MPN are discussed. DAVID analysis of the combined male MPN target list from ComiR yielded 158 clusters, with 25 significantly enriched (score >1). Annotation clusters 1 and 2 (enrichment scores of 2.5) had the gene clusters that belonged to the nuclear-hormone receptor, ligand/DNA-binding receptors families. The pathways included post-trascriptional silencing by small RNAs, nuclear receptor transcription pathway and nuclear receptors among the top six which highlighted the hormone regulatory function of chosen microRNA. The genes chosen for qPCR in each group are shown in **Table 2.5**.

Effects of age and EDC treatment on mRNA expression in the MPN

Because gene targets were different in males and females, analyses were conducted separately for each sex and region. In the female MPN, gene expression decreased with increasing developmental age for *Grin2a* and *Igf1r* ($p<0.005$ for both,

Figure 2.3A, 2.3B). A main effect of treatment was also found for both genes, with decreased expression of *Grin2a* and *Igf1r* in the A1221-treated females compared to DMSO females ($p<0.005$). There were no treatment by age interactions. In the male MPN (**Figure 2.3C, 2.3D, 2.3E**), expression of *Ar, Pgr,* and *Ppara* decreased with developmental age (all $p < 0.005$). A main effect of treatment was found for *Pgr* and *Ppara*, with decreased expression in A1221 compared to DMSO males (both $p < 0.005$). A treatment by age interaction for A1221 was also found, with decreased expression of *Ppara* at P15 ($p<0.005$).

Effects of age and EDC treatment on mRNA expression in the VMN

Age and treatment effects on mRNA gene expression were found in the VMN (**Figure 2.4**; detailed statistics in **Table 2.6**). In the females (**Figure 2.4A, 2.4B, 2.4C, 2.4D**), significant developmental increases were found for *Lepr, Clock,* and *Ppara* (all $p < 0.005$). Main effect of EB treatment were found, with increased gene expression of *Clock* in the female VMN ($p<0.001$). There were also treatment by age interactions in the female VMN for prenatal EB treatment, with increased expression at P45 of *Lepr* and *Clock* (both $p < 0.005$). In the males (**Figure 2.4E, 2.4F**), *Ar* expression increased with age ($p<0.005$) and *Clock* ($p<0.005$) decreased with age. Treatment by age interactions of prenatal A1221 and EB treatments were also found. Specifically, A1221 and EB males had decreased expression of *Ar* ($p<0.0005$), and *Clock* expression was decreased in A1221 males at P90 ($p<0.05$).

Bionetwork analysis of microRNAs, mRNAs, and hormones

Bionetwork analysis was conducted using Pearson's correlation coefficients to enable investigation into relationships among microRNAs, genes, and hormones, and to determine whether there were inverse relationships between microRNAs and their predicted mRNA targets. Only positive correlations were detected, with the correlation strength ranging from 0.5 to 1 (**Figures 2.5, 2.6**). The networks were examined within each region, and the numbers of identical (same as vehicle) and novel (different from vehicle) correlations were counted in A1221 and EB networks. In the females, there were few differences in correlations with treatment in either brain region (**Figure 2.5**). For example, in the female MPN, *mir-132 and mir-145, mir-124a* and *let-7b,* and *mir-9* and *let-7a*, were significantly correlated in all three treatment groups. In the female VMN, *mir-132* and *mir-219, mir-132* and *mir-7*, and others, showed similar correlations across the different treatment networks. By contrast, males showed more differences between networks with treatment (**Figure 2.6**). In the MPN, the A1221 and EB males had correlations not seen in the DMSO males, such as for *mir-124a, mir-7, let-7b, Ar, Ppara,* and *Pgr*. The male VMN networks did not differ substantially by treatment. Interestingly, serum estradiol concentrations were positively correlated with *mir-219* in both the male MPN and VMN of all treatment groups, and estradiol and *mir-132* were correlated in four of these six groups (**Figure 2.6**). In females (**Figure 2.5**) estradiol was also correlated with *mir-219* in the three treatment groups in the MPN, and in the DMSO and A1221 VMN.

Figures and tables

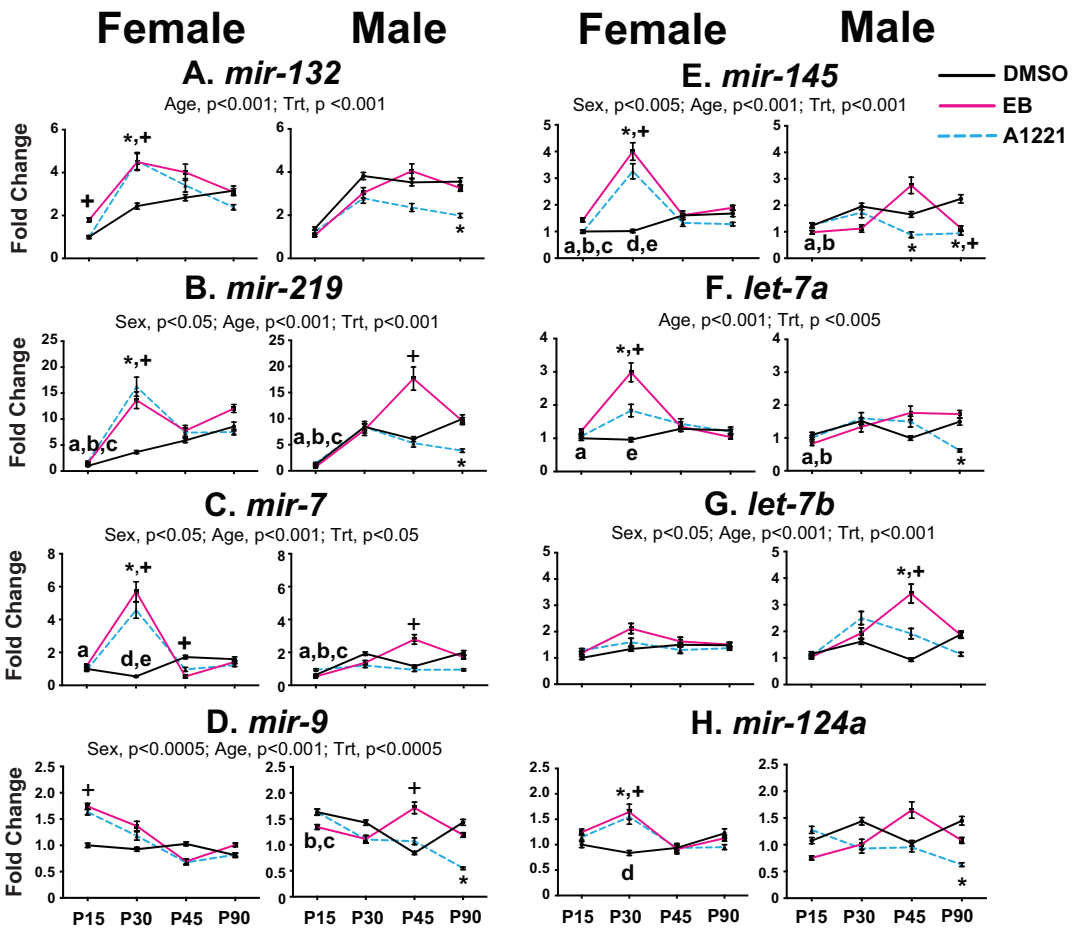

Figure 2.1: Developmental profiles of *mir-132, mir-219, mir-7, mir-9, mir-145, let-7a, let-7b and mir-124a* are shown in the MPN of female and male rats. Main effects of age, sex, and treatment are shown. Post-hoc effects of treatment are indicated when the sex by treatment by age interaction was significant. *p < 0.05, A1221 vs. DMSO at the same age; **+**p < 0.05, EB vs. DMSO at the same age. Significant age effects (p < 0.05) are shown as: **a**, P15 vs P30; **b**, P15 vs P45; **c**, P15 vs P90; **d**, P30 vs P45; **e**, P30 vs P90. Abbreviation: Trt, Treatment.

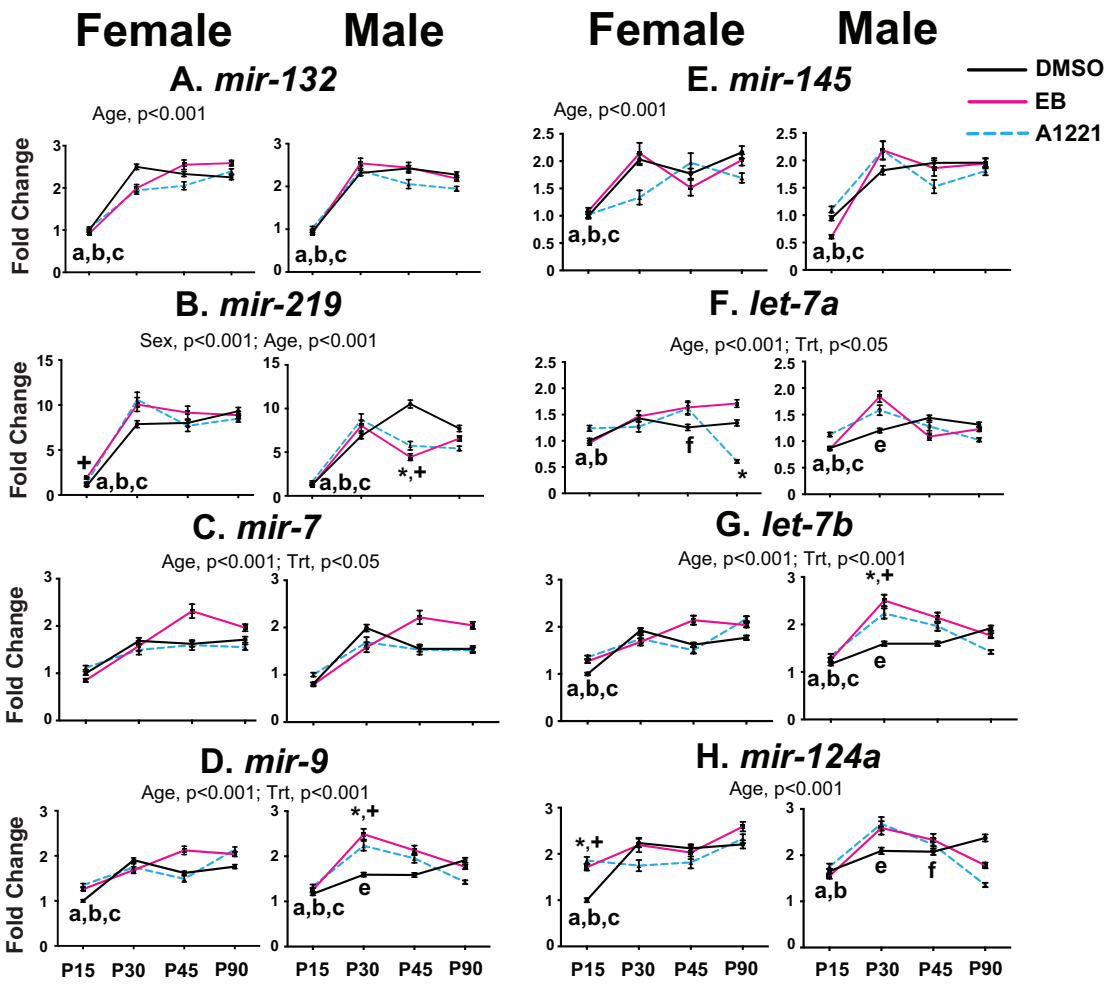

Figure 2.2: Developmental profiles of *mir-132*, *mir-219*, *mir-7*, *mir-9*, *mir-145*, *let-7a*, *let-7a*, and *mir-124a* are shown in the VMN of male and female rats. Main effects of age, sex, and treatment are shown. Post-hoc effects of treatment are indicated when the sex by treatment by age interaction was significant. *$p < 0.05$, A1221 vs. DMSO at the same age; **+**$p < 0.05$ EB vs. DMSO at the same age. Significant age effects are shown as: **a**, P15 vs P30; **b**, P15 vs P45; **c**, P15 vs P90; **e**, P30 vs P90; **f,** P45 vs P90, $p<0.05$ for all. Abbreviations: Trt, Treatment.

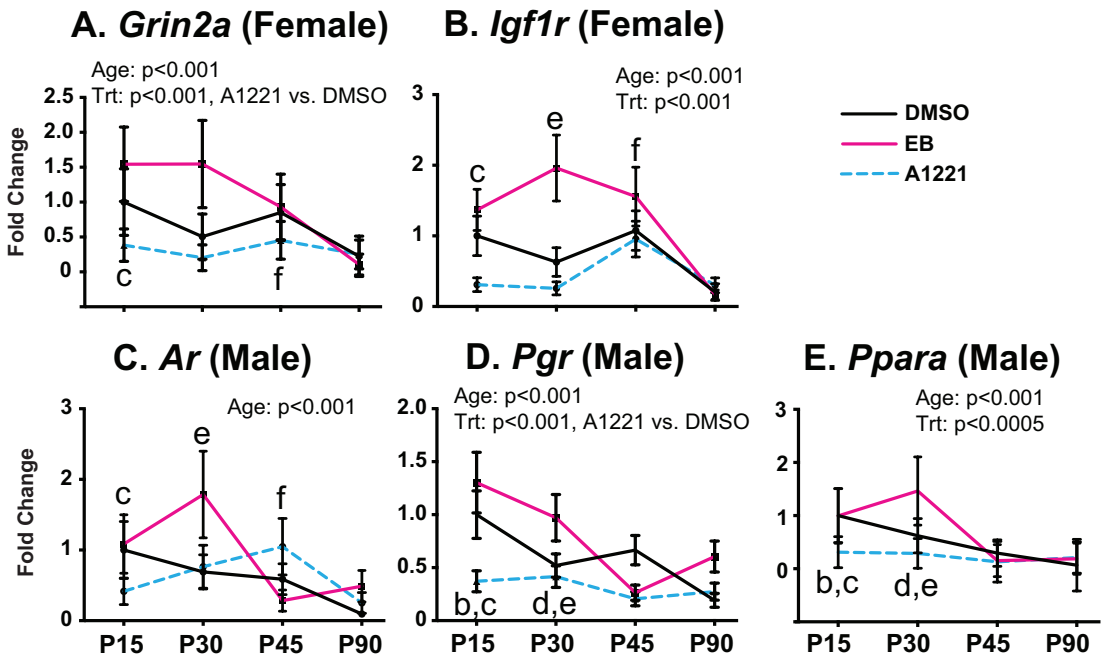

Figure 2.3: mRNA expression is shown in the MPN of females (A-B) and males (C-E). Main effects of age and treatment are shown. Significant age effects ($p < 0.05$) are shown as: **b**, P15 vs P45; **c**, P15 vs P90; **d**, P30 vs P45; **e**, P30 vs P90; **f,** P45 vs P90.

Abbreviations: Trt, Treatment.

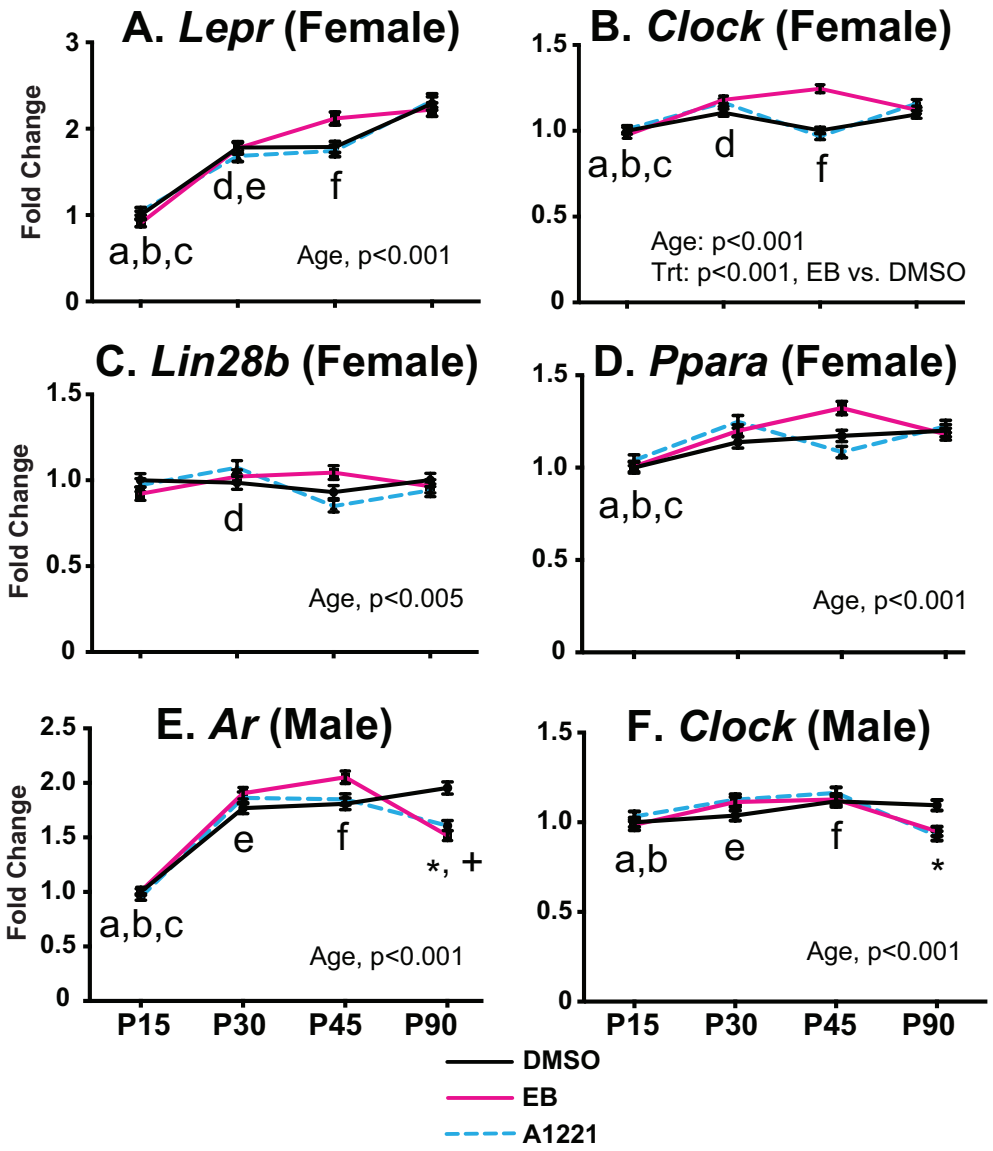

Figure 2.4: mRNA expression is shown in the VMN of females (A-D) and males (E-F). Main effects of age and treatment are shown. Post-hoc effects of treatment are indicated when the treatment by age interaction was significant. *p < 0.05 A1221 vs. DMSO at the same age; +p < 0.05 EB vs. DMSO at the same age. Significant age effects (p < 0.05) are shown as: **a**, P15 vs P30; **b**, P15 vs P45; **c**, P15 vs P90; **d**, P30 vs P45; **e**, P30 vs P90; **f,** P45 vs P90. Abbreviations: Trt, Treatment.

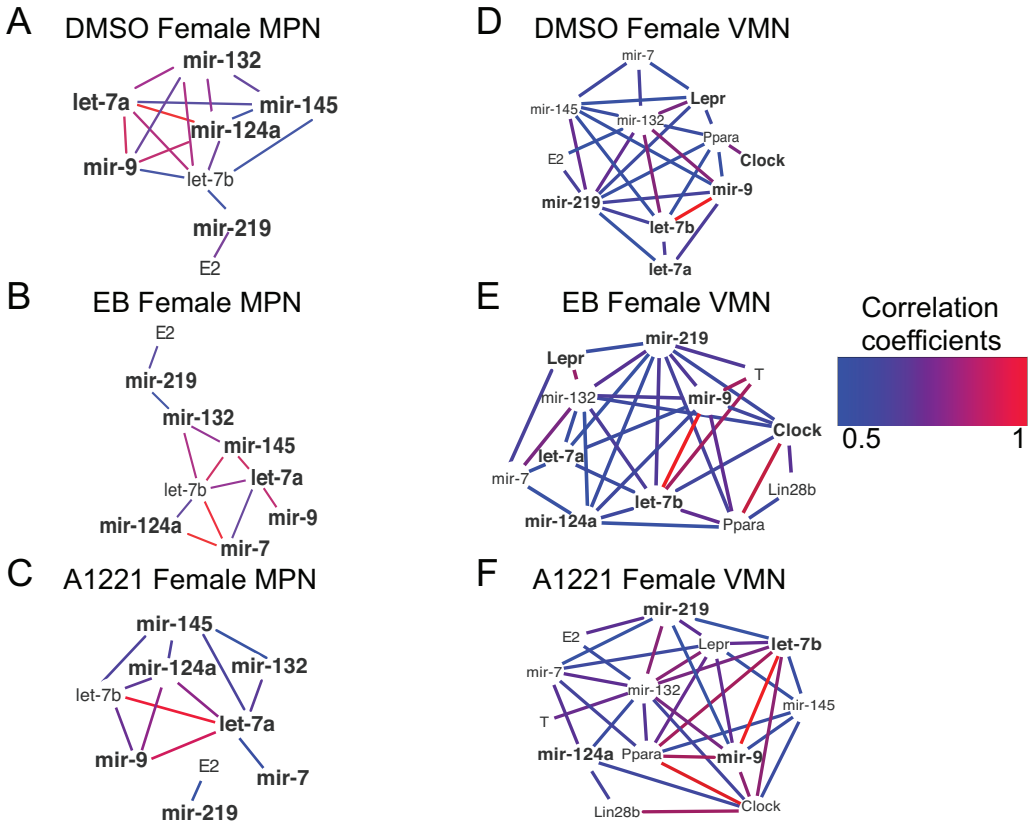

Figure 2.5: Cytoscape analysis of microRNAs, genes, and hormones for the three treatment groups in the females, collapsed across development, in the MPN (A-C) and VMN (D-F). Abbreviations: E2, estradiol, T, testosterone.

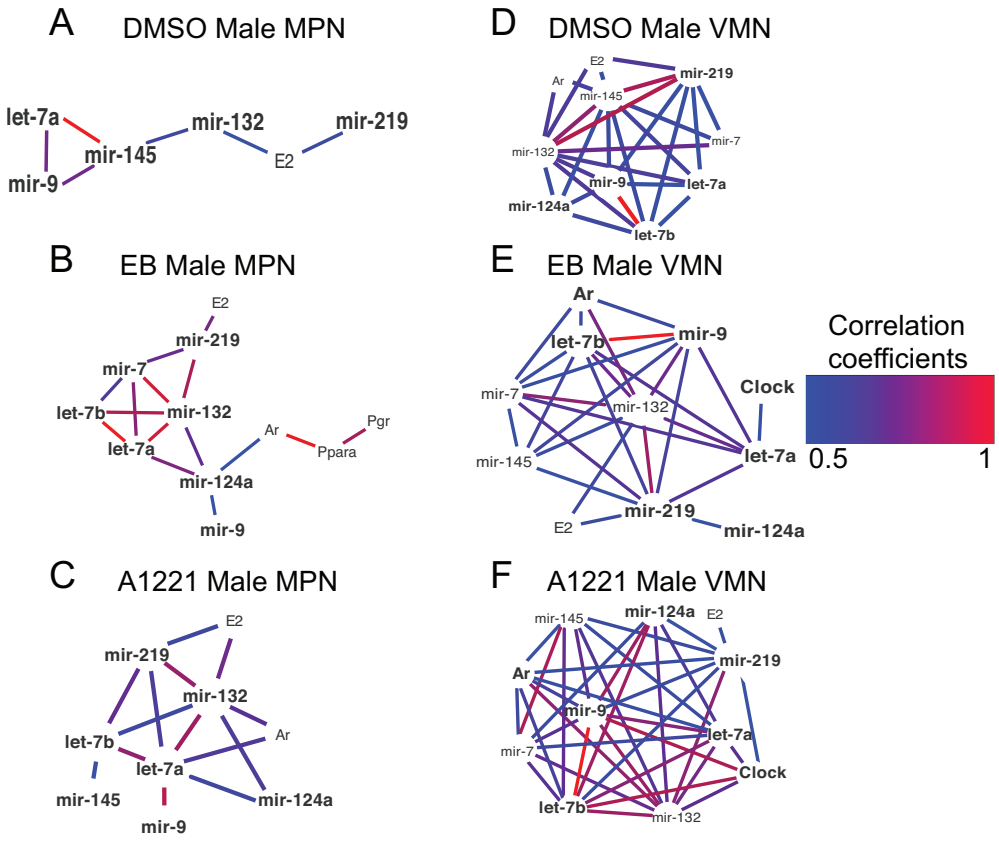

Figure 2.6: Cytoscape analysis of microRNAs, genes, and hormones for the three treatment groups in the males, collapsed across development, in the MPN (A-C) and VMN (D-F). Abbreviations: E2, estradiol, T, testosterone.

