ABSTRACT

SULLIVAN, ALANA WELLS. Early-life Exposures to Estrogenic EDCs Impact Rodent Neuroendocrine Signaling Pathways Involved in Sociosexual Behaviors, Including Anxiety/Activity. (Under the direction of Dr. Heather Patisaul).

The neuroendocrine organization and activation of behavior is governed by sex-steroid hormones and heavily influenced by neuropeptides and respective receptors. Thus, exposure to endocrine disrupting compounds (EDCs) during important developmental windows impacts behavior and the associated underlying neural substrates. Given that "estrogenic" EDCs are ubiquitous in the human and wildlife environment, it is critical to understand how endogenous estrogens orchestrate the organization of the neural architecture involved in sociosexual behavior and how developmental exposure to EDCs may impact it. Recent epidemiological studies in children have correlated prenatal exposure to EDCs with behavioral outcomes including anxiety and deficits in executive functioning and sociality. Similar outcomes have been reported in rodents that have been developmentally exposed to bisphenol-A (BPA), a man-made EDC with estrogenic properties. In other rodent studies a soy-rich diet, or exposure to phytoestrogens found in soy products have affected anxiety and sociosexual behaviors as well, but sometimes in an opposite manner. Importantly both phytoestrogens and BPA are able to bind to nuclear estrogen receptors (ERs). This dissertation focuses on which ERs are involved in sexual differentiation of behaviors, and how developmental exposures to BPA and phytoestrogens found in soy affect the underlying circuitry responsible for sociosexual behavior. Through the use of the prairie vole in conjunction with the rat, I confirmed that developmental exposures to BPA disrupt the mesolimbic pathway involved in sociosexual behaviors and anxiety/activity. The prairie vole is an under-utilized, highly social (prosocial) rodent that has been well- studied due to its unique sociality and likeness to humans. This species is novel to the traditional toxicological study of EDCs but should be considered in the future for further use, as I have demonstrated its efficacy in understanding the perturbance of the neuroendocrine pathways involved in behavior. Moreover, I have shown that developmental exposure to BPA alters behavior across varying rodent species, no matter the degree of sociality and life history thereof. These findings signify the importance of understanding EDCs and in accordance regulatory agencies should consider new techniques (including the use of the prairie vole model) in

testing the effects of EDCs. Because developmental administration of a phytoestrogen found in soy or a soy-rich diet was able to alter sociosexual behaviors and activity, the use of soy-based infant formula should be further explored and future research design should consider the diet of the study animals.

Early-life Exposures to Estrogenic EDCs Impact Rodent Neuroendocrine Signaling Pathways Involved in Sociosexual Behaviors, Including Anxiety/Activity

by
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DEDICATION

To My Progeny

You are what matters most

BIOGRAPHY

According to 23andMe, I am 99.9% European: 48.7% British and Irish, 1.5% French & German, 41.7% Nonspecific Northern European, 0.1 % Iberian, 1.4% Nonspecific Southern European, 6.4% Nonspecific European, and 0.1% Unassigned. In addition, to this information 23andMe estimates that 2.6% of my DNA is from Neanderthals. That is slightly lower than the average of European users. And lucky me—I am probably Norovirus resistant!

ACKNOWLEDGMENTS

TO ALL MY DIFFERENT FAMILIES OLD AND NEW.

To 256, 414, 435, and others……..

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CHAPTER 1: Introduction

The behavioral brain is organized and activated by endogenous gonadal hormones. Most notably, estrogen influences sexual differentiation of reproductive physiology and behavior. While estrogen is necessary for masculinization (Baum, 1979; Beach et al., 1969; McEwen, 1983; McEwen et al., 1977; Morris et al., 2004; Phoenix et al., 1959) and defeminization (Kudwa et al., 2005; Kudwa and Rissman, 2003) in males, the lack of estrogen is necessary in females for feminization. Classically in rodents, estrogens bind to the two nuclear estrogen receptor (ER) subtypes, ER alpha (ERα) and ER beta (ERβ) thus leading to the formation of the male-specific neuroendocrine system (Kuiper et al., 1997; Merchenthaler et al., 2004; White et al., 1987). Through the use of knock-out models, it has been hypothesized that ERα is predominately responsible for masculinization of male-specific reproductive behavior and ERβ is necessary for defeminization, or the loss of lordosis function. Exogenous compounds known as endocrine disrupting compounds (EDCs) are able to interfere with the endocrine system and thus modify either or both the organization and activation of hormone-dependent behaviors. Because developmental exposures to "estrogenic" EDCs impact sociosexual behaviors (Handa et al., 2008; Patisaul et al., 2001; Sullivan et al., 2011; Weiser et al., 2009; Wolstenholme et al., 2013b), it is critical to develop models to elucidate the underpinnings of sociosexual brain organization and EDC effects. This dissertation focuses on the two nuclear ER subtypes, ERα and ERβ because they are known role players in the organization and activation of sociosexual behaviors in rodents, including prairie voles.

"Estrogenic" EDCs examined in this dissertation

Phytoestrogens are plant-derived compounds that possess an estrogen-like structure and action. In the late 1930's naturally occurring phytoestrogens were first studied as an EDC described as "clover disease." Ewes grazing on clover displayed disrupted reproductive cycles and behavior. It was discovered that clover contains a phytoestrogen, coumestrol capable of interfering with estrogen steroid hormone-driven physiology and behavior (Adams, 1978, 1979). Isoflavones are a class of phytoestrogens found in legumes, such as soy. Soy has previously been associated with several health protective effects including lowered risks of breast, prostate, and colon cancers, cardiovascular disease, and bone loss. Because soy is associated with positive health effects and is considered an economical source of protein it has become increasingly popular in human diet as well as animal chow (Lephart et al., 2004). Infant exposure to phytoestrogens has been a concern as isoflavones have been detected in human umbilical cord plasma, amniotic fluid, and breast milk at maternal plasma levels (Todaka et al., 2005). Originally developed as an alternative for babies with milk allergies, a soy-based infant formula can result in daily consumption of 3.9-5.85 mg/kg of genistein (GEN), an isoflavone found in soy (Irvine et al., 1998a; Irvine et al., 1998b; Setchell et al., 1997, 1998). Moreover, infants fed a soy-based infant formula can have an total isoflavones exposure level of 1000ng/ml blood plasma, which is high compared to infants breast-fed or fed a bovine-based infant formula having 4.7 ng/ml and 9.4 ng/ml respectively (Patisaul and Jefferson 2010). Phytoestrogens found in soy products have a binding affinity for estrogen receptors (ERs) (Adlercreutz and Mazur, 1997; Bernal and Jirtle, 2010; Setchell et al., 1997, 1998). Due to their structural conformation, phytoestrogens such as GEN have a higher binding affinity for ERβ than ERα (Kuiper et al., 1998). Furthermore, GEN has a high relative binding affinity of 87 for ERβ, which is critical for the proper expression of sociosexual behavior. Therefore understanding the effects of a soy-rich diet is crucial for addressing a public health concern.

It has been documented that sexually dimorphic behaviors such as anxiety/exploratory activity behaviors and cognition in rats have been altered due to the administration of a diet varied in phytoestrogen content (reviewed in (Lephart et al., 2004)). Both open- and closed-

formula laboratory animal chows have been analyzed and shown to contain phytoestrogens (Thigpen et al., 1999a; Thigpen et al., 1999b). Male rats administered a phytoestrogen-free diet and females administered a phytoestrogen-rich diet learned the maze faster than males or females fed the opposite diet. In another study, males and females fed a phytoestrogen-rich diet displayed less anxiety than those fed a phytoestrogen-free diet, suggesting that diet alone can impact anxiety-related behaviors (Lephart et al., 2004).

Bishpenol-A (BPA) is a synthetic compound first identified in the 1800s, considered as a candidate for birth control in the 1950's and eventually commercially manufactured for use in sealants and plasticizers. Currently, BPA is of concern due to its steroid receptor binding properties and potential epigenetic effects. Consensus statements have been published on the mounting health concerns about developmental BPA exposures resulting in disrupted behavior, reproduction and the onset of adult disease (Vandenberg et al., 2013).

Developmental exposures to BPA has been associated with an increase in anxiety-like behaviors and a loss in sexually dimorphic behaviors important for reproductive success such as male-specific reproductive behaviors, activity, sociality, and cognition. These outcomes have been reported in a wide array of animal models, including humans and socially monogamous mice (*Peromyscus californicus*). Very little mechanistic work, however, has been published in conjunction to elucidate the behavioral outcomes reported.

The Aims of this Dissertation

Early-life exposure to estrogenic EDCs impacts rodent sociosexual behaviors.

Estrogen is involved in the organization and execution of several behaviors important for reproductive success. I plan to address how developmental exposures to EDCs impact sociosexual behaviors and underlying neuroendocrine pathways.

AIM 1 Through the use of knock-out (KO) mice it was hypothesized that ER beta (ERβ) was involved in defeminization while ER alpha (ERα) was crucial for masculinization in rodents (Kudwa et al., 2005; Kudwa et al., 2006). Because KO models cannot address at what point in time which specific ER is necessary, and it has been hypothesized that the two ERs can have a sequential relationship (Rissman, 2008), I found it important to further elucidate the

roles of specific ER agonism in the organization of sex-specific behaviors. I then could address the effects of EDCs capable of agonizing/antagonizing estrogen receptors. For AIM 1 (Published: (Sullivan et al., 2011)) I hypothesized that postnatal exposure to ER-specific EDCs would disrupt male-specific reproductive behavior and success. For Experiment 1, I neonatally administered selective agonists for ERα or ERβ and an estrogen control, estradiol benzoate (EB). Males exposed to EB and diarylpropionitrile (DPN), an ERβ specific ligand, were impaired and unattractive to female conspecifics. I then further hypothesized that this response was dose specific which was confirmed in Experiment 2 that this outcome is dose specific, in a U-shaped manner. GEN is a potent ERβ agonist thus for the final experiment, I hypothesized that neonatal exposure to a human relevant dose of GEN would disrupt male mating behavior in the same manner. I found that neonatal exposure to GEN (at a dose equivalent to that of a traditional Asian or soy-based Western diet) resulted in impairment of reproductive behaviors but not attractiveness.

AIM 2 For my second aim (Published: (Patisaul et al., 2012)) I sought to examine the interaction of BPA and soy on sociosexual behavior. I employed an environmentally relevant exposure paradigm by using concomitant consumption of two EDCs, both with well-known binding affinities for ERβ, at naturally occurring levels of ingestion. I hypothesized that administration of a soy-rich diet would result in anxiolytic effects while BPA exposure would result in anxiety. Furthermore, I hypothesized that gene expression in the amygdala (AMYG), a brain region involved in the limbic fear circuit, would be impacted, particularly ESR2 (ERβ). BPA was administered via the drinking water while GEN was administered via a soy-rich diet, both offered to pregnant rats and their pups thereby administering the EDCs from gestational day (GD) six through weaning, ad libitum. Early-life exposure to BPA resulted in increased anxiety-like and decreased exploratory behavior in juvenile rats while adults showed a loss in important sexually dimorphic behaviors. A soy-rich diet mitigated these effects. A subset of animals tested for behavior were sacrificed on postnatal day (PND) 34, a peri-pubertal window, for brain, blood, and organ collection. The AMYG was analyzed on a 48 gene microarray specific to mesolimbic endocrine functions. Of the 48

genes, 8 had significant changes in expression levels. Interestingly, BPA-exposed animals showed a significant down regulation in gene expression of ESR2 (ERβ) and melanocortin-4 receptor (MCR4). These two genes relegate oxytocin (OT) and vasopressin (AVP) production and secretion in the paraventricular nucleus (PVN), thereby directly regulating fear and anxiety responses and sociality. These findings led me to my third aim, employing a rodent model novel to traditional toxicological studies but better suited for understanding this OT-AVP signaling system.

AIM 3 In my final aim (paper submitted to *Endocrinology*) I wanted to further address how BPA impacts sociosexual behavior. The prairie vole is a prosocial species expressing behaviors similar to those in humans (Modi and Young, 2012). These animals are socially monogamous, alloparental, and exhibit mate guarding, all behaviors strongly tied to the oxytocin-vasopressin (OT-AVP) system. Hence this system has been extensively investigated for decades and recently has been applied to human behavioral conditions (Modi and Young, 2012). I hypothesized that developmental exposure to a range of doses of BPA would disrupt prairie vole sociosexual behavior and the underlying OT-AVP signaling pathway. Data suggested that females were affected more than males. This could be due to the window, or doses used and these variables can be further tested in the future to further elucidate sexual differentiation of prairie vole behavior. Outcomes occurred in a dose-specific manner, with the low dose resulting in hyperactivity. Pair-bonding ability, however, was not significantly disrupted. Behavioral changes were accompanied by altered OT- and AVP-ir cell numbers in subregions of the PVN, and TH-ir cell numbers in the pBNST. OT and AVP immunoreactive neuron number was altered in the female PVN.

Collectively this dissertation work demonstrates that the rodent mesolimbic pathways can be disrupted by environmentally-relevant exposures to EDCs, resulting in impairment of soiciosexual behaviors necessary for reproductive success. Furthermore, dietary composition alone may impact the organization of the sociosexual brain. The prairie vole model was a valuable tool in demonstrating the impacts of BPA on sociosexual behaviors and the

underlying mesolimbic circuitry involved; and thus it should be considered for future use in endocrine disruption work.

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Neonatal agonism of ERβ impairs male reproductive behavior and attractiveness

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ABSTRACT

The organization of the developing male rodent brain is profoundly influenced by endogenous steroids, most notably estrogen. This process may be disrupted by estrogenic endocrine disrupting compounds (EDCs) resulting in altered sex behavior and the capacity to attract a mate in adulthood. To better understand the relative role each estrogen receptor (ER) subtype (ERα and ERβ) plays in mediating these effects, we exposed male Long Evans rats to estradiol benzoate (EB, 10 μg), vehicle, or agonists specific for ERβ (DPN, 1 mg/kg) or ERα (PPT, 1 mg/kg) daily for the first four days of life, and then assessed adult male reproductive behavior and attractiveness via a partner preference paradigm. DPN had a greater adverse impact than PPT on reproductive behavior, suggesting a functional role for ERβ in the organization of these male-specific behaviors. Therefore the impact of neonatal ERβ agonism was further investigated by repeating the experiment using vehicle, EB and additional DPN doses (0.5 mg/kg, 1 mg/kg, and 2 mg/kg bw). Exposure to DPN suppressed male reproductive behavior and attractiveness in a dose dependent manner. Finally, males were exposed to EB or an environmentally relevant dose of genistein (GEN, 10 mg/kg), a naturally occurring xenoestrogen, which has a higher relative binding affinity for ERβ than ERα. Sexual performance was impaired by GEN but not attractiveness. In addition to suppressing reproductive behavior and attractiveness, EB exposure significantly lowered the testis to body weight ratio, and circulating testosterone levels. DPN and GEN exposure only impaired behavior, suggesting that disrupted androgen secretion does not underlie the impairment.

1. Introduction

To display sex-specific reproductive behavior in adulthood, male mammals must undergo both defeminization and masculinization during critical windows of development that span the pre- and postnatal periods. Masculinization is the emergence of male-specific behaviors and the underlying circuitry responsible for those behaviors, while defeminization is the loss of female-specific behaviors and the underlying circuitry necessary for those behaviors. Steroid hormones are required for both the organization and activation of male-specific traits (Baum, 1979; Beach et al., 1969; McEwen, 1983; Morris et al., 2004; Phoenix et al., 1959) and defeminization (Kudwa and Rissman, 2003). In the present study, we explored the hypothesis that exposure to estrogen receptor (ER) agonists, selective for one of the two nuclear estrogen receptor isoforms (ERα and ERβ), or the endocrine disrupting compound (EDC) genistein (Meyers et al., 2001), during the neonatal critical period could disrupt this organizational process, resulting in impaired reproductive behavior and reduced attractiveness to a potential mate.

Classically, in male rodents, estrogens, aromatized from testicular androgens, bind to the two nuclear ER subtypes, ERα and ERβ within the brain to induce male-specific neuroendocrine development (Kuiper et al., 1996; Merchenthaler et al., 2004; White et al., 1987). The specific mechanistic roles these two receptors play in the masculinization and defeminization process, however, remain largely unclear. Based primarily on data obtained from knockout (KO) mice, null for either ERα or ERβ, it has been hypothesized that ERα is primarily responsible for masculinization while ERβ is more important for defeminization (Kudwa et al., 2006). Accordingly, ERαKO mice of both sexes exhibit malformed gonads, infertility, and disrupted reproductive behavior (Lubahn et al., 1993; Rissman et al., 1997), while ERβKO mice develop subfunctional gonads (Krege et al., 1998), but are able to reproduce and display sex-typical reproductive behavior (Kudwa et al., 2005; Ogawa et al., 1999; Temple et al., 2003). Male ERβKO mice, however, display higher levels of lordosis than wild type conspecifics under certain experimental conditions, suggesting that ERβ is important for defeminization (Kudwa et al., 2005). To test this hypothesis, Kudwa and colleagues (Kudwa et al., 2006) injected female mice on postnatal days (PNDs) 1–3 with either a selective ERα agonist, 4,4′,4′-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*-phenol (PPT) or an ERβ selective agonist diarylproprionitrile (DPN) and found that neonatal administration of DPN (but not PPT) significantly reduced lordosis behavior in females, a result which supports a defeminizing role for ERβ during neonatal life. By contrast, to date, few studies have explored how neonatal exposure to ER selective agonists during critical windows of development affect male reproductive behavior or

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neuroendocrine development. Thus, the present study sought to fill this data gap.

Both ER subtypes have been shown to be present in areas of the brain important for male reproductive behavior, such as the medial preoptic area (mPOA), throughout perinatal life and into adulthood (DonCarlos, 1996; Karolczak and Beyer, 1998; Merchenthaler et al., 2004; Shughrue et al., 1998) suggesting that either could be important for crucial aspects of masculinization and defeminization. Notably, ER expression in many hypothalamic regions is sexually dimorphic throughout development (Cao and Patisaul, 2011; Lemmen et al., 1999; Perez et al., 2003). In mice, sex differences in the number of ERβ immunoreactive cells within the mPOA, for example, have been shown to be present both at birth and in adulthood, with males having a greater number than females (Kudwa et al., 2004; Wolfe et al., 2005). This pattern has also been seen in adult rats (Zhang et al., 2002). We hypothesized that agonism of ERβ could disrupt male rat reproductive behavior, and the ability to attract a mate thereby demonstrating that it may play a role in masculinization, as well as defeminization.

To test this hypothesis, we first performed a pilot study, during which we neonatally exposed male rats to vehicle, estradiol benzoate (EB), or agonists selective for ERα (PPT), or ERβ (DPN) to determine which agonist had a more profound impact on the emergence of male sexual behavior and attractiveness. Results from this pilot study suggested that agonism of ERβ could reduce male sexual vigor. Thus, we conducted a subsequent experiment using additional doses of DPN, followed by a third study using the naturally occurring ERβ selective agonist, GEN to determine if the emergence of male sexual behavior is vulnerable to endocrine disruption. GEN is an isoflavone phytoestrogen produced by legumes, such as soy, and commonly found in soy-based food products and beverages, including soy-based infant formula (Dixon and Ferreira, 2002). Developmental GEN exposure has been linked to detrimental changes in female reproductive health (Jefferson et al., 2009; Jefferson et al., 2006, 2007; Kouki et al., 2003; Patisaul et al., 2006) and possibly male reproductive health and sexual differentiation (Atanassova et al., 2000; Bu and Lephart, 2007; Cederroth et al., 2010b; Naciff et al., 2005; Scallet et al., 2004) in rodents. GEN has a nine fold higher relative binding affinity for ERβ than ERα (Barkhem et al., 1998; Kuiper et al., 1998) suggesting it has the potential to interfere with ERβ dependent brain organization.

This series of experiments served to: 1) help elucidate the ER-dependent mechanisms underlying the masculinization of male sex behavior, 2) determine if neonatal exposure to an ERβ selective EDC would have an impact on male sexual behavior and, through partner preference testing, 3) examine how this would impact attractiveness.

2. Materials and methods

2.1. Animals

Long Evans rats were maintained on a soy-free diet (5K96, Purina Test Diets, Richmond, IN) and a reversed (12:12) light cycle at 23 °C and 50% relative humidity for the duration of the experiments. The light cycle for the male animals in Experiment 1 was changed to 13:11 prior to the last round of testing to increase sexual motivation. The animals used in Experiments 1, 2 and 4 were bred in-house, and the dams for Experiment 3 were ordered timed-pregnant from Charles River Laboratories. All pups were weaned on post natal day (PND) 21 into same sex littermate pairs until two months of age, at which time they were singly housed. Animal care and testing procedures followed the relevant guidelines stated in the Animal Welfare Act and U.S. Department of Health and Human Service "Guide for the Care and Use of Laboratory Animals." The protocol was approved by the North Carolina State University Animal Care and use Committee and supervised by animal care personnel and veterinary staff. All animals were tested as young adults, with onset of testing occurring the week of PND 70, but the specific age range varied somewhat between experiments. This was not considered to be a potential confound because male rat reproductive behavior remains relatively consistent until approximately 10 months of age, at which point it begins to decline (Clark, 1995).

2.2. Experiment 1 (pilot). Impact of ERα agonist PPT and ERβ agonist DPN on reproductive behavior, physiology, and attractiveness

On the day of birth, all animals were cross-fostered, as we have done previously (Adewale et al., 2009; Patisaul et al., 2006), to minimize litter effects (as described in (Raubertas et al., 1999) (n = 9 dams). Each litter contained a mixture of animals (n = 12 maximum), only two of which were related to each other. All pups (the females were ultimately used for other projects) were subcutaneously (sc) injected with either a sesame oil based vehicle (OIL), 10 μg EB (Sigma St. Louis IL), 1 mg/kg bw DPN (Tocris Cookson, Ellisville, MO), 1 mg/kg bw PPT (Tocris Biosciences), or a mixture of PPT and DPN (1 mg/kg bw PPT + 1 mg/kg bw DPN (PPT/DPN)) from the day of birth (defined as PND 0) through PND 3. This exposure window was selected because it is well established that this is a critical period of sexual differentiation in the male rat brain (Gore, 2008; McCarthy, 2008; Simerly, 2002). All compounds were first dissolved in 100% ethanol and then sesame oil (Sigma, St. Louis, MO) at a ratio of 10% as we have done previously (Patisaul et al., 2007; Patisaul et al., 2009b). These concentrations of DPN and PPT reflect those successfully used in prior experiments (Donner and Handa, 2009; Handa et al., 2008; Lund et al., 2006; Patisaul et al., 2009a). Male sexual performance was evaluated within PNDs 78–142 with hormone replaced ovariectomized (OVX) females. These males (n = 10 OIL; 4 EB; 10 DPN; 8 PPT; 10 PPT/DPN) were then used as mate choices in a partner preference paradigm during PNDs 155–168 for intact, sexually naïve females (n = 10; age PND 108–123). All males were weighed on PND 182, castrated under isoflurane anesthesia, and returned to the colony for future work beyond the scope of this project. At the time of surgery, the testes were weighed upon removal and blood was collected via tail clip then spun at 4 °C at 13,000 rpm for 10 min to separate the plasma fraction. The plasma was extracted using a transfer pipet and stored at −80 °C until analysis. Plasma testosterone levels were subsequently measured by radioimmunoassay using Siemens Coat-A-Count total testosterone kit with an analytical sensitivity of 4 ng/dl as described previously (Patisaul et al., 2009a).