Table 2.1. Statistical results of multiple comparisons ANOVA using sex, age, and treatment as independent variables for *mir-132, mir-219, mir-7,* and *mir-9* microRNA analysis in the MPN. Significant post-hocs for treatment when main effect of treatment or sex X treatment X age interaction was significant are indicated in bold.

microRNA	Tissue	Main effect/Interaction	F	P	Degree of freedom	Residual degree of freedom
mir-132	MPN	Sex	1.21	0.27	1	207
		Treatment	11.07	0.001	2	207
		Age	134.00	0.001	3	207
		Sex X Treatment	15.20	0.001	2	207
		Sex X Age	0.95	0.41	3	207
		Treatment X Age	2.81	0.01	6	207
		Sex X Treatment X Age	**4.36**	**0.0003**	**6**	**207**
mir-219	MPN	Sex	5.28	0.02	1	207
		Treatment	**13.59**	**0.001**	**2**	**207**
		Age	323.05	0.001	3	207
		Sex X Treatment	18.13	0.001	2	207
		Sex X Age	3.04	0.02	3	207
		Treatment X Age	13.95	0.001	6	207
		Sex X Treatment X Age	**8.33**	**0.001**	**6**	**207**
mir-7	MPN	Sex	4.56	0.03	1	207
		Treatment	3.36	0.001	2	207
		Age	30.61	0.001	3	207
		Sex X Treatment	6.65	0.001	2	207
		Sex X Age	11.27	0.001	3	207
		Treatment X Age	9.54	0.001	6	207
		Sex X Treatment X Age	**24.04**	**0.001**	**6**	**207**
mir-9	MPN	Sex	11.65	0.0007	1	207
		Treatment	7.43	0.0007	2	207
		Age	28.62	0.001	3	207
		Sex X Treatment	4.90	0.008	2	207
		Sex X Age	3.42	0.01	3	207
		Treatment X Age	5.15	0.001	6	207
		Sex X Treatment X Age	**11.54**	**0.001**	**6**	**207**

Table 2.2. Statistical results of multiple comparisons ANOVA using sex, age, and treatment as independent variables for *mir-145*, *let-7a*, *let-7b*, and *mir-124a* microRNA analysis in the MPN. Significant post-hocs for treatment when main effect of treatment or sex X treatment X age interaction was significant are indicated in bold.

microRNA	Tissue	Main effect/Interaction	F	P	Degree of freedom	Residual degree of freedom
mir-145	MPN	Sex	9.18	0.002	1	207
		Treatment	7.61	0.0006	2	207
		Age	22.35	0.001	3	207
		Sex X Treatment	21.84	0.001	2	207
		Sex X Age	5.61	0.001	3	207
		Treatment X Age	10.52	0.001	6	207
		Sex X Treatment X Age	**11.41**	**0.001**	**6**	**207**
let-7a	MPN	Sex	2.30	0.1	1	207
		Treatment	**5.57**	**0.004**	**2**	**207**
		Age	17.70	0.001	3	207
		Sex X Treatment	4.00	0.01	2	207
		Sex X Age	1.08	0.3	3	207
		Treatment X Age	5.61	0.001	6	207
		Sex X Treatment X Age	**9.77**	**0.001**	**6**	**207**
let-7b	MPN	Sex	4.72	0.03	1	207
		Treatment	**10.23**	**0.001**	**2**	**207**
		Age	20.30	0.001	3	207
		Sex X Treatment	0.85	0.4	2	207
		Sex X Age	1.75	0.1	3	207
		Treatment X Age	5.80	0.001	6	207
		Sex X Treatment X Age	**6.70**	**0.001**	**6**	**207**
mir-124a	MPN	Sex	0.79	0.3	1	207
		Treatment	2.50	0.08	2	207
		Age	2.58	0.05	3	207
		Sex X Treatment	9.06	0.0001	2	207
		Sex X Age	3.80	0.01	3	207
		Treatment X Age	5.62	0.001	6	207
		Sex X Treatment X Age	**6.81**	**0.001**	**6**	**207**

Table 2.3. Statistical results of multiple comparisons ANOVA using sex, age, and treatment as independent variables for *mir-132*, *mir-219*, *mir-7*, and *mir-9* microRNA analysis in the VMN. Significant post-hocs for treatment when main effect of treatment or sex X treatment X age interaction was significant are indicated in bold.

microRNA	Tissue	Main effect/Interaction	F	P	Degree of freedom	Residual degree of freedom
mir-132	VMN	Sex	0.06	0.8	1	216
		Treatment	2.46	0.08	2	216
		Age	297.66	0.001	3	216
		Sex X Treatment	0.10	0.9	2	216
		Sex X Age	4.11	0.007	3	216
		Treatment X Age	2.85	0.01	6	216
		Sex X Treatment X Age	2.06	0.05	6	216
mir-219	VMN	Sex	31.43	0.001	1	216
		Treatment	0.46	0.6	2	216
		Age	689.63	0.001	3	216
		Sex X Treatment	16.64	0.001	2	216
		Sex X Age	3.80	0.01	3	216
		Treatment X Age	9.93	0.001	6	216
		Sex X Treatment X Age	**4.76**	**0.0001**	**6**	**216**
mir-7	VMN	Sex	0.55	0.4	1	216
		Treatment	3.73	0.02	2	216
		Age	81.82	0.001	3	216
		Sex X Treatment	0.12	0.8	2	216
		Sex X Age	1.75	0.1	3	216
		Treatment X Age	7.16	0.001	6	216
		Sex X Treatment X Age	0.36	0.8	6	216
mir-9	VMN	Sex	1.60	0.2	1	216
		Treatment	**14.97**	**0.001**	**2**	**216**
		Age	70.03	0.001	3	216
		Sex X Treatment	0.42	0.6	2	216
		Sex X Age	7.16	0.0001	3	216
		Treatment X Age	3.28	0.004	6	216
		Sex X Treatment X Age	**8.14**	**0.001**	**6**	**216**

Table 2.4. Statistical results of multiple comparisons ANOVA using sex, age, and treatment as independent variables for *mir-145, let-7a, let-7b,* and *mir-124a* microRNA analysis in the VMN. Significant post-hocs for treatment when main effect of treatment or sex X treatment X age interaction was significant are indicated in bold.

microRNA	Tissue	Main effect/Interaction	F	P	Degree of freedom	Residual degree of freedom
mir-145	VMN	Sex	0.11	0.7	1	216
		Treatment	0.87	0.4	2	216
		Age	85.02	0.001	3	216
		Sex X Treatment	2.19	0.1	2	216
		Sex X Age	2.98	0.03	3	216
		Treatment X Age	2.98	0.007	6	216
		Sex X Treatment X Age	5.02	0.001	6	216
let-7a	VMN	Sex	1.37	0.2	1	215
		Treatment	3.06	0.04	2	215
		Age	23.78	0.001	3	215
		Sex X Treatment	4.18	0.01	2	215
		Sex X Age	3.21	0.02	3	215
		Treatment X Age	11.86	0.001	6	215
		Sex X Treatment X Age	**5.78**	**0.001**	**6**	**215**
let-7b	VMN	Sex	1.42	0.2	1	216
		Treatment	**14.86**	**0.001**	**2**	**216**
		Age	69.74	0.001	3	216
		Sex X Treatment	0.44	0.4	2	216
		Sex X Age	7.43	0.001	3	216
		Treatment X Age	3.30	0.003	6	216
		Sex X Treatment X Age	**8.49**	**0.001**	**6**	**216**
mir-124a	VMN	Sex	0.79	0.3	1	216
		Treatment	2.30	0.1	2	216
		Age	22.43	0.001	3	216
		Sex X Treatment	2.12	0.1	2	216
		Sex X Age	9.80	0.001	3	216
		Treatment X Age	4.44	0.0002	6	216
		Sex X Treatment X Age	**6.49**	**0.001**	**6**	**216**

Table 2.5. Genes chosen for mRNA expression analysis in the MPN and VMN.			
Gene ID	Gene name	Tissue	Predicted Targeting microRNA (# of binding sites)
Igf1r	Insulin-like growth factor 1 receptor	Female MPN	*mir-145 (2); mir-7 (3)*
Grin2a	Glutamate receptor, ionotropic, N-methyl-D-aspartate 2A	Female MPN	*mir-7 (4); mir-145 (3)*
Ar	Androgen receptor	Male MPN Male VMN	*mir-124a (1)*
Pgr	Progesterone receptor	Male MPN	*mir-9 (2); let-7a (2); mir-124 (1)*
Ppara	Peroxisome proliferator-activated receptor alpha	Female VMN Male MPN	*mir-124a (2); mir-9 (1); let-7a (1); mir-219 (1)*
Lin28b	Lin-28 homolog B	Female VMN	*let7a (4)*
Clock	Clock gene	Female VMN Male VMN	*mir-124a (1)*
Lepr	Leptin receptor	Female VMN	*mir-219 (1)*

Table 2.6. Statistical results of multiple comparisons ANOVA using age and treatment as independent variables for mRNA analysis in the MPN and VMN. Significant post-hocs for treatment when main effect of treatment or treatment X age interaction was significant are indicated in bold.

mRNA	Tissue	Sex	Main effect/Interaction	F	P	Degree of freedom	Residual degree of freedom
Grin2a	MPN	Female	**Treatment**	**7.29**	**0.001**	**2**	**102**
			Age	13.26	0.001	3	102
			Treatment X Age	3.96	0.001	6	102
Igf1r	MPN	Female	**Treatment**	**7.21**	**0.001**	**2**	**102**
			Age	14.09	0.001	3	102
			Treatment X Age	3.57	0.002	6	102
Ar	MPN	Male	Treatment	2.40	0.09	2	105
			Age	11.50	0.001	3	105
			Treatment X Age	**4.15**	**0.0008**	**6**	**105**
Pgr	MPN	Male	**Treatment**	**12.04**	**0.001**	**2**	**105**
			Age	9.88	0.001	3	105
			Treatment X Age	**4.15**	**0.0008**	**6**	**105**
Ppara	MPN	Male	**Treatment**	**8.56**	**0.0003**	**2**	**105**
			Age	31.18	0.001	3	105
			Treatment X Age	**6.11**	**0.001**	**6**	**105**
Clock	VMN	Female	**Treatment**	**7.80**	**0.0006**	**2**	**107**
			Age	17.87	0.001	3	107
			Treatment X Age	**7.56**	**0.001**	**6**	**107**
Lepr	VMN	Female	Treatment	0.54	0.5	2	107
			Age	354.81	0.001	3	107
			Treatment X Age	**5.36**	**0.001**	**6**	**107**
Lin28b	VMN	Female	Treatment	1.14	0.3	2	107
			Age	4.55	0.004	3	107
			Treatment X Age	5.06	0.0001	6	107
Ppara	VMN	Female	Treatment	2.30	0.1	2	107
			Age	27.08	0.001	3	107
			Treatment X Age	**5.11**	**0.0001**	**6**	**107**
Ar	VMN	Male	Treatment	1.22	0.2	2	108
			Age	183.22	0.001	3	108
			Treatment X Age	**4.86**	**0.0001**	**6**	**108**
Clock	VMN	Male	Treatment	0.25	0.7	2	108
			Age	10.42	0.001	3	108
			Treatment X Age	**2.82**	**0.01**	**6**	**108**

Discussion

In the current study we profiled the expression of eight microRNAs in the developing MPN and VMN of the hypothalamus, and determined effects of gestational exposures to PCBs on microRNA expression. We further related changes in microRNA expression to changes in target gene mRNA expression changes in the same animals. Our major findings were that many more of the selected microRNAs were sexually dimorphic, and affected by prenatal EDC treatment in the MPN than the VMN. Importantly, EDC-treated females showed up-regulation of microRNAs at P30, in the midst of pubertal development, whereas males were affected (down-regulated) at P90, in adulthood. Relatively few EDC effects were found on the mRNA targets of these microRNAs, the implications for which are discussed below. As a whole, the results add to knowledge about EDC effects on microRNA expression, and provide new information about sex differences and developmental change in the hypothalamus.

MicroRNA expression in the MPN changes with postnatal development and is sexually dimorphic

The most common finding of our study was a developmental increase in microRNA expression as animals progressed from the juvenile through the pubertal period and into adulthood. In the MPN, six microRNAs (*mir-132, mir-219, mir-7*, *mir-145*, *let-7a,* and *let-7b*) exhibited this pattern. The *lin28/let-7* family has previously been investigated in the preoptic area, for which the expression of *let-7a, let-7b, mir-132,* and *mir-145* increased with postnatal development. Changes in their expression were

suggested to be involved in the mechanisms permitting or leading to puberty onset (Sangiao-Alvarellos *et al*., 2013), as *Lin28* overexpression in mice resulted in delayed puberty and increased body size (Zhu *et al*., 2010). Another previous study showed that seven of the eight microRNAs of the *let-7* family were highly expressed in hypothalamic arcuate and paraventricular nuclei of adult rats (Amar *et al*., 2012). Our finding that *let-7a, let-7b, mir-132* and *mir-145* expression increase with age in the MPN is consistent with those reports (Sangiao-Alvarellos *et al*., 2013) and add greater regional specificity by our focus on the MPN, which is a small sub-region of the entire preoptic area.

To our knowledge, this is the first report on developmental changes in *mir-219* in the brain. Together with *mir-132*, *mir-219* is thought to be involved in the regulation of the biological clock (Cheng *et al*., 2007), with recent reports implicating a role in early development (Hudish *et al*., 2013), oligodendrocyte differentiation (Dugas *et al*., 2010), and modulation of NMDA receptor-mediated effects (Kocerha *et al*., 2009). Interestingly, the *mir-219* and *mir-132* expression patterns were quite similar across development, and most of our bionetwork analyses showed a significant positive correlation between these two microRNAs. Furthermore, these two microRNAs also frequently correlated with serum estradiol levels, especially in males. A previous study comparing *mir-219* and *mir-132* in the fetal, adult, and diseased hippocampus, showed similar expression profiles (Lukiw, 2007), and NMDAR activation downregulated *mir-219* and *mir-132* expression in the adult dentate gyrus *in vivo* (Wibrand *et al*., 2010). Therefore, we speculate that in the MPN, and possibly other regions, *mir-132* and mir-219 are involved in a common signaling pathway.

A developmental decrease in *mir-9* was observed for both sexes. It has been reported that *mir-9* is involved in regulating neurogenesis and maturational events at earlier, fetal stages of brain development (Krichevsky *et al*., 2003; Kapsimali *et al*., 2007; Denli *et al*., 2009). For example, *mir-9* expression was highest in the fetal hippocampus and much lower in adult tissue (Lukiw, 2007). Our results are consistent with that finding.

Several microRNAs in the MPN had sexually dimorphic expression. Females had higher levels of expression of *mir-219, mir-7,* and *mir-145* than males, an effect driven by differences at P30. Although we do not know what this may represent, females mature earlier than males and are farther along in pubertal development than males at this age. There were also sexual dimorphisms in expression of *mir-9* and *let-7b*, with males having higher levels of expression than females at P45, an age when males have just begun to attain adult reproductive function and where serum testosterone concentrations are at their peak (Zanato *et al*., 1994). We suggest that changes in sexually dimorphic microRNAs may be involved in, or reflect, pubertal changes in the MPN, but further work is needed to get at causal relationships.

MicroRNA expression in the MPN increased by A1221 and EB in P30 females, and decreased by A1221 in P90 males

In the females, prenatal A1221 and EB treated rats had increased expression of *mir-219, mir-132, mir-7, mir-145, let-7a,* and *mir-124a* at P30. Similarities between A1221 and EB are consistent with A1221's ability to act via estrogenic mechanisms

(Dickerson *et al*., 2007). Effects of estrogens on neural microRNA expression have primarily been investigated in adults (Rao *et al*., 2013). In the aging female brain, for example, estradiol treatment differentially altered microRNA levels in an age- and brain region-dependent manner (Rao *et al*., 2013). To our knowledge, there are no studies that examined the effect of prenatal estradiol on microRNA expression in hypothalamus; however adult exposure studies in zebrafish and rodents (Cohen *et al*., 2008; Rao *et al*., 2013), in addition to *in vitro* reports on effects of estradiol on microRNA expression in breast cancer cell lines [reviewed in (Klinge, 2009)], underscore the sensitivity of microRNA expression to estradiol treatment.

In the male MPN, expression of all eight microRNAs examined was decreased in prenatally treated A1221 males on P90, an effect not mimicked by EB. This suggests that the mechanism for this A1221 effect in males likely involves a pathway other than through estrogen receptors. A reason for the sex difference may be the already greater exposure of prenatal male than female brains to estradiol (Forest, 1975; Roselli *et al*., 1985), such that the addition of low-levels of exogenous EB had little influence in males compared to females. A1221 is weakly estrogenic but also has been shown to be anti-androgenic at low doses (Bonefeld-Jorgensen *et al*., 2001; Portigal *et al*., 2002) and the decrease in microRNA expression observed in A1221 males may be caused by an antagonistic effect on the androgen receptor.

The finding that prenatal EDC treatment effects are manifested at only a subset of developmental ages is consistent with our previously published study on these same rats, in which we measured mRNA expression in the anteroventral periventricular nucleus

(AVPV) and arcuate nucleus (ARC) (Walker *et al*., 2014). Results from that study showed individual postnatal gene expression profiles that were often affected at one or two ages, but not all ages, in prenatally exposed individuals. In other words, the age of analysis of EDC effects is critically important in determining the outcome. This is not surprising, as the profiles of different genes and proteins in the brain undergo dynamic change throughout postnatal development and may continue to change through aging (Kermath *et al*., 2014).

MicroRNA expression in the VMN changes with postnatal development but have few sex differences or EDC effects

In the VMN, all of the microRNAs studied increased their expression with age in both sexes. Our results for *mir-132, mir-7, mir-145, let-7a,* and *let-7b* are consistent with recent studies on microRNA expression in the medial basal hypothalamus (Sangiao-Alvarellos *et al*., 2013). *Mir-9*, on the contrary, decreased during postnatal development in the medial basal hypothalamus (MBH) (Sangiao-Alvarellos *et al*., 2013), a result that may be explained by our VMN punch being a small sub-region within the entire MBH in which gene expression levels differ from those in neighboring regions.

There were relatively few sex differences or effects of EDCs on microRNA expression in the VMN. In the females, there were modest changes in *let-7a, mir-124a*, and *mir-219* in response to the EDCs that were developmental age-specific. In the male VMN, similarly to the male MPN, A1221 decreased the expression of *mir-124a*. To our

knowledge, there are no other EDC studies examining microRNA expression in the VMN.

mRNA expression of targets of selected microRNAs

We examined the expression levels of mRNAs that were selected based on the microRNA results. In the MPN, few EDC effects were found, but there were several changes with age. In the female MPN, *Igf1r* and *Grin2a* decreased expression with advancing developmental age. Consistent with that result, we reported that *Igf1r* mRNA decreased postnatally from P1 through P60 in the whole preoptic area (POA) of developing male and female rats (Walker *et al*., 2012). However that same study showed a small developmental age-related increase in *Grin2a*, with disparate results from the current study presumably due to differences in the dissection size from the previous (whole POA) and current (MPN) work. In our males, expression of *Ar, Pgr,* and *Ppara* decreased across development. These results were surprising, as the expression of *Ar* in the MPN was reported to increase with age in male hamsters during puberty (Meek *et al*., 1997), and in our earlier rat studies, *Ar* as well as *Pgr* expression underwent significant postnatal developmental increases in the whole POA (Walker *et al*., 2009; Walker *et al*., 2012). We are unaware of any reports on *Ppara* expression in the developing MPN.

Results in the VMN also indicated few gene expression changes, but there were several age effects. In females, an age-related increase in expression of *Lepr, Ppara,* and *Clock* was detected. These genes have not been previously examined in the postnatal developing VMN, to our knowledge. In the male VMN, *Ar* increased with age, consistent

with other work in whole MBH (Walker *et al*., 2012), and *Clock* expression decreased with age. As the only mRNA measured in the VMN of both sexes, *Clock* expression had opposite developmental patterns (increase in females, decrease in males).

Interestingly, for all of the mRNA results in both regions, we did not see a predicted inverse relationship with the microRNA expression. In fact, our network analysis revealed only positive correlations among mRNAs, microRNAs, and hormones. The lack of such a finding is attributable to a number of possibilities. First, we were only able to measure a small number of microRNAs and their mRNA targets. Other microRNAs not measured, and their combinations, may have a stronger association with mRNAs, and vice versa. Second, there are other molecular mechanisms for the regulation of gene expression that include DNA methylation, histone modifications, transcription factors, and post-transcriptional processes, that were not explored in the current study, but which contribute to the absolute expression of any gene. Finally, we have not looked at protein expression, thus changes in microRNA expression might affect protein translation.

Conclusions and implications

Several global conclusions can be drawn for the microRNAs and mRNAs measured herein. First, while both the MPN and VMN are sexually dimorphic in expression of various genes and proteins (Herbison *et al*., 1995; Cao *et al*., 2011), of the microRNAs measured here, expression was sexually dimorphic in the MPN, but not the VMN. Second, the PCB mixture A1221 affected microRNA expression in the MPN, and

to a lesser degree, in the VMN, in a region-, sex-, and age-specific manner. This finding indicates the importance of analyzing endpoints in both sexes and at multiple ages during postnatal development. Third, few treatment changes in the target mRNA expression were found, suggesting that mechanisms of action other than the selected subset of microRNAs are involved in regulation of their overall expression levels. Sex differences in microRNA expression have become an area of investigation for sex-biased neurobiological diseases such as autism, schizophrenia, and stroke (Guan *et al*., 2014; Mundalil Vasu *et al*., 2014; Selvamani *et al*., 2014). Furthermore, an increase in EDC exposures has been linked to playing a role in the rise in these multifactorial disorders (Brown, 2009; Melzer *et al*., 2012; Kalkbrenner *et al*., 2014). While it is premature to draw any strong inferences from these correlations, further research on connections among EDCs, microRNAs, and neurobehavioral outcomes is warranted.

Chapter 3: Sexually dimorphic effects of gestational endocrine-disrupting chemicals on adult behaviors and mRNA expression

Abstract

Prenatal endocrine-disrupting chemicals [EDCs] exposures during critical periods of development may influence reproductive behaviors and the underlying neural architecture later in adulthood. This study examined effects of low-level gestational exposures to environmental EDCs on adult sociosexual behaviors, numbers and spectral characteristics of ultrasonic vocalizations [USVs], and gene expression changes in two hypothalamic nuclei. Pregnant Sprague-Dawley rats were injected on gestational days 16 and 18 with vehicle [DMSO], estradiol benzoate [EB, 50 ug/kg], or a weakly estrogenic mixture of polychlorinated biphenyls [A1221, 1 mg/kg or 0.5 mg/kg]. Littermates were behaviorally characterized on P60. After the behavioral testing was completed, the animals were euthanized on P90. The medial preoptic nucleus [MPN] and ventromedial nucleus [VMN] of the hypothalamus, selected for their roles in reproductive function and behaviors, were microdissected and assayed for expression of 48 hypothalamic genes. The number and bandwidth of ultrasonic vocalizations were sexually dimorphic and sensitive to prenatal 0.5mg/kg A1221 treatment. Principle component analysis identified five sexually dimorphic USV measures that comprised the feminization score, and was used to accurately predict sex effects in the USV experiment. Females, but not males, showed the sociosexual preference for hormone-primed opposite-sex conspecific. Treatment with EB, 0.5mg/kg A1221 or 1mg/kg A1221 decreased nose-touching in

males [salient measure of direct social contact]. In the MPN, steroid hormone receptors and *Kiss1* gene expression was increased in 0.5mg/kg A1221 females, while in the VMN, PCB effects were sex- and treatment-specific on a subset of neuroendocrine genes [*Lepr, Foxp2, Esr1, Drd1a, Avpr1a, Crh, Oxt*]. Bionetwork analysis showed the 0.5mg/kg A1221 group as the most different from DMSO, and, when examined for USV-associated behavioral measures, showed a robust increase in the number of correlations and network complexity. In summary, adult behaviors and selected hypothalamic genes were affected by prenatal EDCs, with 0.5mg/kg A1221 groups showing the most changes.

Introduction

Heightened sensitivity to circulating gonadal hormones during late gestation could sculpt the developing brain into male- or female-typical patterns. Exposure to environmental endocrine-disrupting chemicals [EDCs] during this period can change the neuroendocrine substrates and result in impaired metabolic, reproductive, neurochemical, and hormonal function in adulthood (Steinberg *et al*., 2007; Dickerson *et al*., 2011a; Dickerson *et al*., 2011b; Walker *et al*., 2013; Leon-Olea *et al*., 2014; Walker *et al*., 2014). A discernable consequence of endocrine disruption of the developing brain is the perturbation of adult behaviors. Published data on many EDCs, including polychlorinated biphenyls (PCBs), bisphenol A (BPA), and vinclozolin, show effects on social and sociosexual behavior in a sex and treatment-specific manner (Colbert *et al*., 2005; Ogi *et al*., 2013; Reilly *et al*., under review). Exposure to the single PCB congener 77 diminishes partner preference in female rats for a male over a female stimulus animal

(Cummings *et al*., 2005). Aroclor 1221 (A1221), a more complex mixture of lightly chlorinated PCBs used in the current study, disrupts paced mating behavior in female rats (Steinberg *et al*., 2007) and same-sex social interactions in male rats (Reilly *et al*., under review). BPA exposures in mice decrease social motivational behavior in group setting in females, but increase it in males (Ogi *et al*., 2013), resulting in decreased preference to mate with exposed males (Jasarevic *et al*., 2011), and masculinized play behaviors in treated females (Dessi-Fulgheri *et al*., 2002). Prenatal treatment with anti-androgen vinclozolin increases play behaviors, but reduces copulatory behaviors in male rats (Colbert *et al*., 2005). Interestingly, environmentally low doses of 17alpha-ethinylestadiol also disrupt appetitive components of sexual behavior that influence the rate of copulation (Della Seta *et al*. 2008).

We hypothesized that low doses of estrogenic A1221 would similarly disrupt affiliative components of opposite-sex interactions. The first test examined the number and quality of ultrasonic vocalizations produced after a period of interaction with the opposite-sex animal. A second behavioral test investigated sociosexual preference behaviors, namely whether the rats preferred to spend time with a hormone-implanted opposite-sex rat or a hormone-deprived opposite-sex animal. Finally, we measured hypothalamic gene expression in two sexually-dimorphic, hormone-sensitive hypothalamic nuclei, medial preoptic nucleus (MPN) and ventromedial nucleus (VMN), known to be involved in neuroendocrine control of social and sociosexual behaviors (Mathews *et al*., 1977; Clark *et al*., 1981; Malsbury *et al*., 1977; Hull *et al*., 2006; Hoshina *et al*., 1994, Kato *et al*., 2000).

Materials and methods

Experimental animals

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas at Austin, permit number 201003712. Virgin female (n=40) and male Sprague-Dawley rats (n=15), two-three months of age, were purchased from Harlan Laboratories (Houston, TX). On arrival, animals were housed in same-sex groups, 2-3 animals per cage and provided low phytoestrogen Harlan-Teklad Extruded 2019 Global Rodent diet and water *ad libidum*. Animals were acclimated to housing conditions: temperature (21 – 22 ° C) with a partially reversed 12:12 light cycle (lights on at 12:00 AM). After two weeks of regular estrous cycles, the female rats were impregnated by the males from the same cohort.

In the ultrasonic vocalizations experiment, the stimulus rat for male subjects was an ovariectomized [OVX] + E2 female of confirmed receptivity. The stimulus rat for female subjects was a sexually experienced intact male [age-matched unrelated sire]. Two novel gonadectomized opposite-sex animals (one hormone-implanted [E2-females; T-males] and one without hormone) were used as stimulus animals in the sociosexual preference experiment.

Treatments

On embryonic days 16 and 18 (E1 = day of confirmed sperm presence in vaginal smear), dams were weighed and randomly assigned to one of four treatment groups and injected intraperitoneally with 0.1 ml of vehicle (3% dimethylsulfoxide [DMSO], catalog

no. D4540; Sigma-Aldrich, St. Louis, MO; diluted in sesame oil, catalog no.156621, MP Biomedicals, Carlsbad, CA), 50μg/kg estradiol benzoate [EB] (catalog no. E8515; Sigma-Aldrich) as a positive estrogenic control, 0.5 mg/kg A1221 or 1 mg/kg A1221 (catalog no. C-221N, AccuStandard, New Haven, CT). The dosages are within the range of PCB body burdens found in humans (Mitchell *et al.*, 2012). Previous work in the laboratory using these dosages has been shown to be non-toxic to dams nor cause fetal loss (Steinberg *et al.*, 2007; Dickerson *et al.*, 2011a; Dickerson *et al.*, 2011b); Walker *et al.*, 2013; Walker *et al.*, 2014). On the day after birth (postnatal day [P] 1), litter composition was recorded and the litters culled to equal sex ratios of 4 males and 4 females (8 pups) per litter. Pups were weaned at P21 and rehoused in same-sex dyads where they were monitored daily for signs of pubertal development: vaginal opening (VO) in females and preputial separation (PPS) in males (Steinberg *et al*., 2007; Walker *et al*., 2012). Following VO, daily vaginal smears were taken and cell cytology examined as a measure of estrous cyclicity in the females.

Behavior testing

Beginning at age P60, male and female littermates were tested in the ultrasonic vocalizations and sociosexual behavioral paradigms. Testing was conducted under dim red light, two - six hours after lights off, with experimental females tested on proestrus when confirmed sexually receptive. Due to the extensive behavioral testing and the large number of animals necessary for the study, the animals were tested in eight cohorts (of 10 animals each) with treatments equally assigned to each cohort.

Ultrasonic vocalizations: Ultrasonic vocalizations experiment was a three-day test in a sound-attenuated Plexiglas apparatus with a chamber (23l X 29w X 40h cm) divided by a wire mesh, and a microphone (CM16/CMPA, Avisoft, Germany). Days 1 and 2, the experimental rat was placed into the apparatus, and USVs recorded for 10 minutes to establish a baseline. Day 3, the rat was returned to the chamber and an unfamiliar opposite-sex stimulus animal placed on the other side of the wire mesh. After five minutes, the stimulus animal was removed and USVs recorded from the experimental animal for 10 minutes (McGinnis *et al.*, 2003). USVs were recorded with UltraSoundGate hardware and software and analyzed with Saslab Pro (all Avisoft). Recordings were played at 512 FFT-length and 75% overlap while whistle tracking element separation, optimized to testing conditions, was used to automatically detect and characterize calls. Number of flat and frequency-modulated 50kHz USVs were quantified for Days 1-3. Power spectrum measures (dominant frequency, entropy, bandwidth, internode interval, and duration) were quantified for Day 3 only. Frequency-modulated USVs were further characterized by the type of modulation into rises, trills, and steps (Wright *et al.*, 2010).

Principle component analysis: We performed a principle component analysis on data from vehicle-treated males and females to identify the attributes of vocalizations that loaded strongly on sex. Using JMP 11 statistical software, we scored sex as a dummy variable (-1 = male, +1 = female), and analyzed all of our measures of Day 3 vocalizations. These measures included numbers of vocalizations in each category of USV, as well as bandwidth, entropy, and duration measures in each category. After

varimax rotation of the PCA results, we identified a single factor that loaded highly on sex; we interpret this factor as a measure of feminization/masculinization. We used the factor loadings to create a composite score that could be used to investigate the effects of our treatments on the sex-typical patterns of vocalization.

We calculated the feminization/masculinization PCA score for all animals (all treatments and sexes) by converting each of the variables that loaded highly onto sex into a Z-score, then weighting each of the variables by its loading onto the sex-specific factor identified in the PCA. For simplicity, we included only those variables with a factor loading of magnitude greater than 0.30. The formula for computing the composite score was: PCA score = 0.32 x number of flat calls Z score - 0.34 x duration of rises Z score + 0.47 x duration of steps Z score + 0.46 x bandwidth of steps Z score + 0.42 x bandwidth of trills Z score. To confirm that this score was sexually dimorphic, we first performed a one-way ANOVA for effects of sex on the score, as well as on its component variables. Lastly, we calculated a PCA score for all individuals in our study, and used a two-way ANOVA to calculate the effects of sex, treatment, and sex-by-treatment interactions.

Sociosexual preference behavior: The sociosexual behavior test used a Stoelting Any-Maze three-chamber plexiglas apparatus (100l X 100w X 34.5h cm total size), with restraint cages containing the stimulus animals placed in opposite corners of the side chambers (Moy *et al.*, 2004; Crews *et al.*, 2012). The test took place immediately after USV testing on Day 3 was completed. The experimental animal was habituated in the center compartment for five minutes with the side chamber doors closed. After habituation, the doors were opened, and the experimental animal allowed to explore the

whole arena for 10 minutes. Any-Maze software quantified distance travelled, average speed, time standing still, and time spent near each stimulus animals. Other behaviors (time at plexiglas, time spent investigating stimulus animals enclosure, rearing, grooming, and nose touching) were manually scored by a trained observer blind to treatment.

Tissue collection and storage

Testing was completed by P90 and experimental animals were weighed and euthanized one – to - three hours before lights out via rapid decapitation. Trunk blood samples were collected, allowed to clot, and centrifuged to obtain serum samples. Brains were removed and cut into one mm sections; and one mm micropunches of MPN and VMN were collected using methods reported previously (Walker *et al.*, 2013; Walker *et al.*, 2014). Females exhibiting estrous cycles were euthanized on proestrus based on vaginal cytology. Tissues and organs were stored at -80°C until use.

RNA isolation

Total RNA was isolated from frozen MPN and VMN punches of behaviorally tested male and female rats using a mirVana miRNA isolation kit according to the manufacturer's protocols (catalog no. AM1560, Life Technologies, Carlsbad, CA). All RNA samples were analyzed for quantity by Nanodrop spectrophotometry and run on Bioanalyzer 2100 nanodrop kit (catalog no. 5067-1511, Agilent Technologies, Santa Clara, CA) to assess RNA purity and integrity. Only RNA samples with the RIN of eight

or higher were used in subsequent experiments. Based on this criterion, five samples were excluded from the analysis in the MPN, and eight were excluded from the analysis in the VMN, resulting in a sample size of eight – to – ten animals per group. No animals failed this criterion for both regions.

cDNA synthesis and Taqman microfluidic real-time PCR cards

Total RNA (200 ng total) was used to generate cDNA using high-capacity cDNA reverse transcription kit with RNase inhibitor (catalog no. 4374966, all Life Technologies) according to the manufacturer's recommended protocols. For Taqman Loq Density Array (TLDA) analysis, custom-designed microfluidic 48-gene PCR cards (Life technologies) were used to analyze the chosen mRNAs. Real-time PCR was carried out on an ABI ViiA7 using Taqman universal master mix (catalog no. 4324018, Life Technologies) and the following run parameters: 95 °C for 10 minutes, 50 cycles of 95 °C for 15 seconds, and 60°C for 1 minute. Relative expression was determined for each sample using the comparative cycle threshold (Ct) method (Pfaffl, 2001). The Cts were further calibrated to the median δ-cycle threshold of the DMSO females to determine the relative Ct value for each mRNA.

Statistics

All statistical analyses were conducted using R software. Initial statistical analyses using Analysis of variance (Terranova *et al.*, 1993) were used to identify any potential cohort, litter, and sex effects within groups. ANOVA tests were also used to

compare the treatment groups. All data were normally distributed and homoscedastic. When appropriate, a repeated measures multiple comparisons MANOVA (which addresses the false discovery rate of multiple comparisons) was used to analyze behavioral measures between the lateral chambers in the Anymaze apparatus and the numbers of ultrasonic vocalizations on three days of USV experiment. Grubb's test was used to exclude up to 2 outliers per group in the dataset. Posthoc analyses included t-test for sex effect and Tukey HSD for treatment and interaction effects. All data are presented at mean ± standard error of the mean. Because genes and behavioral measures were specifically chosen based on an *a priori* hypothesis, we report statistical significance as $P < 0.05$ and trends as $P < 0.1$.

Bionetwork analysis

To examine possible relationships among behaviors, genes, and serum corticosterone levels (hormone data published in Reilly *et al.,* under review), the data were analyzed using a bootstrap technique, as previously reported (Walker *et al.*, 2013; Walker *et al.*, 2014). The TLDA data for MPN and VMN were used to generate the networks for each region. The following behavioral data were affected by treatment and selected for network generation: USV numbers and bandwidth on Day 3, time spent near each stimulus animal, stimulus animal enclosure exploration, and nose touching. The sociosexual measures were presented as ratio of GDX+hormone measure divided by sum of GDX+hormone and GDX measures.

Results

There were no effects of litter or cohort on any of the behavioral or gene expression measures examined.

Ultrasonic vocalizations (USV): Females vocalized higher numbers of USVs than males: numbers of flat ($F_{1, 476} = 18.7$, $p < 0.001$), rise ($F_{1, 480} = 4.7$, $p < 0.05$), and step ($F_{1, 477} = 10.5$, $p<0.005$) USVs, but not trill USVs, were significant for the main effect of sex. Number of calls increased across the three-day trial and were significantly higher for all call categories on Day 3 compared to Days 1 or 2 (*females*: **flats**: $F_{2, 148} = 39.2$; **rises**: $F_{2, 152} = 37.5$; **steps**: $F_{2, 151} = 52.7$; **trills**: $F_{2, 148} = 34.2$; *males*: **flats**: day, $F_{2, 144} = 107.7$; **rises**: $F_{2, 145} = 85.5$; **steps**: $F_{2, 149} = 99.6$; **trills**: $F_{2, 147} = 58.2$, all $p < 0.001$). A main effect of prenatal treatment was found, with the 0.5 mg/kg A1221 groups having increased numbers of rise calls in females ($F_{3, 79} = 5.6$, $p < 0.005$) and males ($F_{3, 71} = 9.4$, $p < 0.0005$) compared to their DMSO counterparts. In males, the 0.5 mg/kg A1221 group also had increased numbers of step calls ($F_{3, 75} = 8.0$, $p < 0.001$). Furthermore, the treatment x day interaction was significant with the increase in the numbers of USVs produced by 0.5 mg/kg A1221-treated rats observed on day 3 only for numbers of rises ($F_{6, 152} = 2.8$), steps ($F_{6, 151} = 2.8$, both $p < 0.05$) and trills ($F_{6, 148} = 3.88$, $p < 0.005$) in females and numbers of rises ($F_{6, 145} = 4.95$) and steps ($F_{6, 149} = 5.05$, both $p < 0.0005$) in the males (**Figure 3.1A-H**).

Power spectrum analysis revealed bandwidth of the flat, rise, step, and trill USVs significant for the main effect of sex (**flats**, $F_{1, 78} = 5.5$; **rises**, $F_{1, 72} = 5.0$, both $p < 0.05$; **steps**, $F_{1, 75} = 12.2$; **trills**, $F_{1, 70} = 12.9$, both $p < 0.001$) where the females produced

calls with higher bandwidth compared to males. The entropy was also significant for the main effect of sex, with the females producing calls with higher entropy than males in rise ($F_{1, 74} = 8.7$, $p < 0.005$), step ($F_{1, 72} = 5.7$, $p < 0.05$), and trill ($F_{1, 73} = 7.1$, $p < 0.01$), but not flat USVs. Main effect of prenatal EB and 1 mg/kg A1221 treatment increased bandwidth of male calls with EB increasing it for rise calls ($F_{3, 34} = 3.9$, $p < 0.05$), and 1 mg/kg A1221 increasing it for trill calls ($F_{3, 31} = 4.5$, $p < 0.01$) (**Figure 3.2A-H**). There were no sex effects for duration, internode interval, and dominant frequency. In males, main effect of prenatal treatment with 0.5 mg/kg A1221 increased duration of flat calls ($F_{3, 35} = 3.2$, $p < 0.05$) (**Figure 3.3A-L**).

Principle component analysis (PCA) of acoustic properties and USV numbers: There were 16 components total in the PCA analysis of acoustic properties and USV numbers. Sex loaded onto components 1 (score = 0.49) and 7 (score = 0.46) most strongly. To clarify the interpretation of factors, we performed a varimax rotation of the PCA analysis. After rotation, the factor loadings for 1 and 7 were (0.35) and (0.74), respectively. Because factor 7 loaded most strongly on sex, we interpret it as a measure of feminization/masculinization in USV. We identified five measures in factor 7 with a factor loading of magnitude > 0.30: trill bandwidth (0.42), step bandwidth (0.46), step duration (0.47), rise duration (-0.34), and numbers of flat USVs (0.32). These measures were analyzed in DMSO animals for the effects of sex. Consistent with the signs of their factor loadings, four measures (trill bandwidth, step bandwidth, step duration, and numbers of flat calls) were higher in DMSO females than DMSO males, while duration

of rise calls was higher in DMSO males than DMSO females (all, $p < 0.01$) (**Figure 3.4A-D**).

A PCA score based on our factor loadings was calculated for all animals as described in the methods, and then analyzed by two-way ANOVA for effects of sex, treatment, and their interactions. We found a main effect of sex ($F_{1, 19} = 14.28$, $p < 0.01$), but no treatment or treatment x sex interactions (**Figure 3.4E**).

Sociosexual preference behavior: The main effect of sex was significant in several sociosexual behavior measures. Females, but not males, spent more time near the hormone primed stimulus animal ($F_{1, 65} = 19.1$, $p < 0.0005$), investigating its enclosure ($F_{1, 64} = 13.7$, $p < 0.0005$), and nose-touching with it ($F_{1, 63} = 8.5$, $p < 0.005$) compared to the castrated animal. Males took longer time to start investigating the stimulus animal enclosure ($F_{1, 228} = 19.8$, $p < 0.0005$). Additionally, the main effect of treatment was significant for males treated with prenatal PCBs (1 mg/kg or 0.5 mg/kg A1221) or EB who spent less time nose-touching with stimulus animals, irrespective of the stimulus animal hormone status ($F_{3, 61} = 7.6$, $p < 0.0005$) **(Figure 3.5A-D)**. Distance travelled ($F_{1, 132} = 89.7$, $p < 0.0005$), average speed ($F_{1, 132} = 90.2$, $p < 0.0005$), and time at plexiglas ($F_{1, 132} = 44.0$, $p < 0.0005$) were significant for the main effect of sex with females engaging more time in these activities than males. Compared to females, males spent more time standing still ($F_{1, 129} = 35.2$, $p < 0.0005$) and grooming ($F_{1, 127} = 9.3$, $p < 0.005$). Main effect of treatment was significant for the 1 mg/kg A1221 males who spent less time rearing than DMSO males ($F_{3, 60} = 3.2$, $p < 0.05$) (**Figure 3.6A-F**).

Medial preoptic nucleus TLDA results: In females, main effect of prenatal treatment with 0.5 mg/kg A1221 increased the expression of *Ar* ($F_{3, 35} = 6.7$, $p < 0.05$), *Esr1* ($F_{3, 36} = 7.4$, $p < 0.001$), *Esr2* ($F_{3, 35} = 5.6$, $p < 0.005$), and *Kiss1* ($F_{3, 29} = 6.6$, $p < 0.005$). *Ar* expression was also increased by prenatal 1 mg/kg A1221 treatment in females ($F_{3, 35} = 6.7$, $p < 0.05$). There were no treatment effects in the males compared to the DMSO. *Esr1* ($F_{1, 73} = 4.4$, $p < 0.05$) was significant for the main effect of sex in the MPN with higher levels of expression in the females compared to the males **(Figure 3.7A-D)**. The following genes were also significant for the main effect of sex in the MPN, and had higher levels of expression in females compared to males: *Drd2* ($F_{1, 72} = 4.9$, $p < 0.05$), *Crh* ($F_{1, 71} = 15.2$, $p < 0.0005$), *Egr1* ($F_{1, 68} = 24.6$, $p < 0.0005$), *Igf1* ($F_{1, 72} = 7.0$, $p < 0.01$), *Igr1r* ($F_{1, 70} = 4.7$, $p < 0.05$), and *Per1* ($F_{1, 71} = 6.9$, $p < 0.05$). The genes *Slc6a3* ($F_{1, 64} = 7.7$, $p < 0.01$) and *Oprd1* ($F_{1, 69} = 10.0$, $p < 0.005$) showed main effect of sex with higher levels of expression in males compared to females (**Figure 3.8A-H**).

Ventromedial nucleus TLDA results: In females, the main effect of prenatal EB treatment increased expression of *Esr1* ($F_{3, 34} = 3.9$, $p < 0.05$), while 0.5 mg/kg A1221 decreased expression of *Drd1a* ($F_{3, 37} = 4.9$, $p < 0.01$), and 1 mg/kg A1221 increased expression of *Avpr1a* ($F_{3, 38} = 3.6$, $p < 0.05$). In males, the main effect of prenatal EB and 0.5 mg/kg A1221 increased expression of *Crh* ($F_{3, 32} = 3.8$, $p < 0.05$), while 0.5 mg/kg A1221 increased the expression of *Foxp2* ($F_{3, 36} = 3.3$, $p < 0.05$) and *Lepr* ($F_{3, 36} = 4.9$, $p < 0.01$); 1 mg/kg A1221 increased the expression of *Oxt* ($F_{3, 34} = 4.1$, $p < 0.05$). *Esr1* expression was significant for the main effect of sex in the VMN with higher levels of expression in the females compared to the males **(Figure 3.9A-G)**. The following genes

were also significant for the main effect of sex in the VMN with higher levels of expression in females compared to males: *Pgr* ($F_{1, 80} = 13.6$, $p < 0.0005$), *Drd3* ($F_{1, 78} = 8.1$, $p < 0.01$), *Oprk1* ($F_{1, 78} = 11.7$, $p < 0.005$), and *Per2* ($F_{1, 80} = 8.9$, $p < 0.005$). The genes *Grin2a* ($F_{1, 80} = 11.0$, $p < 0.005$), *Foxp2* ($F_{1, 78} = 11.4$, $p < 0.005$), *Rapgef4* ($F_{1, 80} = 4.3$, $p < 0.05$), and *Shank1* ($F_{1, 80} = 6.0$, $p < 0.05$) were significant for the main effect of sex with higher levels of expression in males compared to females (**Figure 3.10A-H**).

Bionetwork analysis: Bionetwork analysis was conducted using Pearson correlation coefficients to enable investigation into relationships among behaviors, hormones, and gene expression in the MPN. In the female MPN (**Figure 3.11**), all networks showed more positive than negative correlations, however the complexity of the networks (qualitative visual measure of network organization) and the identity of the correlated measures in DMSO and treated networks differed. Relative to DMSO, EB treatment resulted in visually more complex and organized networks with higher number of correlations, and 1mg/kg A1221 resulted in less organized networks, while 0.5mg/kg A1221 did not differ visually. Additionally, female EB networks showed a greater number of positive correlations between genes than DMSO and PCB-treated females.

The effects of treatment on the identity of correlated measures were examined by comparing the correlations formed by bandwidth of rise calls. Bandwidth of flat, rise, step and trill USVs correlated with several interesting measures in the female MPN. While there are too many bandwidth correlations to discuss here, we will provide a detailed description of correlations formed by bandwidth of rise calls. In DMSO females, bandwidth of rise calls formed positive correlations with *Avpr1a, Ar, Foxp2* gene

expression, in addition to positive correlations with the numbers of rise and step USVs, and negatively correlated with *Avp* and *Oxt* gene expression. In EB females, bandwidth of rise calls formed positive correalations with *Oprd1, Esr2, Shank1*, and negatively correlated with *Lepr, Oxtr, and Oprk1*. In 0.5mg/kg A1221 females, bandwidth of rise calls formed positive correlations with gene expression of the following genes in the MPN: *Rapgef4, Oprd1, Oprl1, Fmr1, Grin2b, Grin1, Kiss1, Oxt, Oxtr, Crh, Ar, Nlgn3, Clock, Per2, Nr3c1, Slc6a3, Foxp1*, and negatively correlated with *Drd1a, Bdnf,* and *Th* gene expression. In 1mg/kg A1221 females, bandwidth of rise calls formed one positive correlation with *Th* gene expression (**Figure 3.11A-D**).

In the male MPN (**Figure 3.12**), 0.5mg/kg A1221 treatment, but not EB or 1mg/kg A1221, affected network complexity and resulted in a more organized network with higher number of correlations, compared to DMSO. Despite this differential effect on network organization, all treatments affected the identity of correlated relationships.