2.3. Experiment 2. Dose dependent impact of DPN on reproductive behavior and attractiveness

Males were administered DPN at three different doses (0.5, 1, or 2 mg/kg bw) to establish a dose response on male reproductive behavior and attractiveness. On the day of birth males were cross-fostered as in Experiment 1 (n = 8 dams). All pups were then administered vehicle (OIL, n = 9), EB (10 μg, n = 4), 0.5 mg/kg bw DPN (n = 12), 1 mg/kg bw DPN (n = 7), or 2 mg/kg bw DPN (n = 12) prepared as in Experiment 1, from PND 0 through 3 by sc injection. This dose range is narrow but was selected because these three concentrations of DPN bracket the range of doses successfully used in prior experiments (Donner and Handa, 2009; Handa et al., 2008; Lund et al., 2006; Patisaul and Bateman, 2008; Weiser et al., 2009) and that used in Experiment 1. DPN has a 70-fold higher binding affinity for ERβ than ERα, and the highest dose used was not found to activate ERα dependent gene expression (data not shown). Males were sex tested within PNDs 73–113 with hormone replaced OVX females and then used as mate choices in partner preference over PNDs 113–123 for intact, sexually naïve females age PND 179–187 (n = 12). The males exposed to the low dose (0.5 mg/kg bw DPN) were not tested

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for attractiveness because they showed intromission latencies and counts equivalent to those of the controls.

2.4. Experiment 3. Impact of GEN and EB on reproductive behavior, physiology, and attractiveness

On the day of birth, male animals were again cross-fostered to minimize litter effects (n = 15 dams). All pups were sc injected with either a sesame oil based vehicle (OIL), EB (10 μg, Sigma St. Louis, MO), or GEN (10 mg/kg bw, Indofine Chemical Company Hillsborough NJ) from PND 0 through 3. The dose of GEN used for this experiment is approximately equivalent to the total amount of isoflavone phytoestrogens consumed daily by infants reared exclusively on soy infant formula (Setchell et al., 1998). EB was used as a positive control (Levine and Mullins, 1964). Males (n = 15 GEN; 12 EB; 16 OIL) were sex tested between PNDs 71–85 with age-matched, hormone replaced OVX females and then used as mate choices for age-matched, gonadally intact, sexually naïve females (n = 11) during PNDs 92–156 to assess partner preference. Partner preference testing was performed twice. All animals were novel to one another in both rounds. All males were then weighed on PND 155, and sacrificed by CO_2 asphyxiation followed by rapid decapitation on PND 170 and 171 to collect trunk blood and testes for evaluation. Plasma was extracted and analyzed for circulating testosterone levels as described for Experiment 1, and the testes were weighed upon removal using an electronic balance.

2.5. Experiment 4. Replicate of experiment 3, with no cross-fostering

A second group of animals was used for Experiment 4, which was designed to replicate Experiment 3, but in this case the animals were bred in-house and the pups were not cross-fostered because they were not all born on the same day (n = 10 dams) and a recent study reported that cross-fostering may confound EDC effects (Cox et al., 2010). Thus it became important to determine if the behavioral changes observed after GEN exposure were also evident in animals that had not been cross-fostered. All compounds were prepared and administered as described in Experiment 3. Males (n = 11 GEN; 11 EB; 11 OIL) were sex tested within PND 69–95 with OVX females. These males were then used as mate choices in partner preference during PND 127–173 for age matched, intact, sexually naïve females (n = 12). Partner preference testing was performed twice. All animals were novel to one another in both rounds.

2.5.1. Assessment of reproductive behavior

After the onset of puberty, males in each experiment were tested for reproductive behavior once each week for a total of three (Experiment 3), four (Experiment 2 and 4), or five (Experiment 1) sessions dependent upon vehicle exposed (OIL) male performance. Because inexperienced males often perform poorly at the onset of testing, sexual performance was not statistically compared between exposure groups until ≥25% of vehicle exposed males had intromissions within the session. A fourth round was added to Experiments 2 and 4, while a fifth round was added to Experiment 1 because the vehicle exposed males did not meet this performance criteria in the first four rounds.

In each session, males were placed in a clean, novel cage and introduced to a sexually receptive, hormone replaced, OVX female, a procedure similar to what we and others have done previously (Becker et al., 2005; Hardy and DeBold, 1971; Patisaul et al., 2002). Testing duration was 15 min for each round in Experiments 1, 2, 3, and the first two rounds of 4. Duration was increased to 30 min for the final rounds of Experiment 4 to extend the observation period and better characterize sexual performance. All testing began within two hours of dark onset, under red-light illumination. All stimulus females were deemed receptive via a lordosis response to male contact. Behavior was recorded using a Sony camcorder set on night vision and then hand-scored by an investigator blind to the treatment groups using the behavioral analysis software package Stopwatch (courtesy of David A. Brown, Center for Behavioral Neuroscience, Emory University). Measures included latency to first intromission and total number of intromissions made during the trial because they were robustly displayed in all groups and most reliably captured the sexual motivation of the male subjects. Other measures, most notably number of mounts, and latency to first mount, were not included because they were not consistently performed by all males, particularly after they were sexually experienced.

2.5.2. Assessment of partner preference

After all rounds of sex testing were completed, the same test males were used as choices for intact, sexually naïve, proestrus (determined by vaginal cytology as described in (Becker et al., 2005)) females in a series of partner preference tests. All animals were habituated to the arena prior to testing. The plexiglass testing arena was a total length of 198.12 cm, 30.48 cm deep and 30.48 cm wide and divided into three chambers roughly equal in size with small compartments (17.78 × 30.48 × 30.48 cm) on either side. Each small compartment contained a single male enclosed behind a wire mesh composed of ¾ inch hardware cloth. A control male was placed on one side and an exposed male on the other. Positions were randomized to control for side preference. Females could spend time alone in the central chamber, or interact with one of the males in either of the end chambers though the mesh. Testing began within 2 h of dark onset, under red-light illumination, with each session lasting 20 min. Preference was determined by quantifying the amount of time spent in each chamber next to the stimulus male. In Experiments 3 and 4, the males were presented as mate choices to the females for a first round (3a, 4a) and then again for a second round (3b, 4b) to demonstrate reproducibility. In all cases, all males were novel to the selecting female. Behavior was video recorded and scored by an observer blind to the exposure groups as described above.

2.5.3. Statistical analysis

For the sex behavior tests, differences in number of intromissions and intromission latency were compared by two-way ANOVA with round and exposure as factors. Significant effects were then followed up by one-way ANOVA, first with exposure as a factor for each week, then with week as a factor within each exposure group. Significant effects in the one-way ANOVAs were followed up with Fisher's Least Significant Differences post hoc tests. For all measures, analyses were two tailed and the level of significance was set at $P \leq 0.05$.

For the partner preference data, preference was established by comparing the total time spent in the chamber containing the unexposed control (OIL) male to the time spent with the exposed male using a Fisher's Studentized *t*-test with the level of significance set at $P \leq 0.05$. Because some of the males did not intromit during the sex testing, when analyzing the partner preference results, all data were first analyzed to determine if there was an effect of sexual experience, and no effect was found in any of the experiments (*t*-test; data not shown). The data were also analyzed (*t*-test) for a side preference. A side preference was identified in the second round of Experiment 3, thus that experiment was ultimately repeated (Experiment 4). The SYSTAT software package was used to perform all statistical analyses, and graphs were generated in SigmaPlot.

3. Results

3.1. Experiment 1 (pilot): impact of ERα agonist PPT and ERβ agonist DPN on reproductive behavior, physiology, and attractiveness

3.1.1. Reproductive behavior

Only round 5 met the behavioral threshold for analysis and although a pattern did arise, there was no main effect of exposure

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group on either intromission number ($F(4,36) = 1.125$, $P = 0.360$) or intromission latency ($F(4, 36) = 0.817$, $P = 0.523$; Fig. 1A–B). Analysis of performance among only the intromitting males also failed to reveal a main effect of exposure group for either intromission number ($F(4,10) = 0.508$, $P = 0.731$) or intromission latency ($F(4, 10) = 0.335$, $P = 0.849$). In both analyses, however, a behavioral pattern emerged with the EB and DPN exposed males performing poorly, but not to a significant degree, compared to the other groups. These results constituted the rationale for the subsequent studies.

3.1.2. Partner preference

Females selected against EB exposed males compared to vehicle exposed control males ($P \leq 0.008$; Fig. 1C). In contrast, females showed no clear preference between DPN and vehicle ($P = 0.679$), PPT and vehicle ($P = 0.772$), or PPT/DPN and vehicle ($P = 0.160$) exposed males (Fig. 1C).

3.1.3. Testosterone and testis/body weight

There was a main effect of exposure group on plasma testosterone levels ($F(4, 38) = 3.212$, $P \leq 0.03$). EB exposed males had significantly lower testosterone levels compared to that of the vehicle exposed control (OIL) group ($P = 0.02$), however, none of the other exposure groups had testosterone levels that were significantly different from the controls (Table 1).

Of the four EB exposed males, two had retained testes. This was not seen in any of the other exposure groups. EB exposed males were the lightest and the DPN males were the heaviest (Table 1), but there was only a weak trend for a main effect of exposure on body weight ($P = 0.103$). The testis to body weight ratio, however, was significantly affected by exposure ($F(4, 41) = 8.857$, $P \leq 0.001$; Table 1). The ratio of the EB exposed males was significantly smaller than that of the vehicle exposed (OIL) males ($P \leq 0.001$), but the other groups did not differ from the controls.

Table 1
Experiment 1. Plasma testosterone levels and testes to body weight ratios were significantly lower in the EB exposed males compared to the vehicle exposed (OIL) males. Adult body weight did not significantly differ between groups.

Experiment 1	n	Body wt (kg)	Testes/body wt ratio (kg/g)	Total testosterone (ng/dl)
OIL	10	730.5 ± 17.87	2.61 ± 0.09	16.76 ± 3.35
EB	4	710.5 ± 35.84	1.3 ± 0.41*	2.21 ± 0.72*
DPN	10	785.9 ± 15.48	2.33 ± 0.13	10.16 ± 1.35
PPT	8	737.5 ± 14.2	2.54 ± 0.1	21.64 ± 5.12
PPT/DPN	9	743.78 ± 19.11	2.48 ± 0.1	17.71 ± 4.22

* $P \leq 0.001$.

Collectively the results from this pilot study indicated that neonatal exposure to EB results in significantly lower adult testosterone levels, improper testis development, significantly lower attractiveness, and, potentially, impaired male reproductive behavior. None of these were recapitulated in the group receiving both DPN and PPT. The ERβ agonist, DPN, appeared more effective than PPT in compromising both male sexual behavior and attractiveness.