We will compare the effects of treatment on identity of correlated measures by comparing the correlations formed by bandwidth of rise calls. In DMSO males bandwidth of rise calls did not form any correlations. In EB males, bandwidth of rise calls formed positive correlations with *Bdnf, Gper, Lepr,* and *Drd1a*, and a negative correlation with *Grin2b*. In 0.5mg/kg A1221 males, bandwidth of rise calls formed positive correlations with bandwidth of flat calls and gene expression of Drd1a and negative correlations with *Oprd1, Oprl1, Lepr, Drd2, Kiss1r, Clock, Per1, Nlgn3, Fmr1,* and *Nr3c1*. In 1mg/kg A1221 males, bandwidth of rise calls formed a positive correlation with *Igf1r* and a negative correlation with *Ar* gene expression.

Bionetworks were also used to analyze the relationships among behaviors, hormones, and gene expression in the VMN. In the female VMN (**Figure 3.13**), the complexity of the networks were as follows: EB > DMSO > 0.5mg/kg A1221 > 1mg/kg A1221, where EB treatment resulted in more complex networks, with higher number of correlations, and 0.5mg/kg A1221 and 1mg/kg A1221 networks having fewer number of correlations, all compared to DMSO. In the female VMN, bandwidth of rise calls was differentially affected in treated networks compared to DMSO, and illustrates an example of EDC effects on gene-behavior-hormone relationships. In DMSO females, bandwidth of rise calls formed positive correlations with numbers of rise and step USVs, and *Oprd1*, and formed negative correlations with *Oprk1, Dbh, Drd1a, Foxp1, Crh, Egr1, Nlgn3, Fmr1, Igf1r, Clock,* and *Shank 1*. In EB females, the bandwidth of rise calls formed positive correlations with *Oxt, Drd1a, Pgr, Esr2, Igf1,* and *Grin2a*, and formed negative correlation with *Oprm1*. In 0.5mg/kg A1221 or 1mg/kg A1221 females, bandwidth of rise calls did not form any correlations.

In the male VMN (**Figure 3.14**), treatment with EB or 0.5mg/kg A1221 resulted in visually more complex networks compared to DMSO, while 1mg/kg A1221 networks did not differ from DMSO. However, identity of the correlated relationships was affected by PCB treatment and differed depending on the treatment. In DMSO males, bandwidth of rise calls did not form any correlations, while in EB males bandwidth of rise calls correlated positively with *Pgr* expression and negatively with *Per1* gene expression. In 0.5mg/kg A1221 males, bandwidth of rise calls formed positive correlations with *Ar, Esr1, Kiss1, Lepr, Per2, Per1, Arntl, Drd1a, Pgr, Grin2b,* and *Grin1*, and a negative

correlation with *Foxp2*. In 1mg/kg A1221 males, bandwidth of rise calls formed negative correlations with *Esr2, Arntl,* and *Th.*

Figures

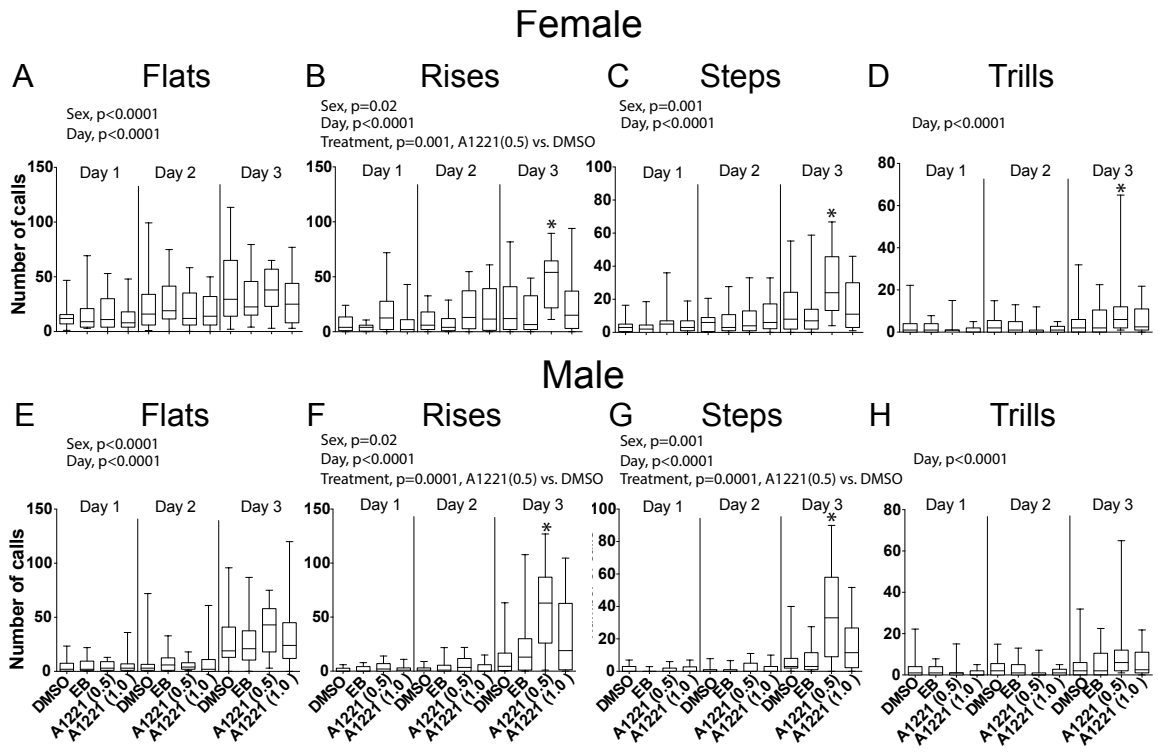

Figure 3.1: Number of flat, rise, step, and trill ultrasonic vocalizations (USVs) in females (**A-D**) and males (**E-H**). Significant main effects of day, sex, and treatment are shown. Significant post-hocs for treatment are indicated when the treatment x day interaction was significant as *$p < 0.05$ 0.5 mg/kg A1221 vs. DMSO on same day. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

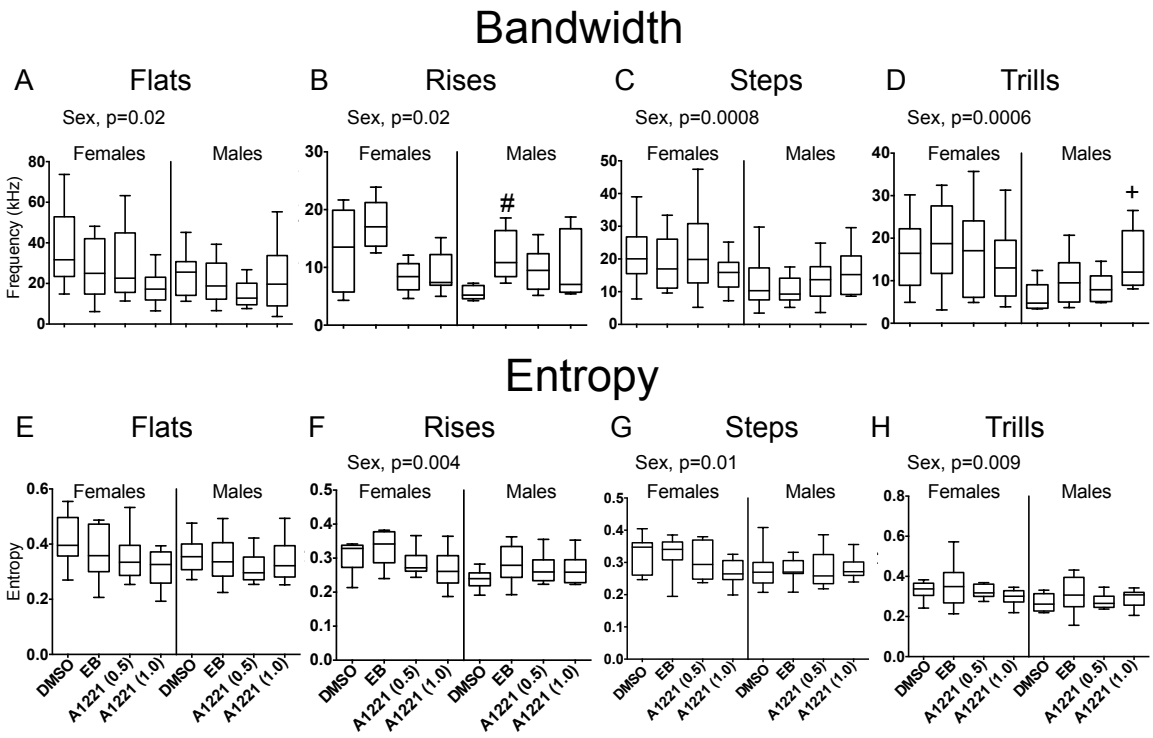

Figure 3.2: Bandwidth (**A-D**) and entropy (**E-H**) of flat, rise, step, and trill ultrasonic vocalizations (USVs) on Day 3 in males and females. Significant main effects of sex are shown. Significant post-hocs for treatment are indicated when the main effect of treatment within each sex was significant as **+**$p < 0.05$ 1 mg/kg A1221 vs. DMSO; #$p < 0.05$ EB vs. DMSO. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

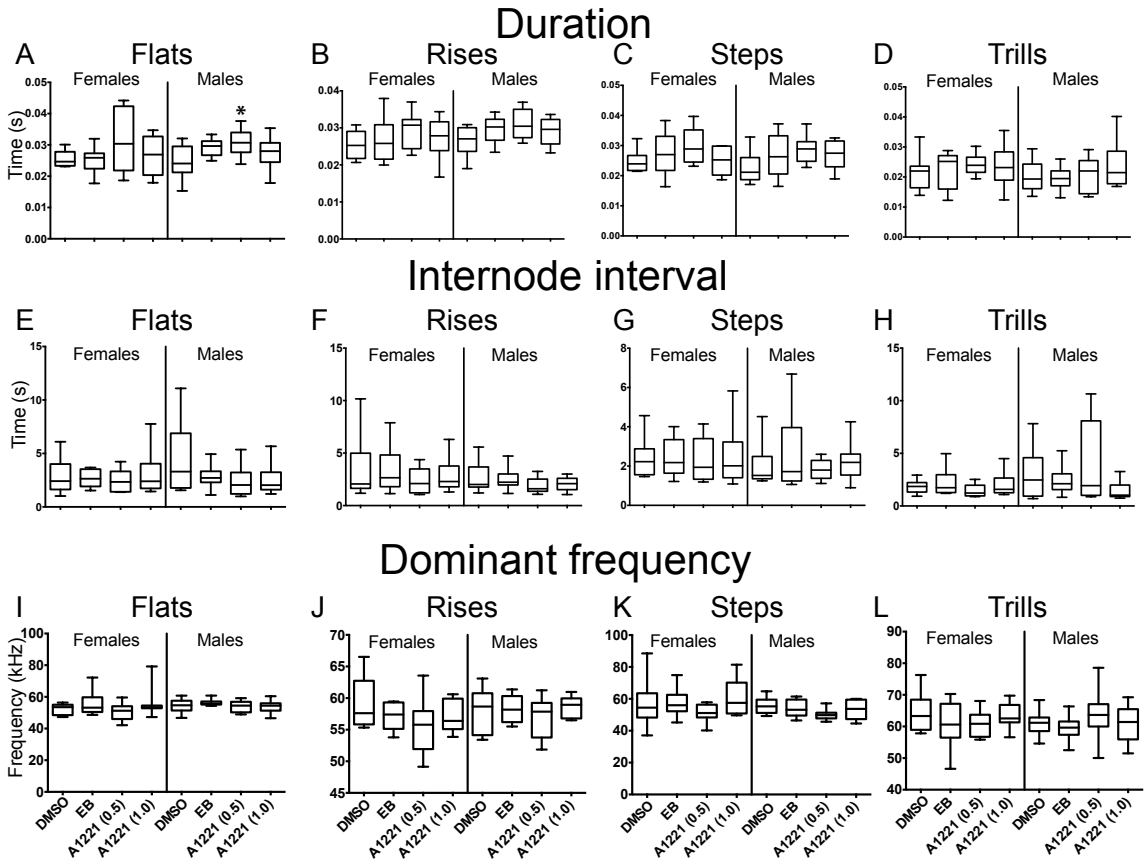

Figure 3.3: Duration (**A-D**), internode interval (**E-H**), and dominant frequency (**I-L**) of flat, rise, step, and trill ultrasonic vocalizations (USVs) on Day 3 in males and females. Significant post-hocs for treatment are indicated when the main effect of treatment within each sex was significant as *$p < 0.05$ 0.5 mg/kg A1221 vs. DMSO. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

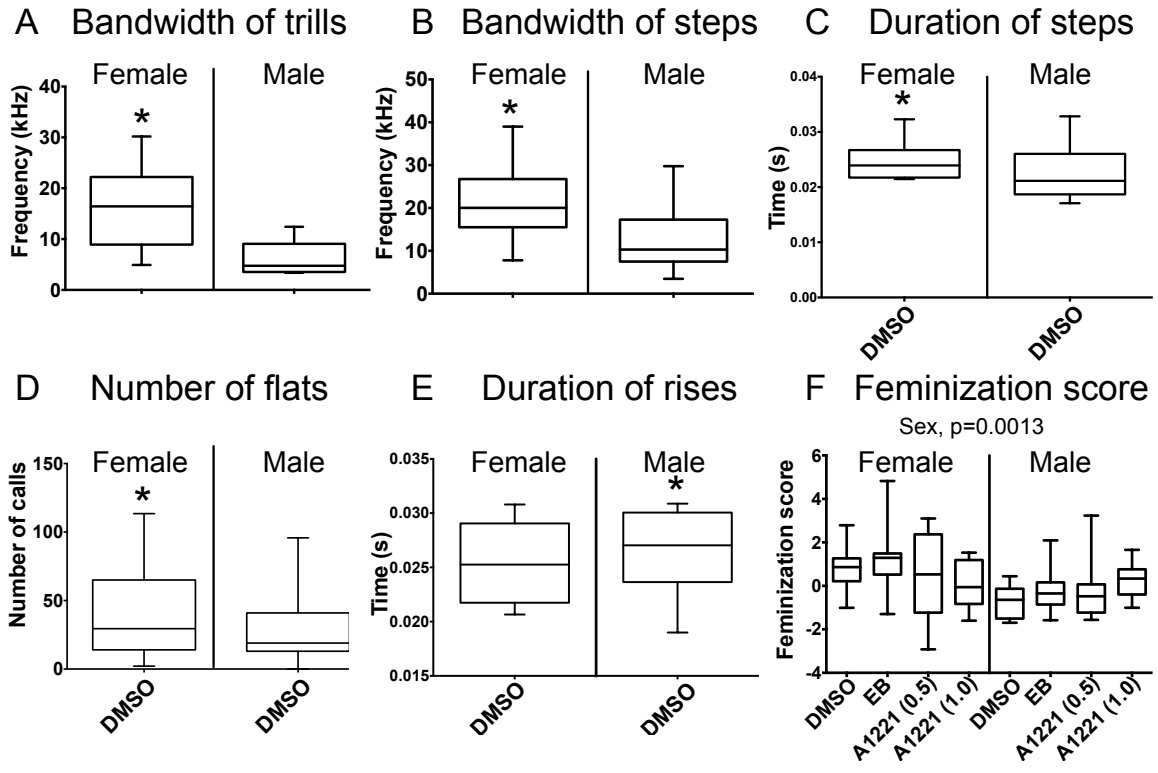

Figure 3.4: Sexually dimorphic USV measures that comprise the feminization score: bandwidth of trills and steps, duration of steps, number of flats, and duration of rises (**A-E**). Feminization score is shown in **F**. Significant main effect of sex is shown for **F** as p-value. Significant effects of sex are shown for **A-E** as *p<0.05 DMSO female vs. DMSO male. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

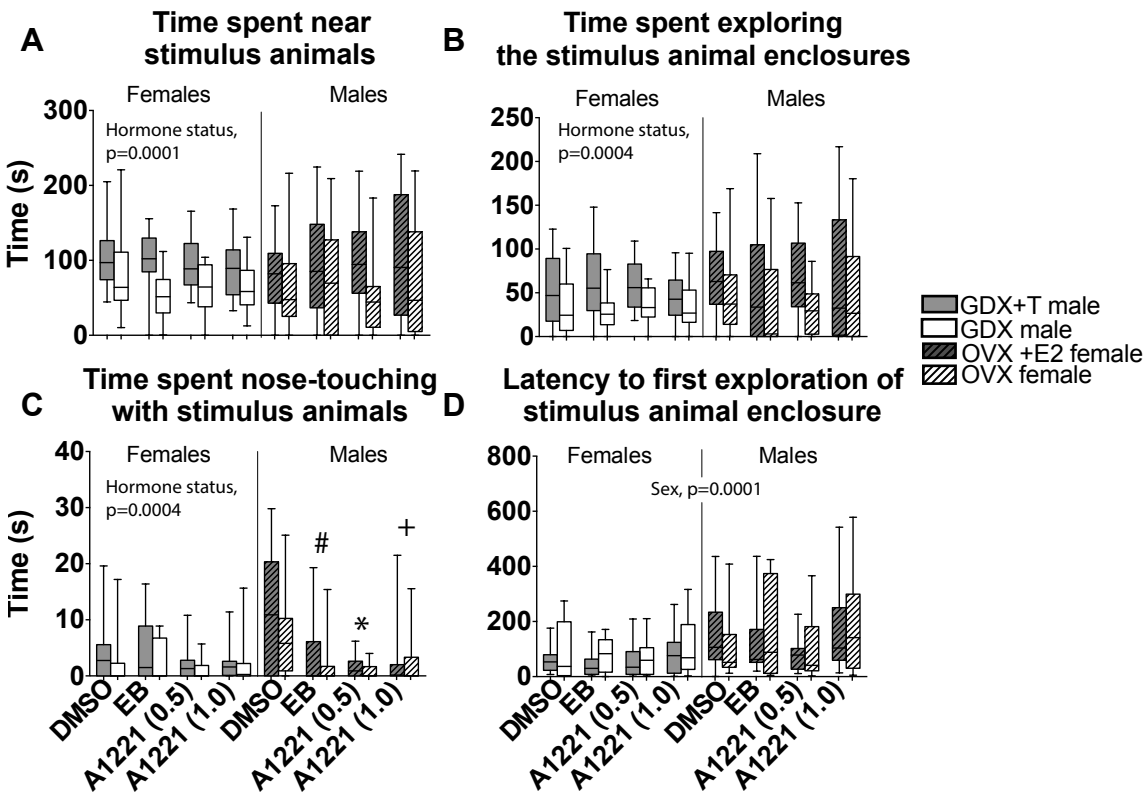

Figure 3.5: Sociosexual preference behaviors: time spent near stimulus animals, exploring their enclosures, and nose-touching with them (**A-C**). Latency to first exploration of stimulus animal enclosure is also shown (**D**). Significant main effects of sex and hormone status in females are shown. Significant post-hocs for treatment are indicated when the main effect of treatment was significant within each sex as *<0.05 0.5 mg/kg A1221 vs. DMSO; #p < 0.05 EB vs. DMSO. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221; GDX male, gonadectomized male; GDX+T male, gonadectomized male with testosterone replacement; OVX female, ovariectomized female; OVX+E2 female, ovariectomized female with estradiol replacement.

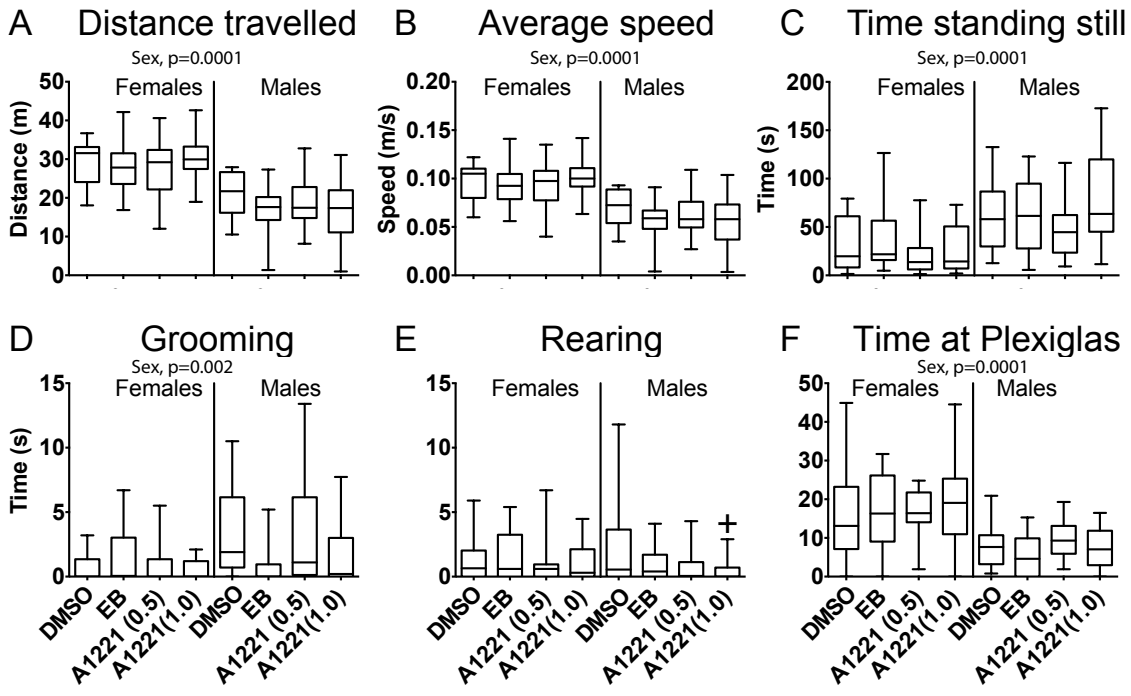

Figure 3.6: Sociosexual preference behaviors: distance travelled, average speed, time standing still, grooming, rearing, and time at Plexiglas (**A-F**). Significant main effects of sex are shown. Significant post-hocs for treatment are indicated when the main effect of treatment within each sex was significant as +$p < 0.05$ 1 mg/kg A1221 vs. DMSO. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

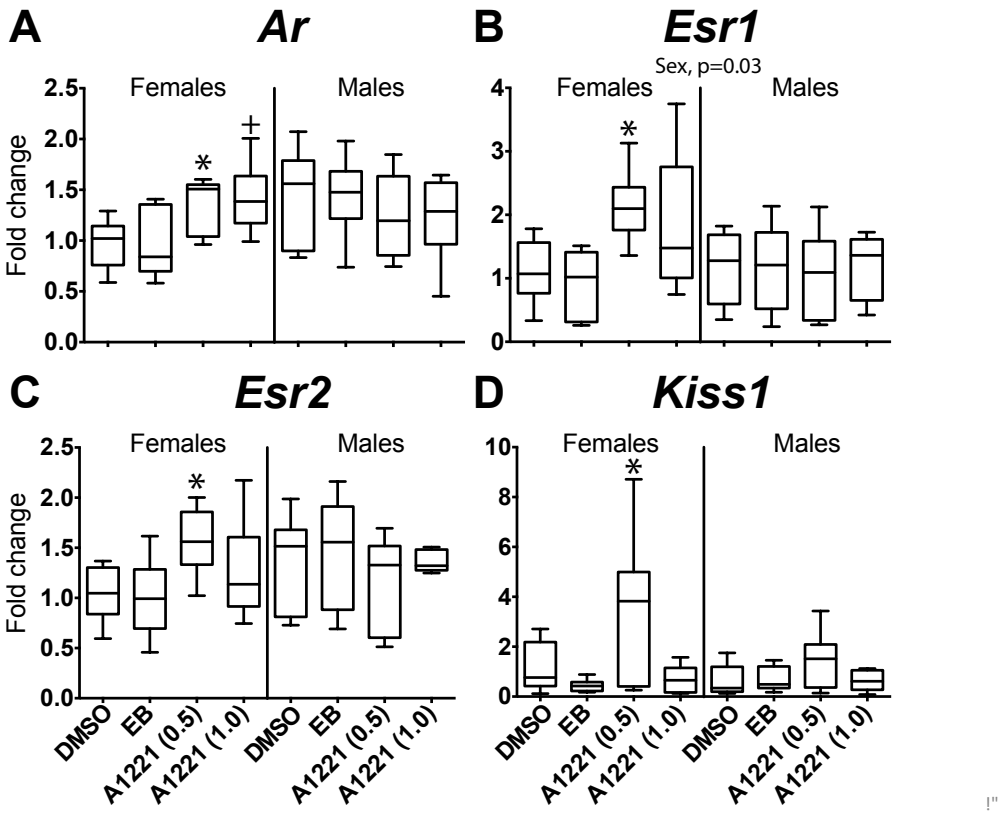

Figure 3.7: Gene expression of androgen receptor [*Ar*], estrogen receptor α [*Esr1*], estrogen receptor β [*Esr2*], and kisspeptin [*Kiss1*] in MPN (**A-D**). Significant main effects of sex are shown. Significant post-hocs for treatment are indicated when the main effect of treatment within each sex was significant as *p < 0.05 0.5 mg/kg A1221 vs. DMSO; **+**p < 0.05 1 mg/kg A1221 vs. DMSO. Abbreviations: A1221 (0.5), 0.5mg/kg A1221; A1221 (1.0), 1mg/kg A1221.