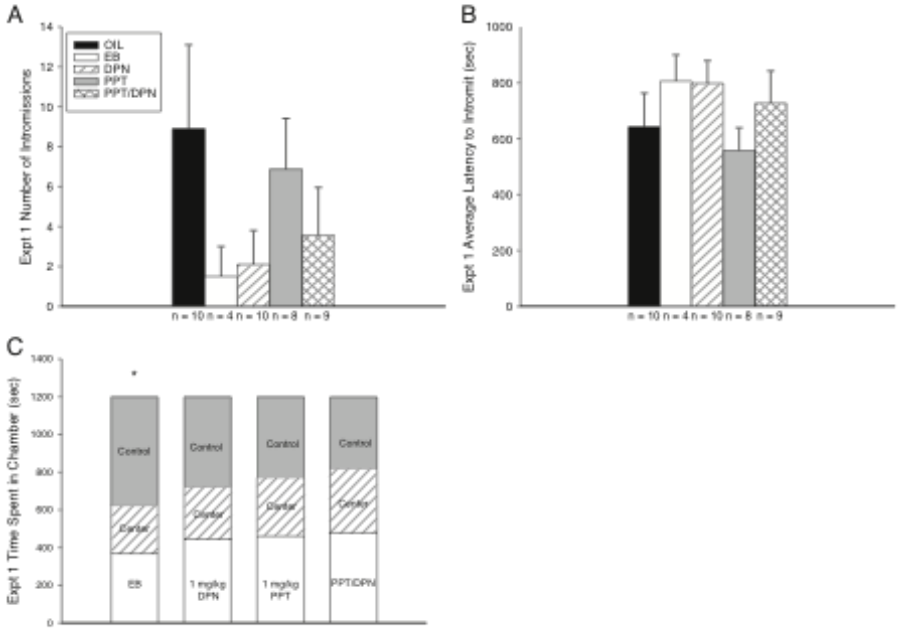

Fig. 1. Experiment 1 sexual behavior and partner preference results. (A–C) Only behavior from the fifth round of testing was analyzed and no significant differences between groups emerged for any endpoint, but the outcome suggested an emasculating effect of neonatal EB or DPN exposure. (A) EB and DPN exposed males had fewer intromissions compared to vehicle exposed (OIL) males. (B) EB and DPN exposed males had longer intromission latencies compared to vehicle exposed males. (C) Females selected against the EB exposed males by spending significantly more time in the chamber containing the vehicle exposed (Control) males. Partner preference was unaffected in the other groups (Mean ± SEM; * $P \leq 0.05$).

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Therefore we chose to further explore the impact of ERβ selective agonism by testing additional doses of DPN.

3.2. Experiment 2: dose dependent impact of DPN on reproductive behavior and attractiveness

3.2.1. Reproductive behavior

Only rounds 3 and 4 met the behavioral threshold for analysis and two-way ANOVA revealed a significant effect of testing round ($F(1,72) = 19.319$, $P \leq 0.001$) as well as exposure ($F(3,72) = 2.874$, $P \leq 0.05$) on the number of intromissions but no significant interaction (Fig. 2A). The vehicle exposed control males (OIL) made significantly more intromissions in round 4 than round 3 ($F(1,16) = 4.630$, $P \leq 0.05$). The 0.5 mg DPN exposed males ($F(1,22) = 4.590$, $P \leq 0.05$) and the 2 mg DPN exposed males ($F(1,22) = 14.288$, $P \leq 0.001$) also significantly improved over time, however the 1 mg DPN exposed males did not ($F(1,12) = 2.548$, $P = 0.136$).

Intromission latency was similarly affected (Fig. 2B). Two-way ANOVA revealed a significant effect of testing round ($F(1,72) = 22.942$, $P \leq 0.001$) as well as exposure ($F(3,72) = 5.772$, $P \leq 0.002$). Latency to intromit decreased in the vehicle exposed males between rounds 3 and 4 ($F(1,16) = 9.634$, $P \leq 0.008$). A similar decrease was observed in the 0.5 mg DPN ($F(1,22) = 4.053$, $P \leq 0.05$) and 2 mg DPN ($F(1,22) = 16.012$, $P \leq 0.001$) exposed groups, whereas the 1 mg DPN exposed ($F(1,12) = 3.243$, $P = 0.136$) males failed to improve (Fig. 2B). Within round 4, a significant difference in intromission latency between exposure groups emerged ($F(3,36) = 4.847$, $P \leq 0.006$). The 1 mg DPN exposed males had a significantly longer latency than the vehicle ($P \leq 0.02$), the 0.5 mg ($P \leq 0.03$), and 2 mg DPN exposed males ($P \leq 0.001$).

3.2.2. Partner preference

The males exposed to the low dose (0.5 mg/kg bw DPN) were not tested for attractiveness because they showed intromission latencies and counts equivalent to those of the vehicle exposed males. Females significantly preferred the vehicle exposed males over the 2 mg DPN exposed males ($P \leq 0.02$; Fig. 2C) while showing no preference between the 1 mg DPN exposed and the vehicle exposed males ($P = 0.452$; Fig. 2C).

3.3. Experiment 3: impact of GEN and EB on reproductive behavior, physiology, and attractiveness

3.3.1. Reproductive behavior

Behavior in the control group did not meet the threshold for statistical analysis until round 2. Therefore, only rounds 2 and 3 were statistically analyzed (Fig. 3A–B). Two-way ANOVA revealed a significant effect of testing round, but not exposure group, on the number of intromissions ($F(1,88) = 4.683$, $P \leq 0.03$) with the number of intromissions increasing over time. Only within the vehicle exposed

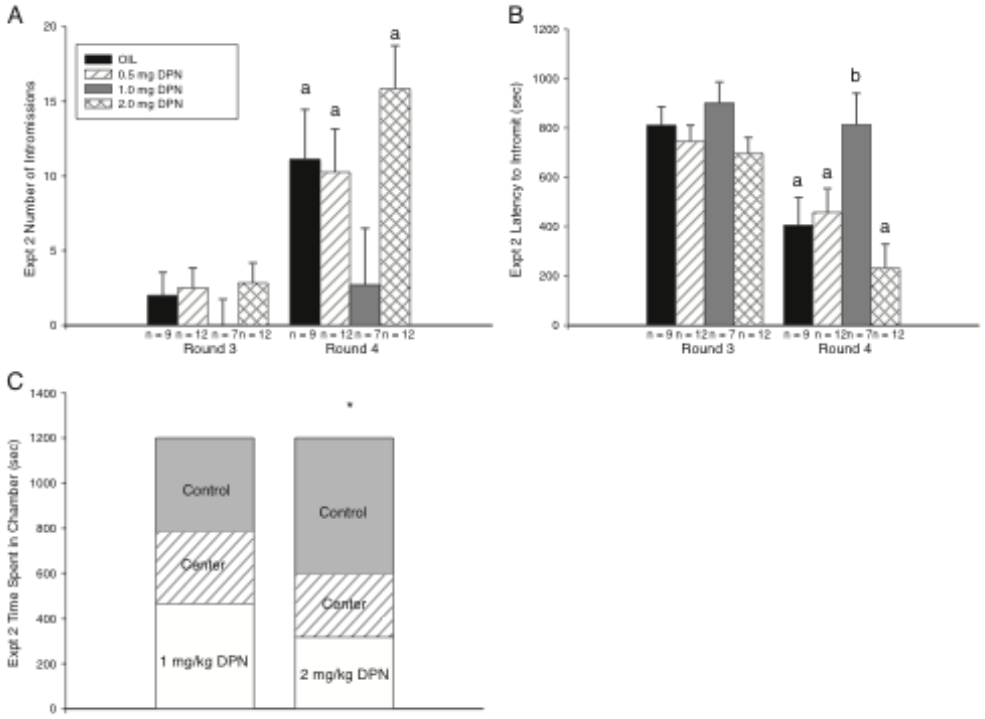

Fig. 2. Experiment 2 sexual behavior and partner preference results. (A) The number of intromissions did not differ between groups in either round but the 1 mg/kg DPN exposed group was the only group that did not display increased numbers of intromissions in Round 4 compared to Round 3. (B) Intromission latency did not initially differ between groups, but significantly dropped over time in all groups except the 1 mg/kg DPN exposed group resulting in significantly longer latencies within this group compared to the vehicle (OIL) exposed controls. (C) Females significantly preferred vehicle exposed (Control) males over 2 mg/kg DPN exposed males but showed no preference between 1 mg/kg DPN exposed males and control males. (Mean ± SEM; * $P \leq 0.05$; a = significant difference between rounds, b = significant difference from OIL controls).

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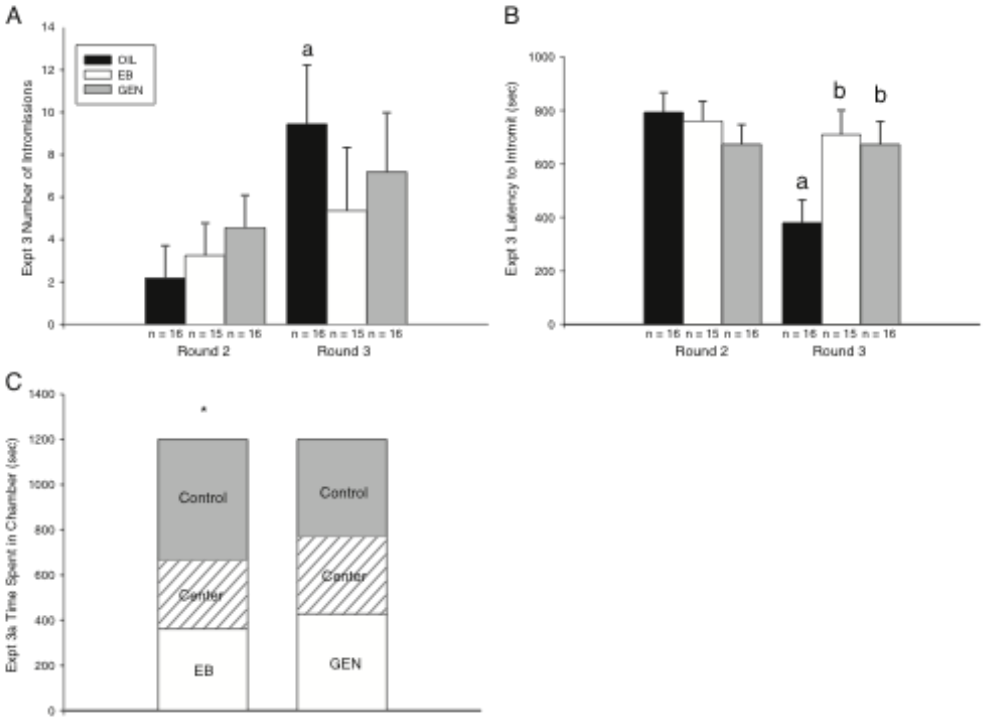

Fig. 3. Experiment 3 sexual behavior and partner preference results. (A) The number of intromissions did not significantly differ between groups in either round. The vehicle exposed group was the only group that had significantly more intromissions over time. (B) Intromission latency was similar across exposure groups in Round 2 but by Round 3, the EB and GEN exposed males had significantly longer latencies compared to controls, with only the controls having shorter latencies in Round 3 compared to Round 2. (C) Females significantly preferred vehicle exposed (Control) males over EB exposed males but showed no preference between GEN exposed and control males. (Mean ± SEM; * $P \leq 0.05$; a = significant difference between rounds, b = significant difference from OIL controls).

group did the number of intromissions significantly increase from round 2 to round 3 ($F(1,30) = 8.168$, $P \leq 0.008$).

Intromission latency was similarly affected (Fig. 3B). Two way ANOVA revealed a significant effect of testing round ($F(1,88) = 5.558$, $P \leq 0.02$) as well as a significant interaction between round and exposure ($F(1,88) = 3.990$, $P \leq 0.02$) but no main effect of exposure ($P = 0.19$). Intromission latency was roughly equivalent within each exposure group in round 2, but significant differences emerged in round 3 ($F(2,43) = 4.356$, $P \leq 0.02$). Intromission latency was significantly shorter in the vehicle exposed males compared to the EB ($P \leq 0.01$) and GEN ($P \leq 0.02$) exposed males (Fig. 3B). Significantly decreased intromission latency between rounds 2 and 3 was only observed in the vehicle exposed males ($F(1,30) = 17.466$, $P \leq 0.001$).

3.3.2. Partner preference

Partner preference was performed twice (2 rounds) with all animals being novel to one another in both rounds. In the first round of testing, females spent significantly more time in the chamber containing the vehicle exposed (Control) male than the EB exposed male ($P \leq 0.006$; Fig. 3C). In contrast, females did not show a preference for vehicle exposed males compared to the GEN exposed males ($P = 0.67$). Results from the second round of testing were confounded by a distinct left side preference and were therefore not analyzed further (data not shown). Instead, the experiment was repeated with a new group of animals to validate the initial findings (Experiment 4).

3.3.3. Testosterone and testis/body weight

Exposure significantly affected plasma testosterone levels ($F(2,39) = 17.320$, $P \leq 0.001$; Table 2). Testosterone levels were significantly lower in the EB exposed males compared to that of the vehicle (OIL) and GEN exposed males ($P \leq 0.001$). In contrast, the plasma testosterone levels in the vehicle exposed males and the GEN exposed males did not significantly differ from each other ($P = 0.578$; Table 2).

During testes collection, it was discovered that 82% (9 out of 11) of the EB exposed males had retained testes. In all cases, both testes were retained. This was not seen in the other exposure groups. Body weight did not significantly differ ($P = 0.392$) between groups. Testis to body weight ratio, however, was significantly affected by exposure ($F(2,39) = 127.984$, $P \leq 0.001$) with the EB exposed males having a smaller ratio than either the vehicle ($P \leq 0.001$) or GEN exposed ($P \leq 0.001$) males (Table 2). There was no significant difference between the ratios of the vehicle and the GEN exposed males ($P = 0.584$).

3.4. Experiment 4: replicate of experiment 3, with no crossfostering

3.4.1. Reproductive behavior

Rounds 3 and 4 met the behavioral threshold for analysis and two-way ANOVA revealed a significant effect of exposure group, but not testing round or an interaction between the two factors, on intromission number ($F(2,60) = 5.312$, $P \leq 0.008$). Significant differences in the number of intromissions made between the groups emerged in

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Table 2
Experiment 3. Plasma testosterone levels and testes to body weight ratios were significantly lower in the EB exposed males compared to the vehicle exposed (OIL) males. Adult body weight did not significantly differ between groups.

Experiment 3	n	Body wt (kg)	Testes/body wt ratio (kg/g)	Total testosterone (ng/dl)
OIL	15	647.13 ± 12.59	2.99 ± 0.09	89.09 ± 7.98
EB	11	649.82 ± 14.99	1.12 ± 0.10*	8.88 ± 5.33*
GEN	13	623.92 ± 15.89	3.06 ± 0.10	81.27 ± 14.34

* P ≤ 0.001.

round 3 ($F(2,30) = 3.414$, $P \leq 0.05$), with the vehicle exposed males intromitting significantly more than the EB ($P \leq 0.02$) or the GEN exposed males ($P \leq 0.05$). Within round 4, a similar performance pattern was observed, but was not found to be statistically significant ($P = 0.09$; Fig. 4A).

Intromission latency was similarly affected (Fig. 4B). Two-way ANOVA only revealed a significant effect of exposure ($F(2,60) = 5.161$, $P \leq 0.01$). A significant difference in intromission latency between groups arose in round 3 ($F(2,30) = 4.231$, $P \leq 0.02$) with the vehicle exposed males having a significantly shorter latency than the EB ($P \leq 0.007$) exposed males. The vehicle exposed males also had a shorter latency than the GEN exposed males (Fig. 4B), however it did not reach statistical significance ($P = 0.08$). There were no significant differences between groups in round 4 ($P = 0.25$).

3.5. Partner preference

Partner preference was performed twice (two rounds) with all animals being novel to one another in both rounds. The females significantly preferred the vehicle (control) males over the EB exposed males in both the first (Fig. 4C; $P \leq 0.01$) and second rounds (Fig. 4D; $P \leq 0.01$). In contrast, the females preferred GEN exposed males over vehicle exposed males in the first round (Expt. 4a) ($P \leq 0.001$), but in the second round (Expt. 4b) they again showed no preference ($P = 0.276$; Fig. 4C–D).

4. Discussion

Here we clearly showed that neonatal exposure to EB negatively impacted male reproductive performance and the ability to attract a mate in adulthood, observations which are consistent with prior studies (Diamond et al., 1973; Zadina et al., 1979). Moreover, we also found that selective agonists for ERβ (DPN and GEN) had an emasculating effect on these behaviors, suggesting that ERβ activation during the neonatal critical period can potentially interfere with the sex specific organization of the neuroendocrine pathways which mediate male reproductive behavior. Reduced testis weight and significantly lower circulating testosterone levels were only observed in the EB exposed group, indicating that the disruption of male reproductive behavior caused by the ERβ agonists was not the result of abnormal testicular development or circulating androgen levels during adulthood. Instead, these observations signify that disruption

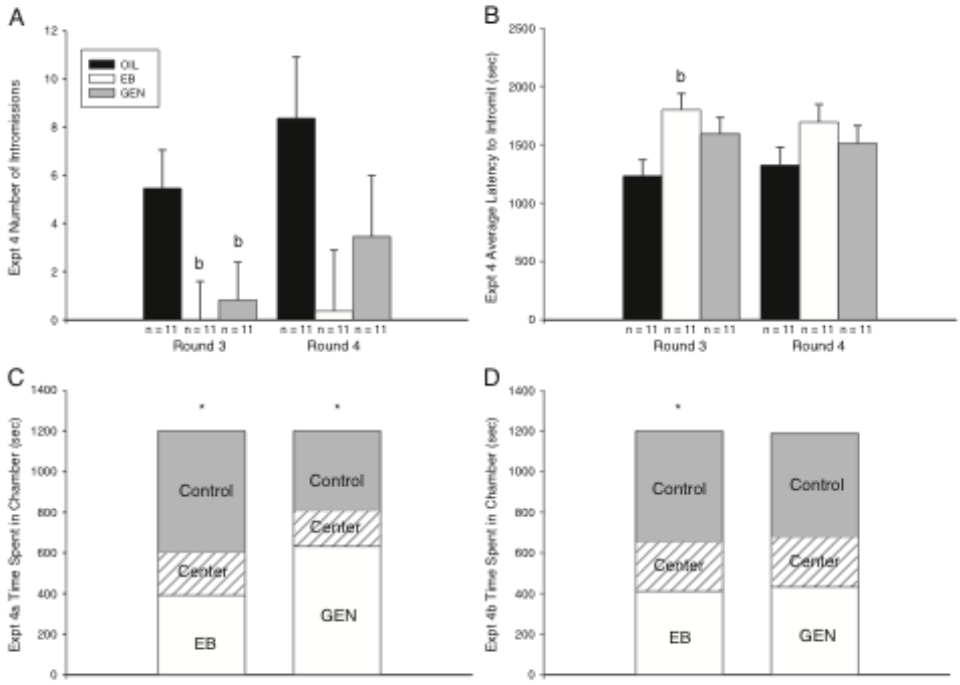

Fig. 4. Experiment 4 sexual behavior and partner preference results. (A) Number of intromissions was significantly lower in the EB and GEN exposed groups compared to the OIL control males in both rounds of testing. Numbers did not significantly increase over time in any group. (B) Latency to intromit was significantly longer in the EB exposed groups compared to the OIL group in Round 3 but this difference was lost in Round 4. (C, D) Partner preference results differed between rounds. In both cases, females significantly preferred control males over EB exposed males. However, females displayed a significant preference for GEN exposed males compared to OIL control males in the first round of partner preference testing (Experiment 4a) but not the second (Experiment 4b). (Mean ± SEM; * $P \leq 0.05$; b = significant difference from OIL controls).

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occurred elsewhere in the neuroendocrine system. Collectively, the partner preference results reveal that compromised reproductive behavior is not always accompanied by a loss of attractiveness, indicating that females may not always be capable of detecting the subtle loss of virility.

Despite the narrow dose range, the effect of DPN on male reproductive behavior was dose specific, but not linear. This non-monotonic effect may not be uncommon for some neuroendocrine endpoints (Kendig et al., 2010), including male reproductive behavior (Jones et al., 2011). This phenomenon has recently emerged as an area of intense interest within the endocrine disruption field because some weakly estrogenic compounds, such as Bisphenol A (BPA), have produced non-linear effects at low doses in a variety of model systems (Vandenberg et al., 2009). Unfortunately, the molecular and biochemical mechanisms associated with this type of dose response curve are poorly understood, but the prevailing hypothesis is that it likely reflects the intersection of multiple mechanisms. For the present study, one possibility is that ERα and ERβ are modulating each other's activity and that this relationship differs at high and low doses of estrogen (or ER-selective agonists). It is now widely recognized that the relationship between ERα and ERβ is dynamic and complex. For example, ERβ activation can antagonize ERα-dependent transcription (Matthews and Gustafsson, 2003; Matthews et al., 2006; Rissman, 2008) but the two ER subtypes can also have synergistic or sequential effects (Rissman, 2008). It is possible that, at the 1 mg/kg dose, agonism of ERβ by DPN dampened the activity of ERα, thereby resulting in reduced virility but that other activities occurred at the 0.5 and 2 mg/kg doses. Further studies using additional doses of DPN and PPT, as well as antagonists for ERα and ERβ would be required to further elucidate these interactions. Moreover, prior work has established that although postnatal administration of testosterone to neonatal castrates can rescue male sexual behavior, exogenous administration to gonadally intact neonates results in compromised male sexual performance (Henley et al., 2010; Pollak and Sachs, 1975; Zadina et al., 1979). Thus there appears to be an "optimal range" in which steroid hormones induce masculinization, and doses above or below that range can result in partial demasculinization. This phenomenon is intrinsically non-monotonic.

The significantly lower circulating testosterone levels observed in the EB exposed animals is likely a substantive contributing factor for compromised reproductive performance and attractiveness in this group (Baum, 2009), but not for the 1 mg/kg DPN group or the GEN groups, as androgen levels in those groups did not significantly differ from controls. Unfortunately, plasma concentrations were not measured in the 2 mg/kg DPN exposed animals but presumably their levels were within the normal range because virility was not found to be compromised. The emasculating effects of EB observed here are consistent with prior studies examining the impact of postnatal estrogen exposure on male reproductive physiology and behavior (Diamond et al., 1973; Zadina et al., 1979) but also a recent study employing the postnatal administration of an aromatizable androgen (Henley et al., 2010). Male sexual performance was significantly compromised and circulating testosterone levels were reduced compared to unexposed control males. Moreover, partner preference was altered such that exposed males spent more time with another male conspecific, when given a choice between a male and an estrous female, than control males. This result was interpreted to indicate partial demasculinization and the development of a bi-sexual social preference, an effect which could reduce reproductive fitness because the hyper-androgenized male would presumably be just as likely to approach a male or a female.

The results from the present study suggest that postnatal agonism of ERβ also induces a partial demasculization. A role for ERβ in the masculinization process is further supported by the discovery that androgen metabolites, most notably 5α-androstane-3β, 17β-diol (3β-Adiol), interact with ERβ in the brain and other organs (Handa et al., 2009; Kuiper et al., 1998; Lund et al., 2006; Weiser et al., 2009). During development, 3β-Adiol is secreted from immature testes and can also be synthesized *de novo* in cells that express 5α-reductase and 17β-hydroxysteroid dehydrogenase type 7 (Weihua et al., 2002). It has previously been shown that 3β-Adiol activates estrogen response element (ERE) dependent ERβ-induced transcription equivalent to that of 17β-estradiol in neural tissue (Pak et al., 2005). Thus it has been proposed that 3β-Adiol is the native ligand for ERβ in perinatal life (Fan et al., 2010). Moreover, expression of ERβ within the mPOA, a region long recognized to be important for both appetitive and consummatory aspects of male sexual behavior (Balthazart et al., 1998; Hull and Dominguez, 2007; McCarthy et al., 2009; Phillips-Farfan and Fernandez-Guasti, 2009), is robust within the neonatal period (Cao and Patisaul, 2011) suggesting that emerging sexual dimorphisms within this region may be particularly sensitive to ERβ agonists during this time. For example, synaptic spine density in the mPOA is positively correlated with male mounting behavior (McCarthy et al., 2009) and the density of dendritic spines in the mPOA is higher in males than females as early as PND 0 (Amateau and McCarthy, 2002). Neonatal agonism of ERβ may have altered the masculinization of this pathway, leading to fewer mPOA dendritic spines and therefore compromised male sexual performance.