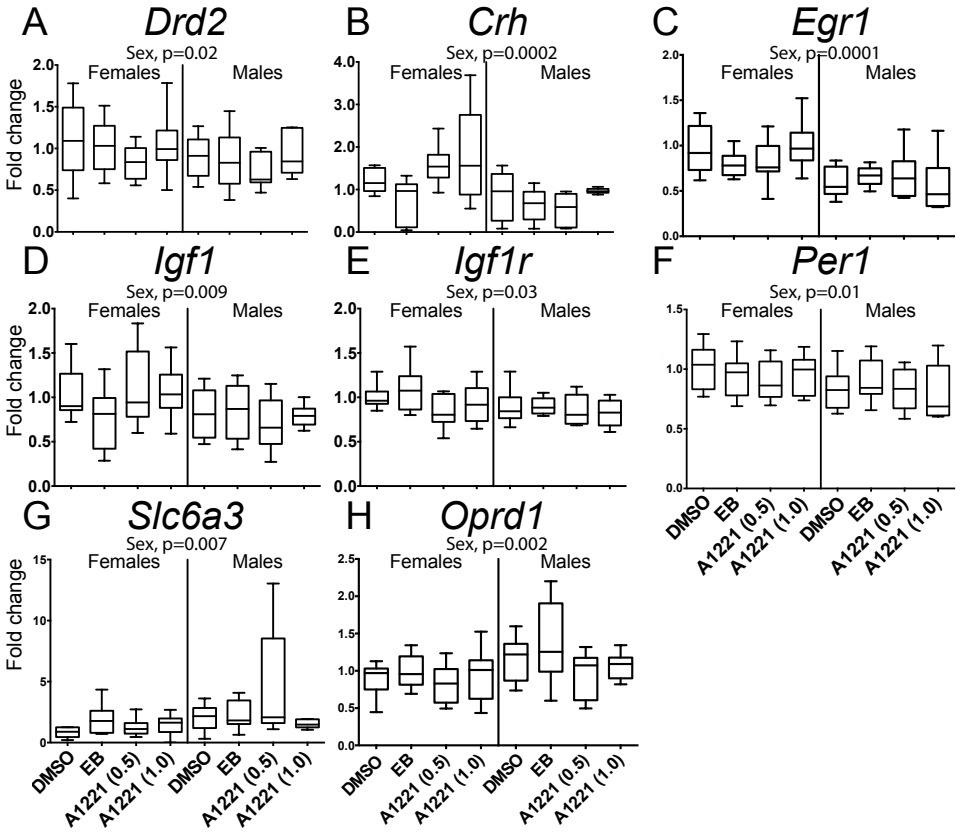

Figure 3.8: Gene expression of sexually dimorphic genes dopamine receptor D2 [*Drd2*], corticotropin releasing hormone [*Crh*], early growth response 1 [*Egr1*], insulin-like growth factor 1 [*Igf1*], insulin-like growth factor 1 receptor [*Igf1r*], period circadian clock 1 [*Per1*], dopamine transporter [*Slc6a3*], δ-opioid receptor [*Oprd1*] (**A-H**) in the MPN. Significant main effects of sex are shown. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

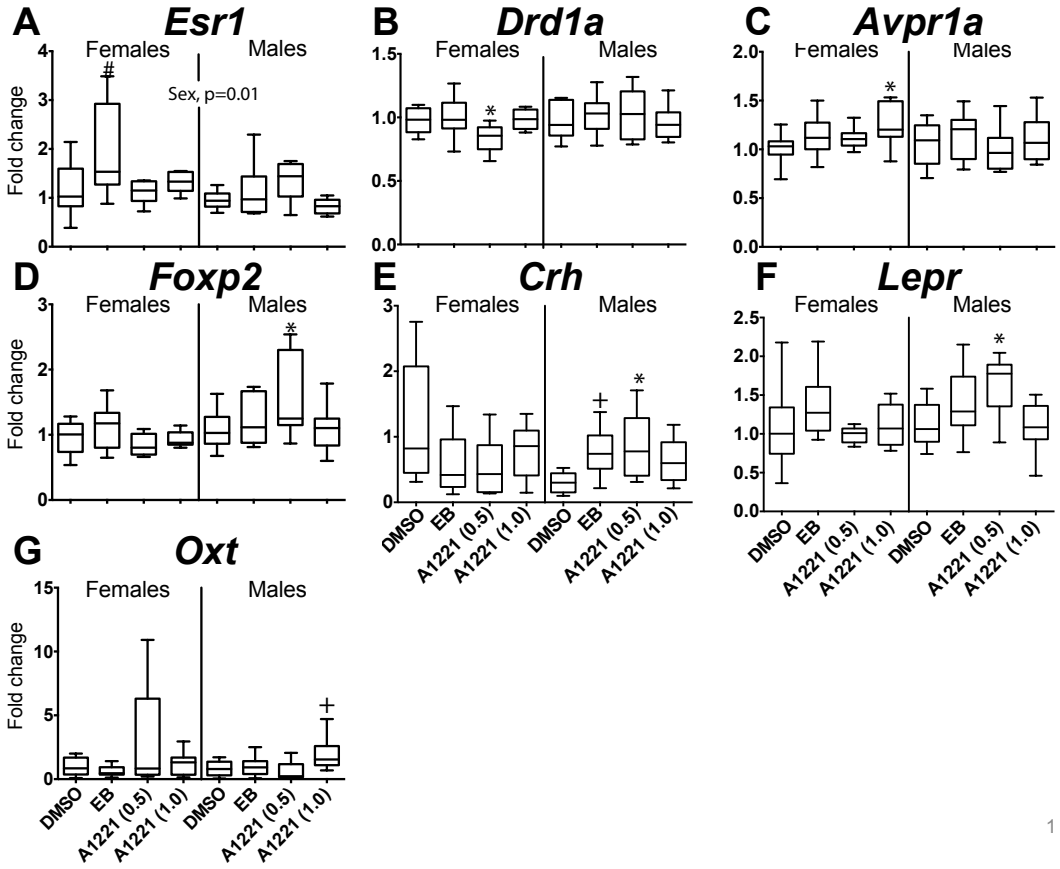

Figure 3.9: Gene expression of estrogen receptor α [*Esr1*], dopamine receptor D1 [*Drd1a*], arginine vasopressin receptor 1a [*Avpr1a*], forkhead box protein P2 [*Foxp2*], corticotropin releasing hormone [*Crh*], leptin receptor [*Lepr*], and oxytocin [*Oxt*] in VMN (**A-G**). Significant main effects of sex are shown. Significant post-hocs for treatment are indicated when the main effect of treatment within each sex was significant as *p < 0.05 0.5 mg/kg A1221 vs. DMSO; **+**p < 0.05 1 mg/kg A1221 vs. DMSO; #p < 0.05 EB vs. DMSO. Abbreviations: A1221 (0.5), 0.5mg/kg A1221; A1221 (1.0), 1mg/kg A1221.

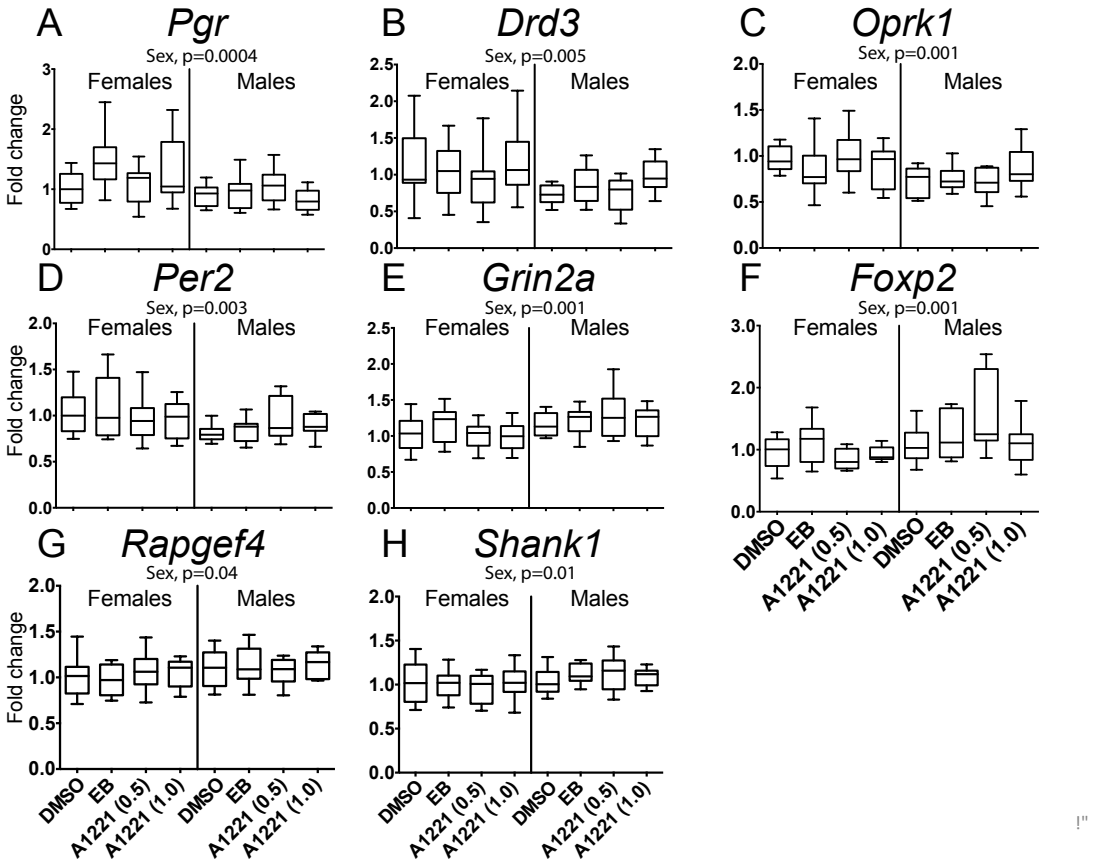

Figure 3.10: Gene expression of sexually dimorphic genes progesterone receptor [*Pgr*], dopamine receptor D3 [*Drd3*], k-opioid receptor [*Oprk1*], period circadian clock 2 [*Per2*], ionotropic glutamate receptor NMDA 2A [*Grin2a*], forkhead box protein P2 [*Foxp2*], Rap guanine nucleotide exchange factor 4 [*Rapgef4*], SH3 and multiple ankyrin repeat domains protein 1(*Shank1*) (**A-H**) in the VMN. Significant main effects of sex are shown. Abbreviations: A1221 (0.5), 0.5 mg/kg A1221; A1221 (1.0), 1 mg/kg A1221.

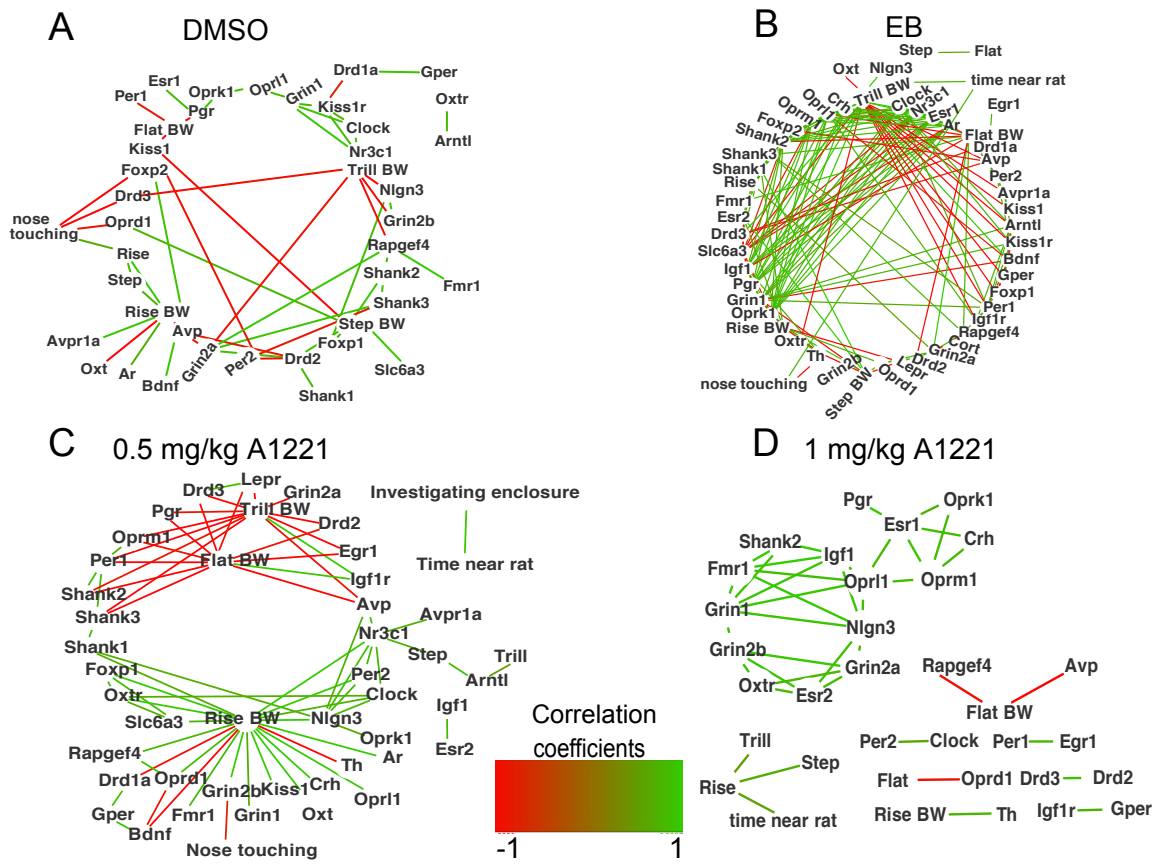

Figure 3.11: Cytoscape analysis of behaviors, genes, and serum corticosterone in the female MPN (**A-D**). Abbreviations: nose touching, time spent nose-touching with hormone-primed rat; investigating enclosure, time spent investigating hormone-primed animal enclosure; time near rat, time spent near hormone-primed rat; flat, number of flat USVs; rise, number of rise USVs; step, number of step USVs; trill, number of trill USVs; flat BW, bandwidth of flat USVS; rise BW, bandwidth of rise USVs; step BW, bandwidth of step USVs; trill USV, bandwidth of trill USVs; cort, corticosterone.

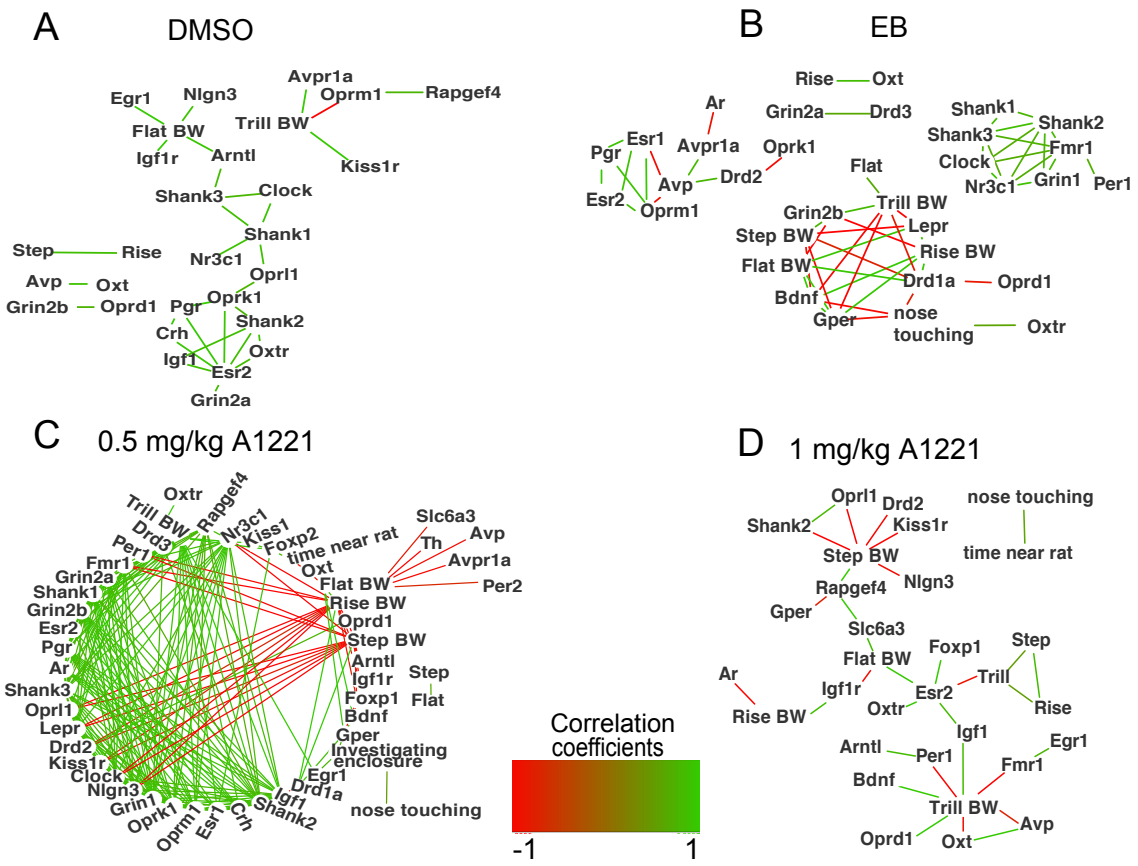

Figure 3.12: Cytoscape analysis of behaviors, genes, and serum corticosterone in the male MPN (**A-D**). Abbreviations: nose touching, time spent nose-touching with hormone-primed rat; investigating enclosure, time spent investigating hormone-primed animal enclosure; time near rat, time spent near the hormone-primed rat; flat, number of flat USVs; rise, number of rise USVs; step, number of step USVs; trill, number of trill USVs; flat BW, bandwidth of flat USVS; rise BW, bandwidth of rise USVs; step BW, bandwidth of step USVs; trill USV, bandwidth of trill USVs; cort, corticosterone.

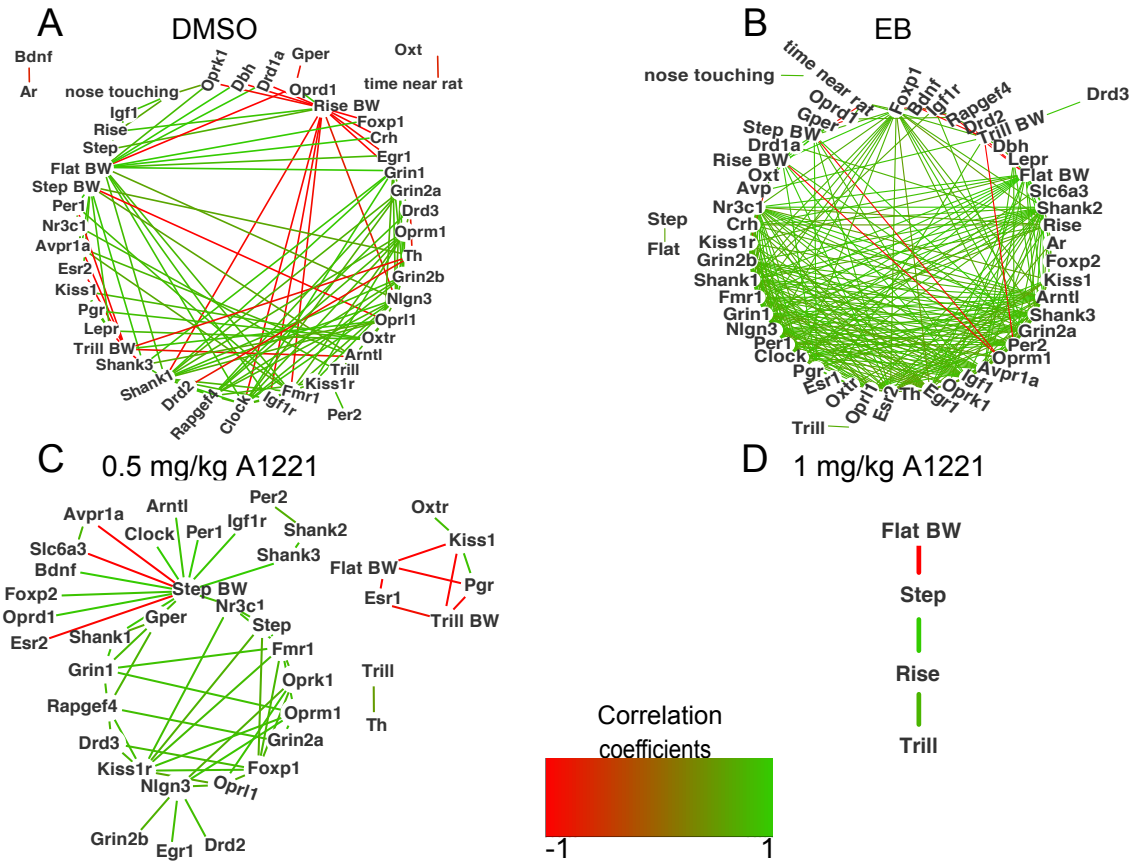

Figure 3.13: Cytoscape analysis of behaviors, genes, and serum corticosterone in the female VMN (**A-D**). Abbreviations: nose touching, time spent nose-touching with hormone-primed rat; investigating enclosure, time spent investigating hormone-primed animal enclosure; time near rat, time spent near hormone-primed rat; flat, number of flat USVs; rise, number of rise USVs; step, number of step USVs; trill, number of trill USVs; flat BW, bandwidth of flat USVS; rise BW, bandwidth of rise USVs; step BW, bandwidth of step USVs; trill USV, bandwidth of trill USVs; cort, corticosterone.

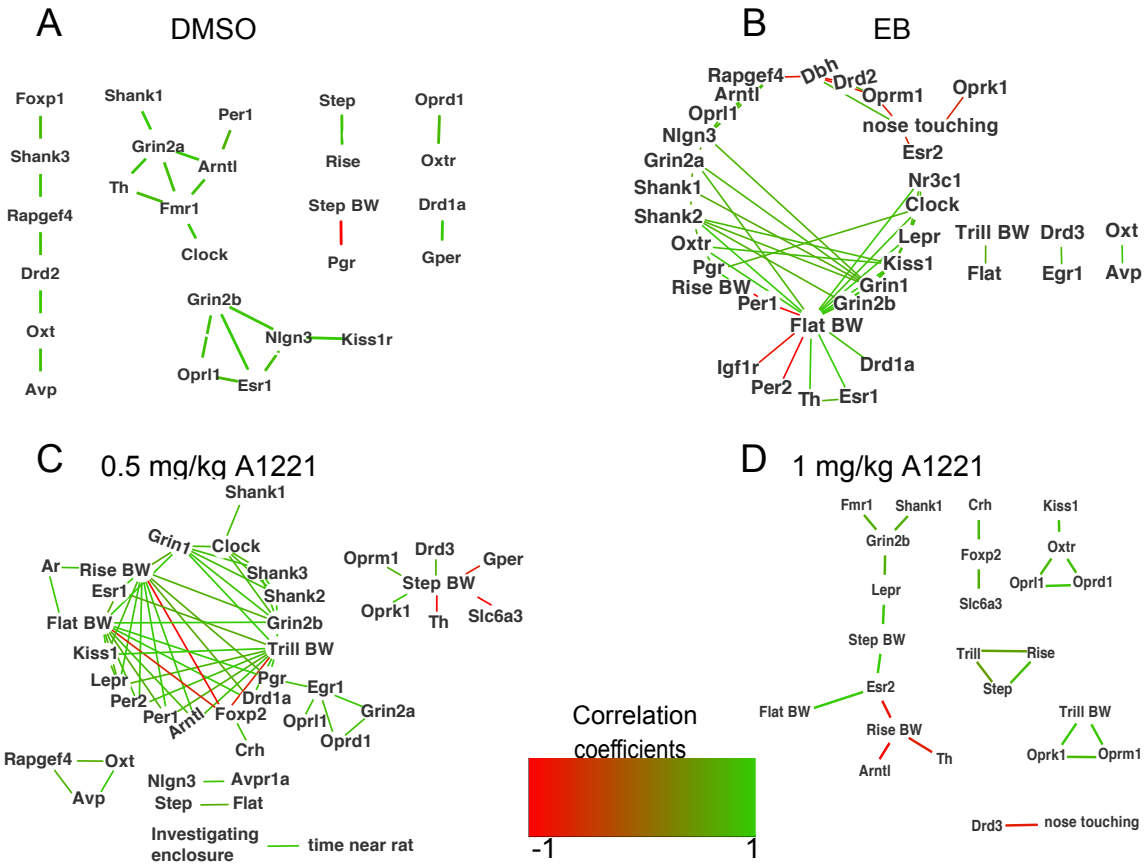

Figure 3.14: Cytoscape analysis of behaviors, genes, and serum corticosterone in the male VMN (**A-D**). Abbreviations: nose touching, time spent nose-touching with hormone-primed rat; investigating enclosure, time spent investigating hormone-primed animal enclosure; time near rat, time spent near the hormone-primed rat; flat, number of flat USVs; rise, number of rise USVs; step, number of step USVs; trill, number of trill USVs; flat BW, bandwidth of flat USVS; rise BW, bandwidth of rise USVs; step BW, bandwidth of step USVs; trill USV, bandwidth of trill USVs; cort, corticosterone.