Paradoxically, females selected against the males neonatally exposed to the high dose (2 mg/kg) of DPN in the partner preference test even though the reproductive behavior of these males did not appear to be compromised. Conversely, females failed to select against the males exposed to the intermediate (1 mg/kg) dose of DPN or GEN, even though both of these groups had lower virility. In one round of Experiment 4, females actually displayed a preference for the GEN exposed males so collectively the data indicate that, if anything, neonatal GEN exposure could slightly enhance attractiveness, rather than reduce it, despite compromised sexual vigor. In all cases, females consistently favored the control males over the EB exposed males, thus it is certain that the females were being selective. It is not clear upon what pheromonal or other cues the females based their preference, but it is possible that females need to physically interact with the males to make a more accurate assessment of virility.

The results observed following neonatal GEN exposure on male reproductive physiology and behaviors are consistent with prior studies. For example, a recent study also failed to find any impact of perinatal GEN exposure, across a wide dose range, on testis weight or blood androgen levels in a variety of species (Cederroth et al., 2010a). Effects on male reproductive parameters may be more subtle, however. A recent study characterized the impact of lifetime (gestation to adulthood) exposure to a soy-rich diet containing GEN on a range of reproductive parameters in male mice and found a 25% decrease in epididymal sperm count as well as a 20% reduction in litter size. Moreover, expression of numerous germ cell specific genes involved in sperm glycolysis and mobility were also significantly lower in the soy-exposed males (Cederroth et al., 2010b). This is important to note in light of the partner preference results because if males exposed to GEN are reproductively impaired, but females continue to select them as a mate, then fitness could be decreased and this may ultimately negatively affect a population.

Only two other published studies have examined the effects of exposure to an environmentally relevant dose of endocrine disrupting chemicals on partner preference (Crews et al., 2007; Markman et al., 2008). Collectively, the results from these two prior studies indicate that EDC exposure might induce multigenerational effects, and a transglobal population decline in a given species. Crews et al. (2007) found that exposure to vinclozolin, an anti-androgenic fungicide, decreased male attractiveness, and that this effect persisted across generations such that females with no exposure history and females three generations removed from the exposure, selected against males with an exposure history. By contrast, males failed to select against

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exposed females. Further assessment revealed a transgenerational impairment in male fertility (Anway et al., 2005) suggesting that vinclozolin exposure induces epigenetic changes within the male germ line that impact both fertility and attractiveness in subsequent generations of males (Anway and Skinner, 2008). Markman et al. (2008) exposed juvenile male song birds to different combinations of estrogenic EDCs, all present in the natural feeding areas of the study population, in a manner consistent with the natural exposure route. A mixture of estradiol, dioctylphthalate, BPA and dibutylphthalate, appeared to act synergistically, producing a hypermasculinized song system, and hence a more attractive male, based on female selection of song quality. Exposure to these EDCs, however, also led to these males being immunocompromised. Therefore, song quality was no longer a reliable indicator of overall fitness which, over time, could contribute to population decline.

Finally, no significant effect of PPT was observed in Experiment 1. It should be noted that, overall, male reproductive performance was poor in Experiments 1 and 4 which contributed to the high degree of variability. This project began and ended during the winter months and, although the environment in our vivarium is tightly controlled, it is possible that there was an effect of season on reproductive performance. To compensate, the experimental paradigm was amended in several ways as the studies progressed. For example, additional rounds of testing were included, the light cycle was extended, and the observational period was lengthened. It is also possible that the dose of PPT was insufficient to induce a response. Because male mounting behavior is compromised by perinatal androgen (Henley et al., 2010) or estrogen (Zadina et al., 1979) administration, it was hypothesized that PPT administration could impair virility. Further work will be needed to adequately test this possibility.

It is important to note that maternal behavior is well recognized to influence the subsequent male reproductive behavior of their offspring, thus EDCs or experimental manipulations that impact maternal care can be potential confounds (Cox et al., 2010; Del Cerro et al., 2010; Rhees et al., 2001). The dams in this study were not dosed directly, as we targeted the developmental window of PNDs 0–3. Therefore if dams were exposed, it would only be minimally via ingestion during the licking and grooming of pups. Prior studies have established that dietary genistein does not significantly impact licking and grooming or nest building in rats (Ball et al., 2010) and either fails to alter (Flynn et al., 2000), or enhances nursing behavior (Ball et al., 2010). Handling stress is a concern when cross-fostering (Caldji et al., 2003; Cox et al., 2010). Thus, to account for that, the GEN exposure was conducted twice, once with cross-fostering and once without. The results were similar in both cases, suggesting that cross-fostering was not a confound and did not significantly modify the effects of GEN.

Collectively, the data indicate that ERβ is involved in the masculinization of neuroendocrine pathways that regulate sex specific behavior, and imply that environmental exposures during critical stages of neuroendocrine development can evoke long term effects on complex behaviors. Our results show that neonatal agonism of ERβ, either with a synthetic agonist or a naturally occurring EDC, can impair reproductive behavior in male rats. This effect, however, does not always accompany decreased capacity to attract a mate. Furthermore, DPN produced a non-monotonic effect on male virility. Because GEN and other EDCs are increasingly ubiquitous components of our environment, a critical next step would be to explore the dose-specific effects of more ERβ agonists like GEN and determine if these effects persist in subsequent generations, potentially through epigenetic mechanisms.

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CHAPTER 3:

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Anxiogenic Effects of Developmental Bisphenol A Exposure Are Associated with Gene Expression Changes in the Juvenile Rat Amygdala and Mitigated by Soy

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Abstract

Early life exposure to Bisphenol A (BPA), a component of polycarbonate plastics and epoxy resins, alters sociosexual behavior in numerous species including humans. The present study focused on the ontogeny of these behavioral effects beginning in adolescence and assessed the underlying molecular changes in the amygdala. We also explored the mitigating potential of a soy-rich diet on these endpoints. Wistar rats were exposed to BPA via drinking water (1 mg/L) from gestation through puberty, and reared on a soy-based or soy-free diet. A group exposed to ethinyl estradiol (50 µg/L) and a soy-free diet was used as a positive estrogenic control. Animals were tested as juveniles or adults for anxiety-like and exploratory behavior. Assessment of serum BPA and genistein (GEN), a soy phytoestrogen, confirmed that internal dose was within a human-relevant range. BPA induced anxiogenic behavior in juveniles and loss of sexual dimorphisms in adult exploratory behavior, but only in the animals reared on the soy-free diet. Expression analysis revealed a suite of genes, including a subset known to mediate sociosexual behavior, associated with BPA-induced juvenile anxiety. Notably, expression of estrogen receptor beta (*Esr2*) and two melanocortin receptors (*Mc3r, Mc4r*) were downregulated. Collectively, these results show that behavioral impacts of BPA can manifest during adolescence, but wane in adulthood, and may be mitigated by diet. These data also reveal that, because ERβ and melanocortin receptors are crucial to their function, oxytocin/vasopressin signaling pathways, which have previously been linked to human affective disorders, may underlie these behavioral outcomes.

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Introduction

In species from rodents to humans, developmental exposure to endocrine disrupting compounds (EDCs) affects the emergence of sexually dimorphic behaviors sensitive to the organizational effects of steroid hormones [1]. Perinatal exposure to the plastics component Bisphenol A (BPA) alters sociosexual and anxiety-related behaviors in rodents (reviewed in [2]) and non-human primates [3], in age- and sex-specific manners. In nearly all of these prior studies, behavior was assessed in adulthood, and the specific mechanisms by which BPA altered behavior were largely unexplored. Emerging studies have associated prenatal BPA exposure with elevated hyperactivity and anxiety in young girls [4,5] suggesting the possibility that developmental exposure to BPA and other EDCs might contribute to the growing prevalence of behavioral- and mood-related disorders in children [6,7]. What remains to be established, and was addressed by the present studies, are (1) how chronic, low dose oral exposure to BPA throughout development impacts affective behavior during adolescence and adulthood; (2) the underlying molecular changes to the nervous system related to these behavioral effects, and (3) the potential for dietary intervention to mitigate BPA effects.

BPA is used in numerous household products including polycarbonate plastic food containers, the epoxy linings of canned foods, and thermal paper receipts [8,9,10]. BPA exposure is nearly ubiquitous in the United States [11] and occurs primarily via consumption of food and beverages from which BPA has leached from the container. BPA is hypothesized to act, primarily, by interacting with estrogen receptors [12], but we have previously shown that the anxiogenic properties of BPA exposure are not consistently recapitulated by estrogen exposure [13]. Other studies demonstrate BPA effects on the thyroid hormone receptor [14] and dopamine signaling pathways [15], indicating that the mechanism of action is more complex. Additionally, work with the viable agouti mouse (Avy) has revealed that the soy

phytoestrogen genistein (GEN) can counter the hypomethylating properties of BPA resulting in a coat color shift, suggesting epigenetic change as an alternative mechanism of action [16].

GEN and other isoflavone phytoestrogens are estrogen-like compounds produced by plants, most notably soy and other legumes. For decades, they have been recognized to interact with the mammalian endocrine system [17,18,19]. Like BPA and other synthetic EDCs, they have historically been thought to act primarily through estrogen receptors [20], but they are also tyrosine kinase inhibitors [21,22], and modulate DNA methylation and chromatin configuration [23]. The observation that GEN can mitigate effects of BPA on DNA methylation [16] suggests that the mechanisms of action of these compounds are not only distinct but can also act in opposition. For example, the impact of BPA on oocyte meiosis depends, at least in part, on diet [24], further emphasizing the interactive potential of anthropogenic and naturally-occurring EDCs.

Although marketed as a therapeutic to alleviate the physiological and mood changes associated with menopause, the experimental evidence for soy phytoestrogen impacts on sociosexual behavior is inconsistent and appears to vary depending on exposure period and sex [25,26,27]. While some studies have shown that life-long soy intake can result in anxiolytic effects in rodents [28,29], others have shown that adult-only intake can be anxiogenic in males yet anxiolytic in females when endogenous estrogen levels are high and exploratory drive is naturally heightened [30].

The current study addressed several important gaps identified above. First, effects of BPA and soy phytoestrogens were tested on sexually dimorphic anxiety-related behaviors as juveniles and adults. This approach enabled us to distinguish the importance of developmental age at testing, and to explore interactions between BPA and phytoestrogens. To gain further insight into what molecular changes might underlie the behavioral phenotype, we used a low-density PCR array to identify transcriptional changes within the amygdala (AMYG), chosen because of its fundamental role in the processing of sociability, fear, anxiety and other emotional responses [31]. The 48 genes selected for analysis in this study were chosen because they are known to influence sociosexual behavior, are modified by estrogen, or are vulnerable to BPA exposure. To ensure that exposure to BPA and the phytoestrogens were in a range considered relevant to humans, internal dose of BPA and GEN were assessed throughout the dosing period.

Methods

Ethics Statement

Animal care, maintenance, and surgery were conducted in accordance with the applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and use of Laboratory Animals" and were approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee (IACUC). All procedures were approved and monitored by the supervising veterinarian for the duration of the project.

Animal Care and Exposure

Wistar rats bred in house and reared on a phytoestrogen-free diet over several generations were used for these experiments. Due to the large number of animals needed and limited space available for breeding, matings were split into 4 cohorts, all bred approximately one month apart. All dams were housed individually in two humidity-and-temperature controlled rooms (segregated by diet), each with a 14 h:10 h light, dark cycle (lights on from 0200 to 1400 h) at 23°C and 50% average relative humidity at the Biological Resource Facility at NCSU.