Discussion

This study was first to report the effects of sex and prenatal PCB disruption on ultrasonic vocalizations in rats. A principle component analysis was used to identify sexually dimorphic USV measures. Additionally, sociosexual preference behaviors were analyzed in the same animals. The changes in neuroendocrine gene expression were examined in the behaviorally tested animals for sexual dimorphisms and effects of PCB exposures. Finally, a bionetwork analysis was conducted on behavioral and molecular endpoints to examine the PCB effects on gene-behavior-hormone relationships.

Ultrasonic vocalizations were sexually dimorphic and sensitive to prenatal PCB disruption

We found that numbers of flat, rise, and step, but not trill USVs were significant for the main effect of sex, with females having higher numbers of USVs than males. We also found that both males and females vocalized higher number of USVs after interaction with an opposite-sex animal. This finding was expected since presence of an opposite-sex conspecific is a known salient cue (Andersson, 1994), and salient cues are known to elicit higher numbers of vocalizations (Ma *et al.,* 2014; Brown *et al.,* 1988). Previous studies show that exposure of adult rats to novel (i.e. salient) social cues evoked differential increases in USVs across sex, with males exhibiting a much more robust increase in USV production both with respect to the frequency and complexity of USV production (Ma *et al.,* 2014). Exposure to an estrous female in particular causes an

extraordinary increase in USVs in male subjects (Ma *et al*., 2014). Others have reported that presence of a partner's odor is sufficient to increase the number of vocalizations (Brown *et al*., 1988). We find that prenatal treatment with 0.5mg/kg A1221 increased the number of rise calls in both males and females, and number of step calls in males. The increase in the numbers of USVs produced by 0.5 mg/kg A1221-treated rats was observed on Day 3 only for the number of rises, steps and trills in females and the number of rises and steps in the males. We speculate that since treatment preferentially affected the frequency-modulated calls, they might be programmed prenatally and, as a result, be more sensitive to hormonal perturbation by EDCs. This is the first report examining the effects of endocrine disruption on numbers of USVs; no comparisons to previous literature can be made. Other studies have investigated the effect of sex hormone (estradiol and testosterone), mediating a clear dependence of USV production on intact hormonal system (reviewed in Yamaguchi *et al*., 2002), with loss of vocalizations in castrated animals with its restoration to pre-gonadectomy levels with testosterone replacement (Ball *et al*., 2004; Bass *et al*., 2008).

Bandwidth of calls was sexually dimorphic and sensitive to disruption with prenatal PCBs

Power spectrum analysis revealed that bandwidth and entropy of rises, steps, and trills were higher for females compared to males. Bandwidth, but not entropy, was higher for female flats compared to male flat USVs. Bandwidth and entropy are related power spectrum measures and similarly measure how energy is dispersed over frequency of the

call (Fernandez-Vargas *et al*., 2015). We suggest that changes in bandwidth and entropy may underlie motivational-structural differences between sexes in USV production. Two conclusions can be made from these results: first, females are more motivated to vocalize (thus the higher bandwidth and entropy of the calls); second, males have more capacity to vocalize (thus the higher bandwidth and entropy are not needed for USV production). We are unable to exclude either, and both could be contributing to the sex differences in bandwidth and entropy. Previous studies have examined bandwidth and entropy in rodents, and show higher entropy and bandwidth in female golden hamster calls (Fernandez-Vargas *et al*., 2015), and minor sex differences in acoustic characteristics in mouse calls (Hammersmidt *et al*., 2012). Prenatal EB and 1mg/kg A1221 treatment increased bandwidth of male rise and trill calls, respectively. This finding was interesting since prenatal treatment increased the bandwidth of male calls, possibly leading to female-like bandwidth levels in treated males. Bandwidth has not been previously shown to be affected by PCB treatment, thus we report it for the first time herein.

Principle component analysis (PCA) identified sexually dimorphic USV measures

Principle component analysis for USV power spectrum and numbers data indicated a score that strongly predicted sex differences. The PCA score was composed of five vocalization measures (trill bandwidth, step bandwidth, duration of rise and step USVs, and numbers of flat USVs). Four of the five measures were found to be higher in DMSO females than DMSO males, while duration of rise calls were found to be higher in DMSO males relative to DMSO females. When we examined the effects of sex and

treatment across all animals (all treatments and sexes), we found significant sex effects, but no treatment or treatment X sex interactions. The significant sex effects confirmed the usefulness of a score as a predictor for sex differences in USV outcomes. We interpreted absence of treatment effects or treatment by sex interactions as a lack of feminizing or masculinizing effects of EDC treatment on the USV outcomes. No such analysis has been previously conducted, thus we are unable to make any previous comparisons to the literature. We suggest that five vocalization measures that comprise the PCA score may underlie the sexually dimorphic nature of USVs in our experimental setup.

Males, but not females, lack preference for hormone-primed conspecific, and are more sensitive to PCB disruption

Females, but not males, spent more time near the gonadectomized, hormone-treated stimulus animal, investigating its enclosure, and nose-touching with it compared to the gonadectomized animal. This suggests that females, but not males, preferred to interact with hormone-primed opposite-sex partner when given a choice of gonadectomized animal with hormone treatment versus without treatment. This finding was unexpected since, based on previous literature (Pankevich *et al.,* 2004), we expected both males and female to show this preference. However, when examined in evolutionary context, this result supports previous findings that females are the "choosier" sex and have higher investment in offspring rearing, thus their preference for the hormone-primed animal might be necessitated by the need to select a more advantageous potential mate (Crews *et al.,* 2007). We also found that males took longer time to begin investigating the

stimulus animal and spent more time standing still and grooming. These data similarly suggest that males are less interested in sociosexual interactions. Males treated prenatally with EB, 0.5mg/kg A1221 or 1mg/kg A1221 spent less time nose-touching with stimulus animals, irrespective of the hormone status of the stimulus animal. The same animals have been previously analyzed in a sociability experiment where males treated with prenatal 0.5mg/kg A1221 exhibited an overall decrease in nose-to-nose investigations (Reilly *et al.,* under review). The differences between Reilly study and this report might stem from the different social context where nose-touch was examined: sociosexual vs. sociability. Nevertheless, nose-touching in males is sensitive to endocrine disruption by PCBs, and in a sociosexual context, PCB might be exerting their effect via estrogenic mechanisms.

Steroid hormone receptors and *Kiss1* gene expression was increased in 0.5mg/kg A1221 in the MPN of females

In the MPN, 0.5mg/kg A1221 treatment increased the gene expression of *Ar, Esr1, Esr2* and *Kiss1* in females, but not males. This finding was unexpected since previously our lab has reported decreased *Ar* expression in preoptic area in adult 1mg/kg A1221 females (Dickerson *et al.,* 2011b). However, the differences in gene expression results might stem from using a lower 0.5mg/kg A1221 dose. A1221 is known to be estrogenic (Dickeron *et al.*, 2007), but at lower doses it has also shown anti-estrogenic activity (Kelce *et al.*, 1997; Schantz *et al.*, 2001). Thus, it is possible that *Ar, Esr1, Esr2* and *Kiss1* gene upregulation in the MPN is due to the anti-estrogenic effects of A1221.

Neuroendocrine genes in the VMN are affected by PCBs in a sex and treatment-specific manner

In the VMN, the effects of PCB treatment were sex- and treatment-specific. In females, EB increased *Esr1* gene expression, while 0.5 mg/kg A1221 decreased expression of *Drd1a*, and 1 mg/kg A1221 increased expression of *Avpr1a*. These genes are involved in neuroendocrine function and have established gene-behavior relationships in social and sociosexual behaviors (De Vries *et al.,* 1984; Bale *et al.,* 1995; Ferguson *et al.*, 2001; Nomura *et al.,* 2002). We suggest that changes to these genes may underlie the increase in ultrasonic vocalizations observed in 0.5mg/kg A1221 females. In males, the main effect of prenatal EB and 0.5 mg/kg A1221 increased expression of *Crh*, 0.5 mg/kg A1221 increased the expression of *Foxp2* and *Lepr*, and 1 mg/kg A1221 increased the expression of *Oxt*. Some of these gene products regulate social behavior (e.g. *Oxt, Crh, Foxp2*) as well as feeding behaviors (*Lepr*). We suggest that changes in the pattern of expression of these genes may underlie changes to sociosexual interactions in males (decreased nose-touching in EB, 1mg/kg A1221 and 0.5mg/kg A1221 males).

0.5mg/kg A1221 group had the highest number of USV-associated correlations in the bionetwork analysis

The networks were examined for organization (total number of correlations) and the identity of relationships. The most complex network organization in both MPN and VMN was exhibited in the 0.5mg/kg A1221 animals. When examined for USV-

associated measures, 0.5mg/kg A1221 networks exhibited a higher number of measures that correlated with bandwidth of rise calls. This finding supports the higher number of USVs in the 0.5mg/kg A1221 males and females. In general, bionetwork findings correlated with the finding that 0.5mg/kg A1221 group had the highest number of behavioral and molecular changes of all treatment groups, compared to DMSO.

Chapter 4: General discussion

In 1962, Rachel Carson in her book "Silent Spring" documented the detrimental effects on the environment – particularly on birds – from the indiscriminate use of pesticides (Carson, 1962). Her book brought environmental concerns to the American public, and inspired an environmental movement that led to the creation of U.S. Environmental Protection Agency. More than 50 years later, endocrinologists have advanced the knowledge on the understanding of how pesticides and other exogenous environmental chemicals influence wildlife and humans. Recent scientific evidence has linked a class of these compounds, known as endocrine-disrupting chemicals (EDCs) to an overall decline in human reproductive health (Chandra *et al.,* 2005). The structures of these compounds enable them to mimic steroid hormones and interact with endocrine systems throughout development and into adulthood. There are documented effects of EDCs on the reproductive, growth, thyroid, stress, and lactotrophic endocrine systems (Dickerson *et al.,* 2007; Zoeller, 2005; Anderson, 2010; Schantz *et al.,* 2001; Scarth 2006). Such effects are particularly detrimental during the critical period of development, when the reproductive organs, genitalia, and the brain are sculpted into sexually dimorphic structures with the help of endogenous hormones. This chapter will review the findings of this dissertation and provide a detailed discussion and implications of the findings.

Gestational PCBs and microRNA expression

In Chapter 2, the expression of eight microRNAs and select relevant target genes were analyzed for effects of sex, age, and gestational PCB treatment during postnatal development at postnatal [P] day 15, P30, P45, and P90 in male and female rats. The relationship between microRNA and mRNA gene expression was examined in the bionetwork analysis.

In the MPN, the most important finding was a developmental increase in microRNA expression with age, during the transition from juvenile (postnatal [P] day 15) to pubertal period (P30). Six microRNAs (*mir-132, mir-219, mir-7*, *mir-145*, *let-7a,* and *let-7b)* showed this increase with age in the MPN. The previous literature has linked the expression of four of the microRNAs (*let-7a, let-7b, mir-132,* and *mir-145)* to mechanisms leading to or permitting the onset of puberty in rats (Sangiao-Alvarellos et al 2012). The expression pattern of *let-7a, let-7b, mir-132,* and *mir-145* had been previously examined in the preoptic area in the hypothalamus of the developing rats, and as in this work, was found to increase with age (Sangiao-Alvarellos *et al.,* 2012). Numerous age-related changes in microRNA expression have also been observed in the female aging rat brain (Rao *et al.,* 2013). This suggests that the developmental effects of age on microRNA expression are important during the transitional hormone-sensitive periods in development, such as puberty.

Puberty is characterized by significant increase in serum hormone levels, which exert effects on reproductive and non-reproductive tissues to prepare the individual for sexual maturity (Matochik *et al.,* 1994). During puberty, which occurs around P30-P45 in

rats, hormone-sensitive changes at all levels of the HPG axis have been reported, including but not limited to changes in gene and protein expression. MicroRNAs regulate target gene expression by inducing mRNA proteolytic degradation, and/or inhibition of protein translation (Kim *et al.,* 2009). This dissertation evaluated the effects of microRNA perturbation by examining target gene mRNA expression in the same-aged animals, and, surprisingly, did not find the predicted inverse relationship with microRNA expression. However, this does not necessarily dictate that microRNAs had no effect on protein expression. As microRNAs exert their influence through reduced translational activity, in addition to mRNA degradation, it is possible that protein expression was reduced with no corresponding decrease in mRNA. Further studies correlating microRNA levels with protein expression should be conducted.

In the MPN, another major finding was the discovery that microRNAs are sexually dimorphic, with the sexual dimorphisms most apparent during puberty. Females had higher levels of expression of *mir-219, mir-7,* and *mir-145* than males, an effect driven by differences at P30. Males, on the other hand, had higher levels of expression of *mir-9* and *let-7b* at P45. Thus, these differences are possibly involved in the pubertal changes in the MPN, and may be the result of hormone-related changes during puberty as the hypothalamus transitions from juvenile quiescence to adult function.

The results of EDC treatment on microRNA expression in the MPN were age and sex specific. In females, prenatal A1221 and EB increased expression of *mir-219, mir-132, mir-7, mir-145, let-7a,* and *mir-124a* at P30. It is worth noting that similarities between A1221 and EB are consistent with their similar actions on estrogenic pathways.

In the male MPN, expression of all 8 miRNAs examined was decreased in prenatally treated A1221 males on P90, an effect not mimicked by EB. In addition to being estrogenic, A1221 is known to be partially anti-estrogenic and anti-androgenic (reviewed in Schantz *et al.,* 2001), thus it is possible that A1221 effects on the male MPN were mediated via its partial antagonistic actions at the estrogen receptor or, perhaps more likely, agonistic actions at the androgen receptor.

In the VMN, expression of all eight examined microRNAs increased with age, with the difference driven by the increase in expression from juvenile [P15] to pubertal [P30] levels. This finding was expected as *mir-132, mir-7, mir-145, let-7a,* and *let-7b* were previously found to increase with age in medial basal hypothalamus (Sangiao-Alvarellos *et al.,* 2013). Similar to the MPN, the microRNA expression profiles in the VMN suggest that microRNA levels vary the most during the transition from juvenile to adult. Interestingly, in the VMN, *mir-9* expression increased with age, despite previous reports on its age-related decrease in medial basal hypothalamus, which contains the VMN (Sangiao-Alvarellos *et al.,* 2013). The finding of age-related increase in *mir-9* expression in the more precise VMN underscores the tissue specificity of microRNA expression.

There were no effects of sex and relatively few effects of EDC treatment on microRNA expression in the VMN. In the females, there were age-specific EDC effects in *let-7a, mir-124a*, and *mir-219* expression; while in the male VMN, A1221 decreased the expression of *mir-124a*. Additionally, since only a handful of microRNAs were examined, it is possible that other microRNAs would have shown more EDC-related

effects in the VMN. It was surprising to observe no sex-related changes in the microRNA expression in the VMN, given the rich literature of sexually dimorphic gene and protein expression in the region (Herbison *et al.,* 1995; Cao *et al.,* 2011).

To ascertain the functional importance of microRNA changes, the expression of several target mRNAs (*Ar, Clock, Lepr, Lin28b, Ppara, Grin2a, Igf1r, Ar, Pgr*) was analyzed in the same animals. Within sex, modest effects of age and EDC effects were observed in both regions. Contrary to predictions, the data did not show an inverse relationship between microRNA and mRNA expression. In fact, in the bionetwork analysis, only positive relationships between microRNAs, mRNAs, and estradiol and testosterone serum hormone levels were observed. Such finding could be due to a number of possibilities. First, out of 2000 microRNAs currently catalogued in mammals (Olive *et al.,* 2015), only eight microRNAs were examined. It is possible that other microRNAs not measured have stronger associations with the examined mRNAs. Second, the effects of the selected microRNAs might not be as strong as anticipated, and thus their effects were masked by additional gene regulatory mechanisms such as DNA methylation, histone modifications, transcriptional factors, and other various forms of regulation. Third, protein expression was not analyzed, thus it is possible that reported microRNA changes contributed to inhibition of protein translation.

This study documented postnatal microRNA expression profiles in two hypothalamic nuclei, MPN and VMN for the effects of age, sex, and EDC treatment. I found that individual microRNAs were affected in both a sexually dimorphic and age-specific manner in prenatally PCB-exposed individuals. Importantly, these results

underlie the importance of age of analysis in examination of gestational PCB effects on adult endpoints. This is not surprising, as the profiles of different genes and proteins in the brain undergo dynamic change throughout postnatal development (Kermath *et al.,* 2014). This study and others (Sangiao-Alvarellos *et al.,* 2013; Rao *et al.,* 2014) also suggest the importance of hormone-sensitive periods in development, such as puberty and menopause. Additionally, these studies point to the possibility that microRNAs are gestationally programmed by estradiol and altered by estrogenic PCBs during embryonic development. Thus, studies to elucidate the timing of organizational events in microRNA programming *in utero* should be undertaken to better understand their importance in neural development.

Gestational PCBs, adult behaviors, and mRNA expression

In Chapter 3, I examined the sex and gestational effects of 50ug/kg EB (positive control), 1mg/kg A1221, or 0.5mg/kg A1221 treatment on two sociosexual behaviors, ultrasonic vocalizations and partner preference. For these behaviors, number and quality of ultrasonic vocalizations produced after an encounter with an opposite-sex partner were analyzed, as was time spent with a hormone-implanted opposite-sex rat or a hormone-deprived opposite-sex animal. Finally, gene expression of 48 candidate neuroendocrine genes was measured in the MPN and VMN of the behaviorally characterized rats. The endpoints were selected to provide a broad assessment of how gestational PCBs alter developing neuroendocrine systems, sex-typical reproductive function, and behaviors in adulthood. This work provides novel insight into the mechanisms through which

gestational low-level exposures to PCBs disrupt neuroendocrine function in adult animals.

Ultrasonic vocalizations were found to be sexually dimorphic and influenced by the animal's sociosexual environment. The number of flat, rise, step USVs, but not trill USVs was sexually dimorphic, with females vocalizing more USVs than males. I also found that both males and females vocalized higher number of USVs after interaction with an opposite-sex animal. Bandwidth and entropy of the USVs were examined and found to be sexually dimorphic, with female calls having higher bandwidth and entropy than male calls. This implies that females have a greater interest in producing vocalizations, or have a greater capacity at vocalizing at higher numbers. Additionally, increases in USVs following exposure to an opposite-sex animal suggest that sociosexual cues are a significant factor for USV production in both sexes.

PCB exposure significantly affected USV production. Prenatal treatment with 0.5mg/kg A1221 increased the number of rise calls in both males and females, and number of step calls in males. Following interaction with an opposite-sex partner, an increase in vocalizations was observed only for numbers of rises, steps and trills in 0.5 mg/kg A1221-treated females and numbers of rises and steps in the 0.5 mg/kg A1221-treated males. In the same animals, the effects of gestational EDCs on bandwidth and entropy were examined. Prenatal EB and 1mg/kg A1221 treatment increased bandwidth of male rise and trill calls, respectively. This finding suggests that USV production is a sensitive measure of endocrine disruption, and sex differences in USVs may be altered during gestation by PCBs. The PCB effect on USV numbers was not mimicked by the EB

treatment, thus it is unlikely PCBs are acting via estrogenic mechanisms. A1221 is known to be anti-estrogenic and androgenic, thus it is possible A1221 effects on USVs occurred via its antagonistic actions at the estrogen receptor, or agonistic action at the androgen receptor. Importantly, gestational PCB exposure in the males resulted in female-like numbers, entropy, and bandwidth of vocalizations. Analysis via a principle component analysis, identified five USV measures that were highly sensitive indicators of sex differences in the examined animals; bandwidth of trill and step USVs, duration of rise and step USVs, and numbers of flat USVs. However, PCB treatment did not produce an overall feminizing effect. The doses of PCBs used in the study are in the very low-level range of exposures, similar to the levels of PCB exposure found in the environment, however the exposure occurred only during gestation, while humans may be exposed to PCBs continually throughout development, and in adulthood. Thus, effects of multiple low-level exposures on vocalizations warrants further investigation. These findings also highlight the extent of the detriment that can be caused by even the lowest levels of exposure to PCBs during pregnancy.

The sociosexual preference behaviors, namely whether the rats preferred to spend time with the gonadectomized, hormone-primed versus gonadectomized hormone-deprived opposite-sex animals, were also examined. Males, but not females, lacked the preference for the hormone-primed partner, and are more sensitive to PCB disruption. For example, females, but not males, spent more time near the hormone primed stimulus animal, investigating its enclosure, and nose-touching with it compared to the castrated animal. This result supports previous findings that females are the “choosier” partners,

due to their higher investment in offspring rearing, thus their preference for the hormone-primed animal might be necessitated by the need to select a more advantageous potential mate. In addition, males were slower to start investigating the stimulus animal enclosure and spent more time standing still and grooming. Thus, these data similarly suggest that males are less interested in sociosexual interactions.

This dissertation also examined a multitude of sociosexual behaviors, and found that nose-touching was the only measure of dyadic interaction that was altered in PCB-treated animals. Gestational exposure to EB, 0.5mg/kg A1221 or 1mg/kg A1221 resulted in decreased nose-touching in treated males. The effects of PCB treatment were mimicked by EB, and thus may be potentiated via estrogenic mechanisms. This also suggests that nose-touching is a sensitive measure of endocrine disruption. In a similar study by Reilly *et al,* nose-touching behavior was similarly decreased in PCB-treated males in a social recognition experiment (Reilly *et al.,* under review). Thus, nose-touching is a sensitive indicator of PCB exposure and in future studies, nose-touching alone may be used to evaluate disruption of sociosexual and social behaviors.

Expression of 48 neuroendocrine genes was examined in the MPN and VMN of the behaviorally characterized animals. In the MPN, steroid hormone receptor (*Ar, Esr1, Esr2)* and *Kiss1* gene expression was increased in 0.5mg/kg A1221 females, while the males were unaffected. Since the effects of PCBs were not mimicked by EB, it is likely that the effect was not mediated via PCB effects on estrogen receptor. Effects of PCB treatment in the VMN were sex- and treatment-specific. In females, EB increased *Esr1* gene expression, 0.5 mg/kg A1221 decreased expression of *Drd1a*, and 1 mg/kg A1221

increased expression of *Avpr1a*. In males, EB and 0.5 mg/kg A1221 increased expression of *Crh*, 0.5 mg/kg A1221 increased the expression of *Foxp2* and *Lepr*, and 1 mg/kg A1221 increased the expression of *Oxt*. It is interesting to note that PCBs affected expression in the MPN and VMN differently. Gene expression, behaviors, and serum corticosterone were examined in the bionetwork analysis. In general, 0.5mg/kg A1221 networks were the most distinct, and different from vehicle. This correlates with the finding that 0.5mg/kg A1221 group had the highest number of behavioral and molecular changes of all treatment groups, compared to vehicle.

Concluding remarks

Taken together, the results of this dissertation provide novel insight into the effects of gestational PCBs on adult reproductive physiology and behavior. The dissertation contains novel findings on PCB effects on first, hypothalamic microRNA and related target mRNA expression during postnatal development, and second, adult reproductive behaviors and candidate neuroendocrine hypothalamic gene expression. In Chapter 2, PCB effects on microRNA expression were region-, sex- and age-specific, with most changes observed in the MPN. In the females, prenatal PCB treatment increased microRNA expression during puberty [P30], and in the males, PCB effects on microRNA expression were observed in adulthood [P90]. In Chapter 3, prenatal PCB treatment increased the number of ultrasonic vocalizations and altered the acoustic qualities of the calls in treated females and males. In the PCB-treated females, but not the males, the gene expression of estrogen receptor alpha, estrogen receptor beta, androgen

receptor, and kisspeptin genes was increased in the MPN. The results of this dissertation research have relevance for human public health and disease, as EDCs are increasingly linked to reproductive decline in humans (Chandra *et al*., 2005). Indeed, a growing body of scientific evidence suggests that early life exposure to EDCs may alter development of reproductive tract and genitalia and hormonal responsiveness in adulthood (reviewed in Diamanti-Kandarakis *et al*., 2009). Coupled with this evidence are a number of disturbing trends in some geographic regions, including reduction in fertility, an increase in hormone sensitive cancers, an earlier age of puberty in girls, and a decrease in the number of boys being born (reviewed in Janssen *et al*., 2007). Additional basic and translations studies that investigate the EDC effects during gestation are needed to uncover the mechanisms behind these disease phenotypes.

Future directions

This dissertation is a precursor to an understanding of the complex regulatory mechanisms underlying effects of endocrine disruption and could be expanded in several ways. For example, using the microRNA candidates that were identified in the dissertation, microRNA mimic and antagomir studies could be conducted using cells expressing target mRNAs (e.g. hypothalamic GT1 cells) to measure gain and loss of microRNA function and demonstrate the specificity of target mRNA and protein downregulation by select microRNAs. Additionally, select microRNA antagomir/mimics could be infused directly into specific nuclei in the brains of prenatally-exposed adult or adolescent rats to ameliorate or reverse the effects of endocrine disruption. Finally, the

dissertation could be expanded by altering the timing of exposure to PCBs allowing us to identify and define a window of susceptibility for PCB exposure, thereby shedding light on the organizational vs. activational effects of PCBs and their long-term consequences on health outcomes.

Bibliography

Alsum, P. and R. W. Goy (1974). "Actions of esters of testosterone, dihydrotestosterone, or estradiol on sexual behavior in castrated male guinea pigs." Horm Behav **5**(3): 207-217.

Alvarez-Saavedra, M., G. Antoun, A. Yanagiya, R. Oliva-Hernandez, D. Cornejo-Palma, C. Perez-Iratxeta, N. Sonenberg and H. Y. Cheng (2011). "miRNA-132 orchestrates chromatin remodeling and translational control of the circadian clock." Hum Mol Genet **20**(4): 731-751.

Amar, L., C. Benoit, G. Beaumont, C. M. Vacher, D. Crepin, M. Taouis and A. Baroin-Tourancheau (2012). "MicroRNA expression profiling of hypothalamic arcuate and paraventricular nuclei from single rats using Illumina sequencing technology." J Neurosci Methods **209**(1): 134-143.

Andersson, M. (1994). Sexual selection, Princeton University Press.

Antoniadis, E. A. and R. J. McDonald (1999). "Discriminative fear conditioning to context expressed by multiple measures of fear in the rat." Behav Brain Res **101**(1): 1-13.

Avissar-Whiting, M., K. R. Veiga, K. M. Uhl, M. A. Maccani, L. A. Gagne, E. L. Moen and C. J. Marsit (2010). "Bisphenol A exposure leads to specific microRNA alterations in placental cells." Reprod Toxicol **29**(4): 401-406.

Bale, T. L. and D. M. Dorsa (1995). "Regulation of oxytocin receptor messenger ribonucleic acid in the ventromedial hypothalamus by testosterone and its metabolites." Endocrinology **136**(11): 5135-5138.

Balfour, M. E., L. Yu and L. M. Coolen (2004). "Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats." Neuropsychopharmacology **29**(4): 718-730.

Ball, G. F. and J. Balthazart (2004). "Hormonal regulation of brain circuits mediating male sexual behavior in birds." Physiol Behav **83**(2): 329-346.

Ball, G. F., C. B. Castelino, D. L. Maney, D. Appeltants and J. Balthazart (2003). "The activation of birdsong by testosterone: multiple sites of action and role of ascending catecholamine projections." Ann N Y Acad Sci **1007**: 211-231.

Barraclough, C. A. (1961). "Production of anovulatory, sterile rats by single injections of testosterone propionate." Endocrinology **68**: 62-67.

Bass, A. H. and L. Remage-Healey (2008). "Central pattern generators for social vocalization: androgen-dependent neurophysiological mechanisms." Horm Behav **53**(5): 659-672.

Bell, H. C., D. R. McCaffrey, M. L. Forgie, B. Kolb and S. M. Pellis (2009). "The role of the medial prefrontal cortex in the play fighting of rats." Behav Neurosci **123**(6): 1158-1168.

Bell, M., communication

Bialy, M., M. Rydz and L. Kaczmarek (2000). "Precontact 50-kHz vocalizations in male rats during acquisition of sexual experience." Behav Neurosci **114**(5): 983-990.

Bizuayehu, T. T., J. Babiak, B. Norberg, J. M. Fernandes, S. D. Johansen and I. Babiak (2012). "Sex-biased miRNA expression in Atlantic halibut (Hippoglossus hippoglossus) brain and gonads." Sex Dev **6**(5): 257-266.

Blanchard, R. J., D. C. Blanchard, R. Agullana and S. M. Weiss (1991). "Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems." Physiol Behav **50**(5): 967-972.

Bleier, R., W. Byne and I. Siggelkow (1982). "Cytoarchitectonic sexual dimorphisms of the medial preoptic and anterior hypothalamic areas in guinea pig, rat, hamster, and mouse." J Comp Neurol **212**(2): 118-130.

Bonefeld-Jorgensen, E. C., H. R. Andersen, T. H. Rasmussen and A. M. Vinggaard (2001). "Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity." Toxicology **158**(3): 141-153.

Borta, A., M. Wohr and R. K. Schwarting (2006). "Rat ultrasonic vocalization in aversively motivated situations and the role of individual differences in anxiety-related behavior." Behav Brain Res **166**(2): 271-280.

Bredfeldt, T. G., K. L. Greathouse, S. H. Safe, M. C. Hung, M. T. Bedford and C. L. Walker (2010). "Xenoestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT." Mol Endocrinol **24**(5): 993-1006.

Breedlove, S. M. (1986). "Cellular analyses of hormone influence on motoneuronal development and function." J Neurobiol **17**(3): 157-176.

Brown, J. S., Jr. (2009). "Effects of bisphenol-A and other endocrine disruptors compared with abnormalities of schizophrenia: an endocrine-disruption theory of schizophrenia." Schizophr Bull **35**(1): 256-278.

Brown, R. E., M. Hauschild, S. D. Holman and J. B. Hutchison (1988). "Mate recognition by urine odors in the Mongolian gerbil (Meriones unguiculatus)." Behav Neural Biol **49**(2): 174-183.

Brudzynski, S. M. (2001). "Pharmacological and behavioral characteristics of 22 kHz alarm calls in rats." Neurosci Biobehav Rev **25**(7-8): 611-617.

Brudzynski, S. M. and A. Pniak (2002). "Social contacts and production of 50-kHz short ultrasonic calls in adult rats." J Comp Psychol **116**(1): 73-82.

Burgdorf, J. and J. Panksepp (2001). "Tickling induces reward in adolescent rats." Physiol Behav **72**(1-2): 167-173.

Cao, J. and H. B. Patisaul (2011). "Sexually dimorphic expression of hypothalamic estrogen receptors alpha and beta and Kiss1 in neonatal male and female rats." J Comp Neurol **519**(15): 2954-2977.

Carpenter, D. O. (1998). "Polychlorinated biphenyls and human health." Int J Occup Med Environ Health **11**(4): 291-303.

Carson, R. (1962). "Silent Spring". 40th anniversary edition. Houston Mifflin, Boston.

Casati, L., R. Sendra, A. Poletti, P. Negri-Cesi and F. Celotti (2013). "Androgen receptor activation by polychlorinated biphenyls: epigenetic effects mediated by the histone demethylase Jarid1b." Epigenetics **8**(10): 1061-1068.

Chakraborty, M. and S. S. Burmeister (2010). "Sexually dimorphic androgen and estrogen receptor mRNA expression in the brain of tungara frogs." Horm Behav **58**(4): 619-627.

Chakraborty, T. R., P. R. Hof, L. Ng and A. C. Gore (2003). "Stereologic analysis of estrogen receptor alpha (ER alpha) expression in rat hypothalamus and its regulation by aging and estrogen." J Comp Neurol **466**(3): 409-421.

Chandra, A., G. M. Martinez, W. D. Mosher, J. C. Abma and J. Jones (2005). "Fertility, family planning, and reproductive health of U.S. women: data from the 2002 National Survey of Family Growth." Vital Health Stat 23(25): 1-160.

Cheng, H. Y., J. W. Papp, O. Varlamova, H. Dziema, B. Russell, J. P. Curfman, T. Nakazawa, K. Shimizu, H. Okamura, S. Impey and K. Obrietan (2007). "microRNA modulation of circadian-clock period and entrainment." Neuron **54**(5): 813-829.

Choi, J. S., J. H. Oh, H. J. Park, M. S. Choi, S. M. Park, S. J. Kang, M. J. Oh, S. J. Kim, S. Y. Hwang and S. Yoon (2011). "miRNA regulation of cytotoxic effects in mouse Sertoli cells exposed to nonylphenol." Reprod Biol Endocrinol **9**: 126.

Chung, W. C., D. F. Swaab and G. J. De Vries (2000). "Apoptosis during sexual differentiation of the bed nucleus of the stria terminalis in the rat brain." J Neurobiol **43**(3): 234-243.

Chung, Y. W., A. A. Nunez and L. G. Clemens (2001). "Effects of neonatal polychlorinated biphenyl exposure on female sexual behavior." Physiol Behav **74**(3): 363-370.

Clancy, A. N., D. Zumpe and R. P. Michael (2000). "Estrogen in the medial preoptic area of male rats facilitates copulatory behavior." Horm Behav **38**(2): 86-93.

Clark, A. S., J. K. Pfeifle and D. A. Edwards (1981). "Ventromedial hypothalamic damage and sexual proceptivity in female rats." Physiol Behav **27**(4): 597-602.

Cohen, A., M. Shmoish, L. Levi, U. Cheruti, B. Levavi-Sivan and E. Lubzens (2008). "Alterations in micro-ribonucleic acid expression profiles reveal a novel pathway for estrogen regulation." Endocrinology **149**(4): 1687-1696.

Colbert, N. K., N. C. Pelletier, J. M. Cote, J. B. Concannon, N. A. Jurdak, S. B. Minott and V. P. Markowski (2005). "Perinatal exposure to low levels of the environmental antiandrogen vinclozolin alters sex-differentiated social play and sexual behaviors in the rat." Environ Health Perspect **113**(6): 700-707.

Colciago, A., L. Casati, O. Mornati, A. V. Vergoni, A. Santagostino, F. Celotti and P. Negri-Cesi (2009). "Chronic treatment with polychlorinated biphenyls (PCB) during pregnancy and lactation in the rat Part 2: Effects on reproductive parameters, on sex behavior, on memory retention and on hypothalamic expression of aromatase and 5alpha-reductases in the offspring." Toxicol Appl Pharmacol **239**(1): 46-54.

Colciago, A., P. Negri-Cesi, A. Pravettoni, O. Mornati, L. Casati and F. Celotti (2006). "Prenatal Aroclor 1254 exposure and brain sexual differentiation: effect on the expression of testosterone metabolizing enzymes and androgen receptors in the hypothalamus of male and female rats." Reprod Toxicol **22**(4): 738-745.

Collins, S. A. (2004). Vocal fighting and flirting: the functions of birdsong. Nature's music: the science of birdsong. a. H. S. P. Marler. San Diego, Elsevier Academic Press**:** 39-79.

Conaco, C., S. Otto, J. J. Han and G. Mandel (2006). "Reciprocal actions of REST and a microRNA promote neuronal identity." Proc Natl Acad Sci U S A **103**(7): 2422-2427.

Corey, D. A., L. M. Juarez de Ku, V. P. Bingman and L. A. Meserve (1996). "Effects of exposure to polychlorinated biphenyl (PCB) from conception on growth, and development of endocrine, neurochemical, and cognitive measures in 60 day old rats." Growth Dev Aging **60**(3-4): 131-143.

Coronnello, C. and P. V. Benos (2013). "ComiR: Combinatorial microRNA target prediction tool." Nucleic Acids Res **41**(Web Server issue): W159-164.

Coronnello, C., R. Hartmaier, A. Arora, L. Huleihel, K. V. Pandit, A. S. Bais, M. Butterworth, N. Kaminski, G. D. Stormo, S. Oesterreich and P. V. Benos (2012). "Novel modeling of combinatorial miRNA targeting identifies SNP with potential role in bone density." PLoS Comput Biol **8**(12): e1002830.

Crews, D., A. C. Gore, T. S. Hsu, N. L. Dangleben, M. Spinetta, T. Schallert, M. D. Anway and M. K. Skinner (2007). "Transgenerational epigenetic imprints on mate preference." Proc Natl Acad Sci U S A **104**(14): 5942-5946.

Crews, D. (2012). "The (bi)sexual brain. Science & Society Series on Sex and Science." EMBO Rep **13**(9): 779-784.

Crews, D., R. Gillette, S. V. Scarpino, M. Manikkam, M. I. Savenkova and M. K. Skinner (2012). "Epigenetic transgenerational inheritance of altered stress responses." Proc Natl Acad Sci U S A **109**(23): 9143-9148.

Cummings, J. A., L. G. Clemens and A. A. Nunez (2008). "Exposure to PCB 77 affects partner preference but not sexual behavior in the female rat." Physiol Behav **95**(3): 471-475.

Cummings, J. A., A. A. Nunez and L. G. Clemens (2005). "A cross-fostering analysis of the effects of PCB 77 on the maternal behavior of rats." Physiol Behav **85**(2): 83-91.

Davidson, J. M. (1966). "Activation of the male rat's sexual behavior by intracerebral implantation of androgen." Endocrinology **79**(4): 783-794.

Davila, B., K. W. Whitford, and E. S. Saylor (1993). "Technology alternatives for the remediation
of PCB-contaminated soil and sediment." EPA Engineering Issue.

Davis, C. J., J. M. Clinton and J. M. Krueger (2012). "MicroRNA 138, let-7b, and 125a inhibitors differentially alter sleep and EEG delta-wave activity in rats." J Appl Physiol (1985) **113**(11): 1756-1762.

Davis, E. C., P. Popper and R. A. Gorski (1996). "The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area." Brain Res **734**(1-2): 10-18.

de Vries, G. J., R. M. Buijs and A. A. Sluiter (1984). "Gonadal hormone actions on the morphology of the vasopressinergic innervation of the adult rat brain." Brain Res **298**(1): 141-145.

Denison, M. S. and S. R. Nagy (2003). "Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals." Annu Rev Pharmacol Toxicol **43**: 309-334.

Denli, A. M., X. Cao and F. H. Gage (2009). "miR-9 and TLX: chasing tails in neural stem cells." Nat Struct Mol Biol **16**(4): 346-347.

Dessi-Fulgheri, F., S. Porrini and F. Farabollini (2002). "Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats." Environ Health Perspect **110 Suppl 3**: 403-407.

Diamanti-Kandarakis, E., J. P. Bourguignon, L. C. Giudice, R. Hauser, G. S. Prins, A. M. Soto, R. T. Zoeller and A. C. Gore (2009). "Endocrine-disrupting chemicals: an Endocrine Society scientific statement." Endocr Rev **30**(4): 293-342.

Dickerson, S. M., S. L. Cunningham and A. C. Gore (2011a). "Prenatal PCBs disrupt early neuroendocrine development of the rat hypothalamus." Toxicol Appl Pharmacol **252**(1): 36-46.

Dickerson, S. M., S. L. Cunningham, H. B. Patisaul, M. J. Woller and A. C. Gore (2011b). "Endocrine disruption of brain sexual differentiation by developmental PCB exposure." Endocrinology **152**(2): 581-594.

Dickerson, S. M. and A. C. Gore (2007). "Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle." Rev Endocr Metab Disord **8**(2): 143-159.

Dolinoy, D. C., D. Huang and R. L. Jirtle (2007). "Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development." Proc Natl Acad Sci U S A **104**(32): 13056-13061.

Dugas, J. C., T. L. Cuellar, A. Scholze, B. Ason, A. Ibrahim, B. Emery, J. L. Zamanian, L. C. Foo, M. T. McManus and B. A. Barres (2010). "Dicer1 and miR-219 Are required for normal oligodendrocyte differentiation and myelination." Neuron **65**(5): 597-611.

Emerson, S. B. (2001). Male advertisement calls: Behavioral variation and physiological processes. Anuran communication. M. J. Ryan. Washington, DC, Smithsonian Institution Press**:** 36-44.

Feder, H. H. (1981). Estrous cyclicity in mammals. New York, Plenum Press.

Ferguson, J. N., J. M. Aldag, T. R. Insel and L. J. Young (2001). "Oxytocin in the medial amygdala is essential for social recognition in the mouse." J Neurosci **21**(20): 8278-8285.

Fernandez-Vargas, M. and R. E. Johnston (2015). "Ultrasonic Vocalizations in Golden Hamsters (Mesocricetus auratus) Reveal Modest Sex Differences and Nonlinear Signals of Sexual Motivation." PLoS One **10**(2): e0116789.

Filipowicz, W., L. Jaskiewicz, F. A. Kolb and R. S. Pillai (2005). "Post-transcriptional gene silencing by siRNAs and miRNAs." Curr Opin Struct Biol **15**(3): 331-341.

Fisher, A. E. (1956). "Maternal and sexual behavior induced by intracranial chemical stimulation." Science **124**(3214): 228-229.

Forest, M. G. (1975). "Differentiation and development of the male." Clin Endocrinol Metab **4**(3): 569-596.

Gentner, T. Q., S. H. Hulse, D. Duffy and G. F. Ball (2001). "Response biases in auditory forebrain regions of female songbirds following exposure to sexually relevant variation in male song." J Neurobiol **46**(1): 48-58.

Gooren, L. (1989). "Androgens and estrogens in their negative feedback action in the hypothalamo-pituitary-testis axis: site of action and evidence of their interaction." J Steroid Biochem **33**(4B): 757-761.

Gore, A. C. (2010). "Neuroendocrine targets of endocrine disruptors." Hormones (Athens) **9**(1): 16-27.

Gorski, R. A., J. H. Gordon, J. E. Shryne and A. M. Southam (1978). "Evidence for a morphological sex difference within the medial preoptic area of the rat brain." Brain Res **148**(2): 333-346.

Gorski, R. A., R. E. Harlan, C. D. Jacobson, J. E. Shryne and A. M. Southam (1980). "Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat." J Comp Neurol **193**(2): 529-539.

Guan, F., B. Zhang, T. Yan, L. Li, F. Liu, T. Li, Z. Feng, B. Zhang, X. Liu and S. Li (2014). "MIR137 gene and target gene CACNA1C of miR-137 contribute to schizophrenia susceptibility in Han Chinese." Schizophr Res **152**(1): 97-104.

Hammerschmidt, K., K. Radyushkin, H. Ehrenreich and J. Fischer (2012). "The structure and usage of female and male mouse ultrasonic vocalizations reveal only minor differences." PLoS One **7**(7): e41133.

Hany, J., H. Lilienthal, A. Sarasin, A. Roth-Harer, A. Fastabend, L. Dunemann, W. Lichtensteiger and G. Winneke (1999). "Developmental exposure of rats to a reconstituted PCB mixture or aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior." Toxicol Appl Pharmacol **158**(3): 231-243.

Heeb, M. M. and P. Yahr (1996). "c-Fos immunoreactivity in the sexually dimorphic area of the hypothalamus and related brain regions of male gerbils after exposure to sex-related stimuli or performance of specific sexual behaviors." Neuroscience **72**(4): 1049-1071.

Herbison, A. E. (2008). "Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V)." Brain Res Rev **57**(2): 277-287.

Herbison, A. E., J. E. Robinson and D. C. Skinner (1993a). "Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone." Neuroendocrinology **57**(4): 751-759.

Herbison, A. E. and D. P. Spratt (1995). "Sexually dimorphic expression of calcitonin gene-related peptide (CGRP) mRNA in rat medial preoptic nucleus." Brain Res Mol Brain Res **34**(1): 143-148.

Herbison, A. E. and D. T. Theodosis (1993b). "Absence of estrogen receptor immunoreactivity in somatostatin (SRIF) neurons of the periventricular nucleus but sexually dimorphic colocalization of estrogen receptor and SRIF immunoreactivities in neurons of the bed nucleus of the stria terminalis." Endocrinology **132**(4): 1707-1714.

Hermanson, M. H., RA (1989). "Long-term measurement of atmosheric polychlorinated biphenyls in the vicinity of Superfund dumps." Environ Sci Technol **23**: 1253-1258.

Hoke, K. L., S. S. Burmeister, R. D. Fernald, A. S. Rand, M. J. Ryan and W. Wilczynski (2004). "Functional mapping of the auditory midbrain during mate call reception." J Neurosci **24**(50): 11264-11272.

Hoshina, Y., T. Takeo, K. Nakano, T. Sato and Y. Sakuma (1994). "Axon-sparing lesion of the preoptic area enhances receptivity and diminishes proceptivity among components of female rat sexual behavior." Behav Brain Res **61**(2): 197-204.

Huang da, W., B. T. Sherman and R. A. Lempicki (2009a). "Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists." Nucleic Acids Res **37**(1): 1-13.

Huang da, W., B. T. Sherman and R. A. Lempicki (2009b). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." Nat Protoc **4**(1): 44-57.

Hudish, L. I., A. J. Blasky and B. Appel (2013). "miR-219 regulates neural precursor differentiation by direct inhibition of apical par polarity proteins." Dev Cell **27**(4): 387-398.

Hull, E. M. and J. M. Dominguez (2006). "Getting his act together: roles of glutamate, nitric oxide, and dopamine in the medial preoptic area." Brain Res **1126**(1): 66-75.

Imamura, T. (2011). "Epigenetic setting for long-term expression of estrogen receptor alpha and androgen receptor in cells." Horm Behav **59**(3): 345-352.

Janssen, S., Fujimoto, VY, Giudice, LC. (2007). "Endocrine disruption and reproductive outcomes". In Gore, AC, ed. "Endocrine Disrupting chemicals: From basic research to clinical practice". Totowa, NJ. Humana Press.

Jasarevic, E., P. T. Sieli, E. E. Twellman, T. H. Welsh, Jr., T. R. Schachtman, R. M. Roberts, D. C. Geary and C. S. Rosenfeld (2011). "Disruption of adult expression of sexually selected traits by developmental exposure to bisphenol A." Proc Natl Acad Sci U S A **108**(28): 11715-11720.

Johansen, J. A., C. L. Jordan and S. M. Breedlove (2004). "Steroid hormone masculinization of neural structure in rats: a tale of two nuclei." Physiol Behav **83**(2): 271-277.

Jolous-Jamshidi, B., H. C. Cromwell, A. M. McFarland and L. A. Meserve (2010). "Perinatal exposure to polychlorinated biphenyls alters social behaviors in rats." Toxicol Lett **199**(2): 136-143.

Kalkbrenner, A. E., R. J. Schmidt and A. C. Penlesky (2014). "Environmental chemical exposures and autism spectrum disorders: a review of the epidemiological evidence." Curr Probl Pediatr Adolesc Health Care **44**(10): 277-318.

Kapsimali, M., W. P. Kloosterman, E. de Bruijn, F. Rosa, R. H. Plasterk and S. W. Wilson (2007). "MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system." Genome Biol **8**(8): R173.

Kato, A. and Y. Sakuma (2000). "Neuronal activity in female rat preoptic area associated with sexually motivated behavior." Brain Res **862**(1-2): 90-102.

Kauffman, A. S. (2009). "Sexual differentiation and the Kiss1 system: hormonal and developmental considerations." Peptides **30**(1): 83-93.

Kaya, H., J. Hany, A. Fastabend, A. Roth-Harer, G. Winneke and H. Lilienthal (2002). "Effects of maternal exposure to a reconstituted mixture of polychlorinated biphenyls on sex-dependent behaviors and steroid hormone concentrations in rats: dose-response relationship." Toxicol Appl Pharmacol **178**(2): 71-81.

Kelce, W. R., C. R. Lambright, L. E. Gray, Jr. and K. P. Roberts (1997). "Vinclozolin and p,p'-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism." Toxicol Appl Pharmacol **142**(1): 192-200.

Kermath, B. A., P. D. Riha, M. J. Woller, A. Wolfe and A. C. Gore (2014). "Hypothalamic molecular changes underlying natural reproductive senescence in the female rat." Endocrinology **155**(9): 3597-3609.