Exposure began on gestational day (GD) 6 and continued until postnatal day (PND) 40 (GD 0 defined as day of sperm plug detection; PND 0 defined as day of birth) so that exposure covered post-implantation gestation through peri-puberty. On PND 21 pups were weaned into same sex and exposure groups (3–5 pups per cage), and ear punched for identification. At 3 months of age they were separated into same-sex pairs. There were a total of 5 exposure groups: **Soy** (soy diet); **BPA + Soy** (soy diet plus water containing BPA); **Soy-free** (soy-free diet); **BPA** (soy-free diet plus water containing BPA) and **EE** (soy-free diet plus water containing EE). The soy-based diet was Purina 5001 [32] and the soy-free diet was Teklad 2020 (Harlan). Phytoestrogen levels in the Purina 5001 diet [33,34] approximate levels in a traditional Asian diet or consumed by infants reared on soy formula [26]. The pharmacokinetics of GEN and other phytoestrogens delivered by this diet have been well characterized in the rat [34,35]. Dams were placed on their respective diet at least a week prior to mating and remained on their assigned diet for the duration of the experiment. BPA ((2,2-bis(4-hydroxyphenyl)propane; CAS No. 80-05-7; Lot 11909; USEPA/NIEHS standard provided to HBP); 1 mg/L of water) and the positive control ethinyl estradiol (EE; 50 µg/L) were administered via drinking water. This dose of BPA was chosen based on prior studies utilizing this method of exposure [36,37,38] to produce serum levels in the human range. The scale of the study precluded us from examining more than one dose of BPA. Each water solution (BPA and EE) was prepared initially as a 10× stock solution. Working solutions were prepared by adding 50 ml of stock solution to 450 ml filtered water (500 ml per bottle total). Cohort 1 EE dams drank less than controls or dams consuming BPA. To encourage them to drink, for cohorts 2 and 3, 1 g of sucrose was added to the 10× EE stock solution to increase palatability.

Water consumption for each cage was measured bi-weekly by weighing the bottles empty, full (500 ml) and then after a few days to assess intake. For the pups, estimated intake per pup was estimated by dividing consumption by the number of pups in the cage. Daily intake was used to estimate daily ingestion of BPA or EE (Tables 1 and 2).

Serum Analysis of BPA and GEN

BPA, GEN and the glucuronide metabolite of GEN were isolated and identified in the dams and PND 12 animals as described previously [39] (See Supporting Information S1). An attempt was made to quantify levels in a subset of juveniles but those results could not be reported with confidence.

Behavior

Juvenile behavior was assessed after weaning but prior to puberty (PNDs 24–28) using the light/dark box (L/D box) and elevated plus maze (EPM) as previously described [40]. Adult behavior was assessed from PNDs 60–70 using the EPM. Because it is well established that female exploratory behavior is cycle-dependent [30,41], all females were tested in estrus. Cycle phase was identified by vaginal lavage [42]. All behavior was video recorded and subsequently scored by an observer, blind to the treatment groups, using Stopwatch (courtesy of the Center for Behavioral Neuroscience, Emory University, Atlanta, GA, USA).

Gene Expression Analysis

A subset of animals tested for juvenile behavior was sacrificed on PND 34, and the brains removed and flash frozen on powdered dry ice. Using a cryostat, each brain was sectioned to reveal the

Table 1. Mean Dam Fluid Intake (ml) and Exposure Levels (µg) of BPA or EE.

Dam Exposure Group	Mean Daily Gestational Intake (ml)	Mean Daily Gestational Exposure to BPA or EE (µg)	Mean Daily Lactational Intake (ml)	Mean Daily Lactational Exposure to BPA or EE (µg)
Soy-free	60.6±1.7	0	57.9±2.4	0
Soy	58.7±2.3	0	86.1±17.3	0
BPA	35.2±2.2	35.2±2.2	71.8±18.5	71.8±18.5
BPA + Soy	55.6±4.5	55.6±4.5	105.6±5.7	105.6±5.7
EE	21.0±4.9	1.1±0.3	28.8±5.1	1.4±0.3

Average daily water consumption was quantified from the dams during mid-gestation and mid-lactation, from which average daily BPA or EE was calculated. Soy exposure occurred via diet only and therefore is not included.

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caudal border of the amygdala was visible (identified with the assistance of a brain atlas (Paxinos and Watson plates 49–58), removed via micropunch, and shipped to Dr. Gore at University of Texas at Austin for further analysis according to their established protocols [43,44] (See Supporting Information S1). Relative expression was determined using the comparative Ct method [45,46,47], with each sample normalized to *Gapdh*, and data calibrated to the median **Δ**Ct for the group with the lowest expression set at 1.0 [44]) to determine fold change in expression for each sample.

Data Analysis

Because the EE group was included to specifically test the hypothesis that any observed BPA effects were estrogenic, it was not incorporated in the overall analysis, but rather compared to the group of interest by a t-test, when appropriate. Behavior reported as a percent was analyzed by logistic regression. For the remaining behavioral and gene expression endpoints, three-way ANOVA with gender, BPA exposure, and diet as factors, was used to identify main effects and interactions. No effect of gender was found for juvenile behavior so the data were collapsed across gender for all subsequent analyses. Group differences between groups maintained on the same diet were then identified using post-hoc two-sample separate variance t-tests. A similar approach was used to analyze behavioral endpoints in the adults, but the analysis was conducted within sex.

For gene expression data, relative expression was analyzed by three-way ANOVA with gender, BPA exposure and diet as factors to determine main effects and interactions. Data were first tested for outliers using the z-score of the residuals from the initial regression and was considered an outlier if it was greater than 2.5 standard deviations from the initial line of best fit. Confirmed outliers were excluded from final analysis. If expression data did not meet the assumptions for ANOVA, the data were transformed (ln) and reanalyzed. In a few cases, transformed data did not meet the assumptions for parametric testing. In those cases, non-parametric tests were used to determine differences between the groups (See Table S1). Because interactions of variables cannot be determined using non-parametric testing, data were coded according to each variable or combination of variables and analyzed using a Kruskal-Wallis or Mann-Whiney U test. To identify group differences within sex for the post-hoc analysis, two-sample t-tests were performed with separate variance.

Results

Litter Data

Average litter size ($P = 0.381$), and sex ratio within the litters ($P = 0.886$) did not significantly differ across treatment groups.

Exposure and Internal dose of BPA and GEN

Dam water consumption was subdivided into amounts consumed during mid-gestation and mid-lactation (Table 1). One-way

Table 2. Mean Pup Intake (ml) and Exposure Levels (µg) of BPA or EE.

Pup Exposure Group	Sex	Mean Daily Intake (ml)	Mean Daily Exposure to BPA or EE (µg)
Soy-free	Female	11.9±0.1	0
	Male	11.8±2.0	0
Soy	Female	18.7±2.8	0
	Male	16.8±1.7	0
BPA	Female	22.4±2.8	22.4±2.8
	Male	18.2±2.0	18.2±2.0
BPA + Soy	Female	23.2±1.7	23.2±1.7
	Male	24.8±2.1	24.8±2.1
EE	Female	16.9±1.8	0.9±0.1
	Male	18.5±5.1	0.9±0.3

Average daily water consumption was quantified from the pups during postnatal days 21–40, from which average daily BPA or EE was calculated. Soy exposure occurred via diet only and therefore is not included.

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ANOVA indicated a significant effect of exposure group ($P \leq 0.001$). Subsequent analysis with a Fisher's LSD post-hoc test revealed that during gestation, BPA exposed dams drank significantly less water than all other groups ($P \leq 0.001$), and EE exposed females drank significantly less across gestation ($P < 0.001$) and lactation ($P \leq 0.003$) compared to controls. Importantly however, intake in these groups was sufficiently high enough to ensure that dehydration was not a potential confound. Pup intake over PNDs 21–40 was estimated for each sex (Table 2) and analyzed in a two-way ANOVA with gender and exposure as factors. A significant exposure effect was identified ($P \leq 0.004$) with BPA-exposed pups of both sexes (regardless of diet) drinking significantly more water than unexposed groups ($P \leq 0.05$; Table 2). An estimate of daily BPA and EE intake was then calculated for the dams and pups from these water intake levels (Tables 1 and 2).

Internal dose was established by quantifying free BPA and GEN, as well as conjugated GEN plasma levels in a subset of PND 12 and all dams on the day of weaning (Table S1 A–B). Samples from the pups were pooled to obtain sufficient volume for assessment. For all groups, free BPA levels were less than 2 ng/ml; a range that approximates the current estimated mean serum levels in humans [48]. Levels in the pups were lower than in the dams, a result which is consistent with prior work indicating that it does not lactationally transfer efficiently [49]. Dam GEN levels were in the range of vegetarians and other populations that regularly consume soy-rich foods (Table S1), but well below those seen in soy-formula fed infants [26]. GEN levels were lowest in the PND 12 pups, reflecting poor lactational transfer [49,50]. An attempt was made to quantify serum levels in the juveniles but those results could not be reported with confidence because the values were near the limit of detection and other technical issues. Trace levels of free BPA were found in some of the unexposed controls a result which may reflect contamination (from the collection materials or diet), or an artifact since the levels were close to the limit of detection (0.1 ng/ml).

Juvenile Exploratory and Anxiety-like Behavior

Juvenile behavior was first assessed in the Light/Dark box, an apparatus that appraises an animal's motivation to leave the reassuring confines of a darkened enclosure to explore an aversive, brightly lit chamber [51]. No significant effect of sex was found for any of the assessments, nor any significant interactions with sex; so, for subsequent analyses, the data were collapsed across gender. Among the animals maintained on a soy-free diet, the percent of BPA and EE exposed animals entering the lit chamber was significantly lower than for the control animals ($P \leq 0.05$; Figure 1A) indicating a higher level of anxiety and reluctance to explore the novel environment in the treatment groups. No effect of BPA was found among the soy-fed animals. A significant effect of BPA exposure was found on latency to enter the lit chamber (Figure 1B), but only among animals maintained on the soy-free diet ($P \leq 0.05$) with BPA exposed animals taking longer to enter the lit chamber. A diet by BPA exposure interaction ($P \leq 0.05$) was identified for the overall amount of time spent in the lit chamber, with BPA exposed animals on the soy-free diet spending significantly less time in the lit chamber than control animals on the same diet (Figure 1C; $P \leq 0.05$). Among the animals maintained on the soy-based diet, however, BPA exposure elevated the time spent in the lit chamber indicating a significant modifying effect of diet. Collectively, these results show that developmental low dose BPA exposure induces anxiogenic responses and that concurrent consumption of a soy-based diet is capable of counteracting these effects. EE exposure did not recapitulate the behavioral effects of BPA exposure, suggesting that the mechanism is not classically estrogenic.