Kim, D. H., P. Saetrom, O. Snove, Jr. and J. J. Rossi (2008). "MicroRNA-directed transcriptional gene silencing in mammalian cells." Proc Natl Acad Sci U S A **105**(42): 16230-16235.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat Rev Mol Cell Biol **10**(2): 126-139.

Kliese, N., P. Gobrecht, D. Pachow, N. Andrae, A. Wilisch-Neumann, E. Kirches, M. Riek-Burchardt, F. Angenstein, G. Reifenberger, M. J. Riemenschneider, E. Meese, D. Panayotova-Dimitrova, D. H. Gutmann and C. Mawrin (2013). "miRNA-145 is downregulated in atypical and anaplastic meningiomas and negatively regulates motility and proliferation of meningioma cells." Oncogene **32**(39): 4712-4720.

Klinge, C. M. (2009). "Estrogen Regulation of MicroRNA Expression." Curr Genomics **10**(3): 169-183.

Knutson, B., J. Burgdorf and J. Panksepp (1998). "Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats." J Comp Psychol **112**(1): 65-73.

Knutson, B., J. Burgdorf and J. Panksepp (1999). "High-frequency ultrasonic vocalizations index conditioned pharmacological reward in rats." Physiol Behav **66**(4): 639-643.

Kocerha, J., M. A. Faghihi, M. A. Lopez-Toledano, J. Huang, A. J. Ramsey, M. G. Caron, N. Sales, D. Willoughby, J. Elmen, H. F. Hansen, H. Orum, S. Kauppinen, P. J. Kenny and C. Wahlestedt (2009). "MicroRNA-219 modulates NMDA receptor-mediated neurobehavioral dysfunction." Proc Natl Acad Sci U S A **106**(9): 3507-3512.

Komada, M., Y. Asai, M. Morii, M. Matsuki, M. Sato and T. Nagao (2012). "Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses." Toxicology **295**(1-3): 31-38.

Kovanecz, I., R. Gelfand, M. Masouminia, S. Gharib, D. Segura, D. Vernet, J. Rajfer, D. K. Li, K. Kannan and N. F. Gonzalez-Cadavid (2014). "Oral Bisphenol A (BPA) given to rats at moderate doses is associated with erectile dysfunction, cavernosal lipofibrosis and alterations of global gene transcription." Int J Impot Res **26**(2): 67-75.

Krichevsky, A. M., K. S. King, C. P. Donahue, K. Khrapko and K. S. Kosik (2003). "A microRNA array reveals extensive regulation of microRNAs during brain development." RNA **9**(10): 1274-1281.

Kudwa, A. E., C. Bodo, J. A. Gustafsson and E. F. Rissman (2005). "A previously uncharacterized role for estrogen receptor beta: defeminization of male brain and behavior." Proc Natl Acad Sci U S A **102**(12): 4608-4612.

Kuiper, G. G., B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson and J. A. Gustafsson (1997). "Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta." Endocrinology **138**(3): 863-870.

Lauber, A. H., C. V. Mobbs, M. Muramatsu and D. W. Pfaff (1991). "Estrogen receptor messenger RNA expression in rat hypothalamus as a function of genetic sex and estrogen dose." Endocrinology **129**(6): 3180-3186.

Lee, R. C., R. L. Feinbaum and V. Ambros (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-854.

Leon-Olea, M., C. J. Martyniuk, E. F. Orlando, M. A. Ottinger, C. S. Rosenfeld, J. T. Wolstenholme and V. L. Trudeau (2014). "Current concepts in neuroendocrine disruption." Gen Comp Endocrinol **203**: 158-173.

Lesiak, A., M. Zhu, H. Chen, S. M. Appleyard, S. Impey, P. J. Lein and G. A. Wayman (2014). "The environmental neurotoxicant PCB 95 promotes synaptogenesis via ryanodine receptor-dependent miR132 upregulation." J Neurosci **34**(3): 717-725.

Lichtensteiger, W., Ceccatelli, R., Faass, O., Fleischmann, I., and Schlumpf, M (2003). "Effects of polybrominated diphenylether (PBDE) on reproductive organ and brain development and gene expression in rats." Toxicological Sciences **72**: 133-133.

Lukiw, W. J. (2007). "Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus." Neuroreport **18**(3): 297-300.

Ma, S. T., S. L. Resendez and B. J. Aragona (2014). "Sex differences in the influence of social context, salient social stimulation and amphetamine on ultrasonic vocalizations in prairie voles." Integr Zool **9**(3): 280-293.

Malsbury, C. W., L. M. Kow and D. W. Pfaff (1977). "Effects of medial hypothalamic lesions on the lordosis response and other behaviors in remale golden hamsters." Physiol Behav **19**(2): 223-237.

Mathews, D. and D. A. Edwards (1977). "Involvement of the ventromedial and anterior hypothalamic nuclei in the hormonal induction of receptivity in the female rat." Physiol Behav **19**(2): 319-326.

Matochik, J. A., R. J. Barfield and J. Nyby (1992). "Regulation of sociosexual communication in female Long-Evans rats by ovarian hormones." Horm Behav **26**(4): 545-555.

Matochik, J. A. and R. J. Barfield (1994). "Dissociation of androgen-dependent sociosexual behaviors in response to castration in Long-Evans rats." Physiol Behav **55**(3): 533-536.

Matz, M. V., R. M. Wright and J. G. Scott (2013). "No control genes required: Bayesian analysis of qRT-PCR data." PLoS One **8**(8): e71448.

McGinnis, M. Y., M. C. Mirth, A. F. Zebrowski and R. M. Dreifuss (1989). "Critical exposure time for androgen activation of male sexual behavior in rats." Physiol Behav **46**(2): 159-165.

McGinnis, M. Y. and M. Vakulenko (2003). "Characterization of 50-kHz ultrasonic vocalizations in male and female rats." Physiol Behav **80**(1): 81-88.

Meek, L. R., R. D. Romeo, C. M. Novak and C. L. Sisk (1997). "Actions of testosterone in prepubertal and postpubertal male hamsters: dissociation of effects on reproductive behavior and brain androgen receptor immunoreactivity." Horm Behav **31**(1): 75-88.

Mellios, N., M. Galdzicka, E. Ginns, S. P. Baker, E. Rogaev, J. Xu and S. Akbarian (2012). "Gender-specific reduction of estrogen-sensitive small RNA, miR-30b, in subjects with schizophrenia." Schizophr Bull **38**(3): 433-443.

Melzer, D., N. J. Osborne, W. E. Henley, R. Cipelli, A. Young, C. Money, P. McCormack, R. Luben, K. T. Khaw, N. J. Wareham and T. S. Galloway (2012). "Urinary bisphenol A concentration and risk of future coronary artery disease in apparently healthy men and women." Circulation **125**(12): 1482-1490.

Miller, B. H., Z. Zeier, L. Xi, T. A. Lanz, S. Deng, J. Strathmann, D. Willoughby, P. J. Kenny, J. D. Elsworth, M. S. Lawrence, R. H. Roth, D. Edbauer, R. J. Kleiman and C. Wahlestedt (2012). "MicroRNA-132 dysregulation in schizophrenia has implications for both neurodevelopment and adult brain function." Proc Natl Acad Sci U S A **109**(8): 3125-3130.

Mitchell, M. M., R. Woods, L. H. Chi, R. J. Schmidt, I. N. Pessah, P. J. Kostyniak and J. M. LaSalle (2012). "Levels of select PCB and PBDE congeners in human postmortem brain reveal possible environmental involvement in 15q11-q13 duplication autism spectrum disorder." Environ Mol Mutagen **53**(8): 589-598.

Moore, F. L., S. K. Boyd and D. B. Kelley (2005). "Historical perspective: Hormonal regulation of behaviors in amphibians." Horm Behav **48**(4): 373-383.

Morgan, C. P. and T. L. Bale (2011). "Early prenatal stress epigenetically programs dysmasculinization in second-generation offspring via the paternal lineage." J Neurosci **31**(33): 11748-11755.

Morgan, C. P. and T. L. Bale (2012). "Sex differences in microRNA regulation of gene expression: no smoke, just miRs." Biol Sex Differ **3**(1): 22.

Morgantaler, A. and D. Crews (1978). "Role of the anterior hypothalamus-preoptic area in the regulation of reproductive behavior in the lizard, Anolis carolinensis: implantation studies." Horm Behav **11**(1): 61-73.

Morse, D. C., R. F. Seegal, K. O. Borsch and A. Brouwer (1996). "Long-term alterations in regional brain serotonin metabolism following maternal polychlorinated biphenyl exposure in the rat." Neurotoxicology **17**(3-4): 631-638.

Moy, S. S., J. J. Nadler, A. Perez, R. P. Barbaro, J. M. Johns, T. R. Magnuson, J. Piven and J. N. Crawley (2004). "Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice." Genes Brain Behav **3**(5): 287-302.

Mundalil Vasu, M., A. Anitha, I. Thanseem, K. Suzuki, K. Yamada, T. Takahashi, T. Wakuda, K. Iwata, M. Tsujii, T. Sugiyama and N. Mori (2014). "Serum microRNA profiles in children with autism." Mol Autism **5**: 40.

Murphy, S. J., T. A. Lusardi, J. I. Phillips and J. A. Saugstad (2014). "Sex differences in microRNA expression during developmentin rat cortex." Neurochem Int **77**: 24-32.

Neal, J. K. and J. Wade (2007). "Effects of season, testosterone and female exposure on c-fos expression in the preoptic area and amygdala of male green anoles." Brain Res **1166**: 124-131.

Neugebauer, J., J. Wittsiepe, M. Kasper-Sonnenberg, N. Schoneck, A. Scholmerich and M. Wilhelm (2015). "The influence of low level pre- and perinatal exposure to PCDD/Fs, PCBs, and lead on attention performance and attention-related behavior among German school-aged children: results from the Duisburg Birth Cohort Study." Int J Hyg Environ Health **218**(1): 153-162.

Nomura, M., E. McKenna, K. S. Korach, D. W. Pfaff and S. Ogawa (2002). "Estrogen receptor-beta regulates transcript levels for oxytocin and arginine vasopressin in the hypothalamic paraventricular nucleus of male mice." Brain Res Mol Brain Res **109**(1-2): 84-94.

Nordeen, E. J., K. W. Nordeen, D. R. Sengelaub and A. P. Arnold (1985). "Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus." Science **229**(4714): 671-673.

Nordeen, E. J. and P. Yahr (1983). "A regional analysis of estrogen binding to hypothalamic cell nuclei in relation to masculinization and defeminization." J Neurosci **3**(5): 933-941.

Ogi, H., K. Itoh and S. Fushiki (2013). "Social behavior is perturbed in mice after exposure to bisphenol A: a novel assessment employing an IntelliCage." Brain Behav **3**(3): 223-228.

Olive, V., A. C. Minella and L. He (2015). "Outside the coding genome, mammalian microRNAs confer structural and functional complexity." Sci Signal **8**(368): re2.

Olsen, L., M. Klausen, L. Helboe, F. C. Nielsen and T. Werge (2009). "MicroRNAs show mutually exclusive expression patterns in the brain of adult male rats." PLoS One **4**(10): e7225.

Pankevich, D. E., M. J. Baum and J. A. Cherry (2004). "Olfactory sex discrimination persists, whereas the preference for urinary odorants from estrous females disappears in male mice after vomeronasal organ removal." J Neurosci **24**(42): 9451-9457.

Panksepp, J. and J. Burgdorf (2000). "50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables." Behav Brain Res **115**(1): 25-38.

Panksepp, J. and J. Burgdorf (2003). ""Laughing" rats and the evolutionary antecedents of human joy?" Physiol Behav **79**(3): 533-547.

Paredes, R. G. (2003). "Medial preoptic area/anterior hypothalamus and sexual motivation." Scand J Psychol **44**(3): 203-212.

Pasch, B., A. S. George, H. J. Hamlin, L. J. Guillette, Jr. and S. M. Phelps (2011). "Androgens modulate song effort and aggression in Neotropical singing mice." Horm Behav **59**(1): 90-97.

Pfaff *et al.,* S.-G. S., McCarthy MM, Kow LM (1994). Cellular and molecular mechanisms of female reproductive behaviors. New York, Raven press.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Phoenix, C. H., R. W. Goy, A. A. Gerall and W. C. Young (1959). "Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig." Endocrinology **65**: 369-382.

Platonow, N. S. and E. B. Meads (1975). "Distribution and excretion of two chlorinated biphenyl isomers: 4-chlorobiphenyl and decachlorobiphenyl in lactating bovine." Can J Comp Med **39**(1): 104-106.

Portigal, C. L., S. P. Cowell, M. N. Fedoruk, C. M. Butler, P. S. Rennie and C. C. Nelson (2002). "Polychlorinated biphenyls interfere with androgen-induced transcriptional activation and hormone binding." Toxicol Appl Pharmacol **179**(3): 185-194.

Rao, Y. S., N. N. Mott, Y. Wang, W. C. Chung and T. R. Pak (2013). "MicroRNAs in the aging female brain: a putative mechanism for age-specific estrogen effects." Endocrinology **154**(8): 2795-2806.

Reilly, M. W., CD; Topper, VY; Thompson, LM; Crews, DP; Gore, AC (under review). "The effects of prenatal PCBs on adult social behavior in rats ".

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz and G. Ruvkun (2000). "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans." Nature **403**(6772): 901-906.

Roselli, C. E., L. E. Horton and J. A. Resko (1985). "Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system." Endocrinology **117**(6): 2471-2477.

Sakai, A., F. Saitow, N. Miyake, K. Miyake, T. Shimada and H. Suzuki (2013). "miR-7a alleviates the maintenance of neuropathic pain through regulation of neuronal excitability." Brain **136**(Pt 9): 2738-2750.

Sangiao-Alvarellos, S., M. Manfredi-Lozano, F. Ruiz-Pino, V. M. Navarro, M. A. Sanchez-Garrido, S. Leon, C. Dieguez, F. Cordido, V. Matagne, G. A. Dissen, S. R. Ojeda, L. Pinilla and M. Tena-Sempere (2013). "Changes in hypothalamic expression of the Lin28/let-7 system and related microRNAs during postnatal maturation and after experimental manipulations of puberty." Endocrinology **154**(2): 942-955.

Scarth, J. P. (2006). "Modulation of the growth hormone-insulin-like growth factor (GH-IGF) axis by pharmaceutical, nutraceutical and environmental xenobiotics: an emerging role for xenobiotic-metabolizing enzymes and the transcription factors regulating their expression. A review." Xenobiotica **36**(2-3): 119-218.

Schantz, S. L. and J. J. Widholm (2001). "Cognitive effects of endocrine-disrupting chemicals in animals." Environ Health Perspect **109**(12): 1197-1206.

Seegal, R. F., K. O. Brosch and R. J. Okoniewski (2005). "Coplanar PCB congeners increase uterine weight and frontal cortical dopamine in the developing rat: implications for developmental neurotoxicity." Toxicol Sci **86**(1): 125-131.

Selvamani, A., M. H. Williams, R. C. Miranda and F. Sohrabji (2014). "Circulating miRNA profiles provide a biomarker for severity of stroke outcomes associated with age and sex in a rat model." Clin Sci (Lond) **127**(2): 77-89.

Shaked, I., A. Meerson, Y. Wolf, R. Avni, D. Greenberg, A. Gilboa-Geffen and H. Soreq (2009). "MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase." Immunity **31**(6): 965-973.

Simerly, R. B. (2002). "Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain." Annu Rev Neurosci **25**: 507-536.

Simerly, R. B., C. Chang, M. Muramatsu and L. W. Swanson (1990). "Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study." J Comp Neurol **294**(1): 76-95.

Steinberg, R. M., T. E. Juenger and A. C. Gore (2007). "The effects of prenatal PCBs on adult female paced mating reproductive behaviors in rats." Horm Behav **51**(3): 364-372.

Steinberg, R. M., D. M. Walker, T. E. Juenger, M. J. Woller and A. C. Gore (2008). "Effects of perinatal polychlorinated biphenyls on adult female rat reproduction: development, reproductive physiology, and second generational effects." Biol Reprod **78**(6): 1091-1101.

Stewart, P. W., J. Reihman, E. I. Lonky, T. J. Darvill and J. Pagano (2003). "Cognitive development in preschool children prenatally exposed to PCBs and MeHg." Neurotoxicol Teratol **25**(1): 11-22.

Sumida, H., M. Nishizuka, Y. Kano and Y. Arai (1993). "Sex differences in the anteroventral periventricular nucleus of the preoptic area and in the related effects of androgen in prenatal rats." Neurosci Lett **151**(1): 41-44.

Takeda, H., G. Chodak, S. Mutchnik, T. Nakamoto and C. Chang (1990). "Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor." J Endocrinol **126**(1): 17-25.

Tan, S. L., T. Ohtsuka, A. Gonzalez and R. Kageyama (2012). "MicroRNA9 regulates neural stem cell differentiation by controlling Hes1 expression dynamics in the developing brain." Genes Cells **17**(12): 952-961.

Taylor, A. M., and D. Reby (2010). "Contribution of the source-filter theory to the study of mammal vocal communication." Journal of Zoology (London) **280**: 221-236.

Teng, Y., T. T. Manavalan, C. Hu, S. Medjakovic, A. Jungbauer and C. M. Klinge (2013). "Endocrine disruptors fludioxonil and fenhexamid stimulate miR-21 expression in breast cancer cells." Toxicol Sci **131**(1): 71-83.

Terranova, M. L., G. Laviola and E. Alleva (1993). "Ontogeny of amicable social behavior in the mouse: gender differences and ongoing isolation outcomes." Dev Psychobiol **26**(8): 467-481.

Tilghman, S. L., M. R. Bratton, H. C. Segar, E. C. Martin, L. V. Rhodes, M. Li, J. A. McLachlan, T. E. Wiese, K. P. Nephew and M. E. Burow (2012). "Endocrine disruptor regulation of microRNA expression in breast carcinoma cells." PLoS One **7**(3): e32754.

Tokumoto, T., M. Tokumoto and P. Thomas (2007). "Interactions of diethylstilbestrol (DES) and DES analogs with membrane progestin receptor-alpha and the correlation with their nongenomic progestin activities." Endocrinology **148**(7): 3459-3467.

Uotinen, N., R. Puustinen, S. Pasanen, T. Manninen, M. Kivineva, H. Syvala, P. Tuohimaa and T. Ylikomi (1999). "Distribution of progesterone receptor in female mouse tissues." Gen Comp Endocrinol **115**(3): 429-441.

Vivian, J. A. and K. A. Miczek (1993). "Diazepam and gepirone selectively attenuate either 20-32 or 32-64 kHz ultrasonic vocalizations during aggressive encounters." Psychopharmacology (Berl) **112**(1): 66-73.

Wade, J., J. M. Huang and D. Crews (1993). "Hormonal control of sex differences in the brain, behavior and accessory sex structures of whiptail lizards (Cnemidophorus species)." J Neuroendocrinol **5**(1): 81-93.

Wagner, C. K. (2006). "The many faces of progesterone: a role in adult and developing male brain." Front Neuroendocrinol **27**(3): 340-359.

Walker, D. M., B. M. Goetz and A. C. Gore (2014). "Dynamic postnatal developmental and sex-specific neuroendocrine effects of prenatal polychlorinated biphenyls in rats." Mol Endocrinol **28**(1): 99-115.

Walker, D. M., T. E. Juenger and A. C. Gore (2009). "Developmental profiles of neuroendocrine gene expression in the preoptic area of male rats." Endocrinology **150**(5): 2308-2316.

Walker, D. M., B. A. Kermath, M. J. Woller and A. C. Gore (2013). "Disruption of reproductive aging in female and male rats by gestational exposure to estrogenic endocrine disruptors." Endocrinology **154**(6): 2129-2143.

Walker, D. M., D. Kirson, L. F. Perez and A. C. Gore (2012). "Molecular profiling of postnatal development of the hypothalamus in female and male rats." Biol Reprod **87**(6): 129.

Wallen, K. (2009). "The Organizational Hypothesis: Reflections on the 50th anniversary of the publication of Phoenix, Goy, Gerall, and Young (1959)." Horm Behav **55**(5): 561-565.

Wang, X. Q., J. Fang, A. A. Nunez and L. G. Clemens (2002). "Developmental exposure to polychlorinated biphenyls affects sexual behavior of rats." Physiol Behav **75**(5): 689-696.

Westneat, D. F., A. Walters, T. M. McCarthy, M. I. Hatch and W. K. Hein (2000). "Alternative mechanisms of nonindependent mate choice." Anim Behav **59**(3): 467-476.

Wibrand, K., D. Panja, A. Tiron, M. L. Ofte, K. O. Skaftnesmo, C. S. Lee, J. T. Pena, T. Tuschl and C. R. Bramham (2010). "Differential regulation of mature and precursor microRNA expression by NMDA and metabotropic glutamate receptor activation during LTP in the adult dentate gyrus in vivo." Eur J Neurosci **31**(4): 636-645.

Wightman, B., I. Ha and G. Ruvkun (1993). "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans." Cell **75**(5): 855-862.

Wilczynski, W. and M. J. Ryan (2010). "The behavioral neuroscience of anuran social signal processing." Curr Opin Neurobiol **20**(6): 754-763.

Wohr, M., A. Borta and R. K. Schwarting (2005). "Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: a dose-response study in the rat." Neurobiol Learn Mem **84**(3): 228-240.

Wohr, M., B. Houx, R. K. Schwarting and B. Spruijt (2008). "Effects of experience and context on 50-kHz vocalizations in rats." Physiol Behav **93**(4-5): 766-776.

Woolley, S. C. and A. J. Doupe (2008). "Social context-induced song variation affects female behavior and gene expression." PLoS Biol **6**(3): e62.

Wright, J. M., J. C. Gourdon and P. B. Clarke (2010). "Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context." Psychopharmacology (Berl) **211**(1): 1-13.

Yamaguchi, A., and D. B. Kelley (2002). Hormonal mechanisms of acoustic communication. New York, Springer.

Yin, W. and A. C. Gore (2010). "The hypothalamic median eminence and its role in reproductive aging." Ann N Y Acad Sci **1204**: 113-122.

Zanato, V. F., M. P. Martins, J. A. Anselmo-Franci, S. O. Petenusci and T. L. Lamano-Carvalho (1994). "Sexual development of male Wistar rats." Braz J Med Biol Res **27**(5): 1273-1280.

Zhang, B. and X. Pan (2009). "RDX induces aberrant expression of microRNAs in mouse brain and liver." Environ Health Perspect **117**(2): 231-240.

Zhu, H., S. Shah, N. Shyh-Chang, G. Shinoda, W. S. Einhorn, S. R. Viswanathan, A. Takeuchi, C. Grasemann, J. L. Rinn, M. F. Lopez, J. N. Hirschhorn, M. R. Palmert and G. Q. Daley (2010). "Lin28a transgenic mice manifest size and puberty phenotypes identified in human genetic association studies." Nat Genet **42**(7): 626-630.

Ziats, M. N. and O. M. Rennert (2014). "Identification of differentially expressed microRNAs across the developing human brain." Mol Psychiatry **19**(7): 848-852.

Zoeller, R. T., R. Bansal and C. Parris (2005). "Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain." Endocrinology **146**(2): 607-612.

Zoeller, R. T., T. R. Brown, L. L. Doan, A. C. Gore, N. E. Skakkebaek, A. M. Soto, T. J. Woodruff and F. S. Vom Saal (2012). "Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society." Endocrinology **153**(9): 4097-4110.

Zucchi, F. C., Y. Yao, I. D. Ward, Y. Ilnytskyy, D. M. Olson, K. Benzies, I. Kovalchuk, O. Kovalchuk and G. A. Metz (2013). "Maternal stress induces epigenetic signatures of psychiatric and neurological diseases in the offspring." PLoS One **8**(2): e56967.

Vita

Viktoria Yuryevna Topper (née Davaeva) was born in Elista, Russia on June 11th, 1986. She traveled during her years in middle school and high school, and chose to pursue further education in the United States. She attended Lanphier High School in Springfield, Illinois. In 2004, she began her undergraduate career at the College of Saint Elizabeth in Morristown, New Jersey. She received her Bachelor of Science degree in biochemistry and mathematics from the College of Saint Elizabeth in 2008. She then entered the MD/PhD program at the University of Texas at Austin and University of Texas Medical Branch. At the University of Texas, she attended a doctoral program at the Institute of Cellular and Molecular Biology and worked under the advisory of Dr. Andrea Gore.

This dissertation was typed by the author. She can be reached by permanent email at toppervikto ria@gmail.com.