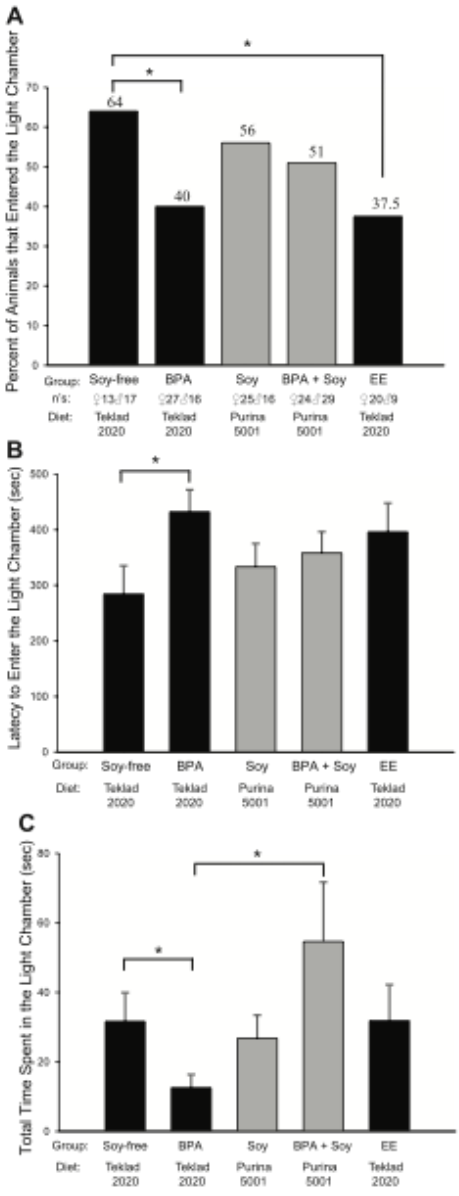

Figure 1. BPA, EE and dietary effects on juvenile Wistar rat Light/Dark Box activity. No significant effect of gender was identified, therefore the data were collapsed across sexes. Animals maintained on the soy-based diet are depicted in gray and animals maintained on the soy-free diet are depicted in black. (A) BPA and EE exposure significantly decreased the percent of animals entering the lit

chamber compared to controls on the same diet (Soy-free). (B) BPA exposure significantly increased latency to enter the lit chamber compared to the Soy-free controls. (C) Animals exposed to BPA and maintained on the soy-free diet spent significantly less time in the lit chamber than the Soy-free controls, and also the BPA-exposed animals maintained on the soy-based diet (BPA + Soy). Graphs depict mean ± SEM, *$P \leq 0.05$.
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The animals were then subjected to the elevated plus maze (EPM) as an additional assessment of anxiety. This maze has two arms with high protective walls, and two arms with no walls. This apparatus assesses the animal's reluctance to leave the relative safety of the walled arms to explore the more aversive, open arms [52]. Activity in the closed arms is not mood dependent and thus regarded as a marker of general activity rather than anxiety (See Figure S1). The percent of BPA exposed males entering the open arms was significantly lower than all other groups ($P \leq 0.001$; Figure 2A) with only 75% of males entering the open arms. For the remaining measures, the data were collapsed across gender because no main effect of sex was identified. Latency to enter the open arms was not significantly different between groups (Figure 2B). Two-way ANOVA revealed a main effect of diet ($P \leq 0.002$) on number of open arm entries, with the soy-fed animals making more entries than those maintained on soy-free diet regardless of BPA exposure (Figure 2C). Follow-up t-tests revealed that BPA exposed animals on the soy-free diet made significantly fewer open arm entries than control animals on the same diet ($P \leq 0.05$), while EE exposure had no effect. Of the animals exposed to BPA, those maintained on the soy-based diet made significantly more entries ($P \leq 0.001$) indicating a protective effect of soy diet. Two-way ANOVA also revealed a significant main effect of diet on time spent on the open arms ($P \leq 0.001$; Figure 2D) with animals on the soy-free diet showing less activity on the open arms than those maintained on the soy-based diet. BPA had no effect on this measure, regardless of diet. These data are consistent with those obtained from the Light/Dark box in that they reveal the anxiogenic effects of BPA exposure, and the capacity of a soy-based diet to mitigate this behavioral change.

Gene Expression Changes in the Juvenile Amygdala Associated with Disrupted Behavior

A subset of the juveniles was sacrificed on PND 34 to identify gene expression levels in the amygdala associated with BPA-induced behavioral change. Of the 48 genes selected for analysis, a main effect of BPA exposure and/or an interaction with BPA was found for 13 genes when compared by 3-way ANOVA (with factors of sex, BPA and soy; see Table S2). No effects survived a false-discovery rate correction [53,54]. Two housekeeping genes were included on the array (*18s* and *Gapdh*). Both displayed a small but significant effect of diet on relative expression (~20% when collapsed across all other variables). Thus, in order to maintain consistency with previous publications [44], the data were normalized to *Gapdh* and, as a conservative approach, only genes displaying greater than 20% change in expression were considered in the subsequent analysis to identify expression changes associated with BPA and/or soy intake. This approach identified 8 genes from the list of 13 (Figure 3).

Generally, BPA exposure decreased expression by about 1.5-fold. Four genes (*Esr2 (ERβ)*, *Mc4r*, *Mc3r*, *Tac2*) exhibited a BPA by diet interaction ($P \leq 0.05$), with soy diet mitigating the effect of BPA, and all four decreased ~1.5–2 fold with BPA exposure alone. These candidate 8 genes were then analyzed within sex using a two-sample t-test. *Bdnf* and *Kiss1* expression were affected by BPA exposure in a sex specific manner. Both genes were sexually dimorphic with *Kiss1* being ~9-fold greater in males than females and *Bdnf* expression being ~1.5-fold lower in males when compared to females. These sex differences were eliminated by BPA exposure. In both sexes, *Esr2* and *Gal2* expression were significantly down-regulated by BPA compared to Soy-free controls ($P \leq 0.05$). *Tac2* and *Mc4r* ($P \leq 0.05$) were significantly down-regulated by BPA exposure only in females. Among the soy-fed animals, no significant effect of BPA was identified for any gene, suggesting a protective effect of soy. None of these BPA-associated gene expression changes were recapitulated by EE demonstrating that they do not likely result from strictly estrogenic effects.

Adult Exploratory and Anxiety-Related Behavior

Adult exploratory behavior was assessed between PNDs 60–70 in the EPM. EE exposed animals were not included because it produced no significant behavioral effects in the juveniles and all of these animals were sacrificed for inclusion in the gene expression analysis. Adult exploration of both EPM arm types is typically lower than that of juveniles and sexually dimorphic, with females displaying more open arm activity than males [55]. All females were tested on the day of behavioral estrus to control for ovulatory cycle effects, because females are typically most active on the EPM when they are sexually receptive and thus naturally more exploratory [56,57]. For both sexes, the percent of animals entering the open arms was lowest in the group reared on the soy-based diet, but no significant overall effect of diet, BPA or gender was found for this specific measure (Figure 4A). As expected [57], three-way ANOVA identified a significant effect of sex ($P \leq 0.001$) on all other EPM activity (Figure 4B-D) with females being more exploratory than males. Because of the strong gender effect, the data were analyzed within sex.

Latency to enter the open arms (Figure 4B) was highly sexually dimorphic, with males taking nearly four times longer to enter the more aversive open arm than females ($P \leq 0.05$). A significant BPA by diet interaction ($P \leq 0.02$) was found in females with BPA and soy diet increasing latency when administered independently, but not in combination. In contrast, a significant main effect of BPA exposure ($P \leq 0.05$) was found in males with BPA feminizing this behavior regardless of diet. Confirmatory t-tests identified a significant sex difference in latency to enter the open arms for only the controls reared on soy-free diet, demonstrating the loss of this sex difference by BPA and soy intake. In general, activity on the open arms was sexually dimorphic, as expected, but this difference was eliminated by BPA exposure in the animals maintained on the soy-free diet, and preserved in the animals consuming the soy-rich diet (Figures 4C and D). Two-way ANOVA (BPA and diet as factors) revealed a significant BPA by diet interaction on the amount of time females spent on open arms ($P \leq 0.03$), and post-hoc t-tests showed that females spent significantly more time exploring open arms than males, except in the BPA exposed group reared on the soy-free diet ($P \leq 0.05$; Figure 4D). Sex differences in closed arm activity were also impacted by BPA (see Figure S2). Collectively, these data show that aspects of the anxiogenic action of developmental BPA exposure persist into adulthood, resulting in the loss of behavioral sex differences, but that a soy-rich diet is protective.

Discussion

The present study reveals that early life exposure to BPA elevates juvenile anxiety in rats of both sexes, but that these effects can be mitigated by concomitant intake of a soy-rich diet. Our

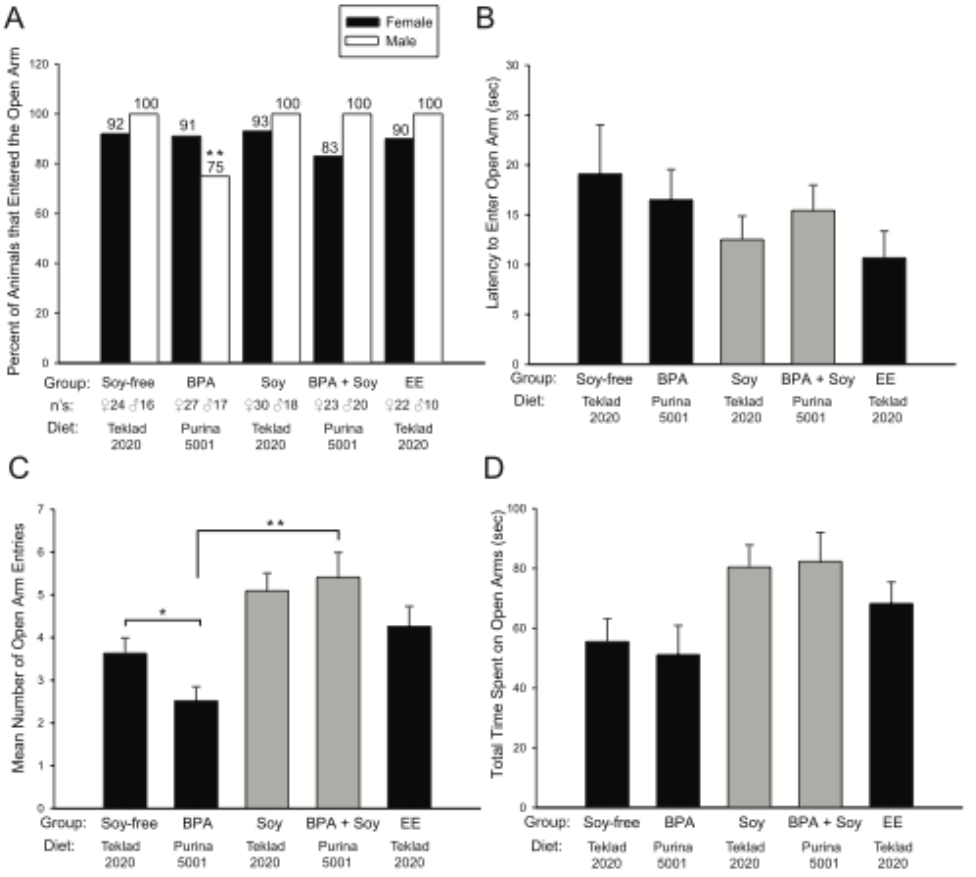

Figure 2. BPA, EE and dietary effects on juvenile Elevated Plus Maze (EPM) activity. (A) BPA exposure significantly decreased the percent of males that entered an open arm. No significant effect of gender was found for any subsequent measure, therefore the data were collapsed across sexes. (B) There were no significant group differences in latency to enter the open arms. (C) A main effect of diet on mean number of open arm entries was identified (not depicted), with soy-fed animals making significantly more entries. BPA exposed animals on the soy-free diet (BPA) made fewer entries compared to the diet matched controls (Soy-free) and also the BPA exposed animals on the soy diet (BPA + Soy). (D) Soy-based diet significantly increased time spent on the open arms, regardless of BPA exposure. EE had no significant effect on any EPM endpoint examined. Graphs depict mean ± SEM, *$P \leq 0.05$, **$P \leq 0.001$, # main effect of diet; $P \leq 0.05$.
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data are novel in that they evaluated outcomes of exposure at adolescence, a life stage of great hormonal and neurobiological change, and identified specific gene targets in the amygdala. Furthermore, our data are consistent with recent studies reporting that developmental exposure to BPA is associated with hyperactivity and increased anxiety in young girls [4,5], but indicate that diet, or other factors, could alleviate these effects.

The behavioral outcomes described here are consistent with prior reports showing that developmental BPA exposure has anxiogenic effects in juvenile and adult C57BL/6J mice [58] and deer mice (*Peromyscus maniculatus*) [59]; and abrogates behavioral sex differences in adult CD-1 mice [60,61]. A notable difference between the present and prior studies, however, is that here the overall anxiogenic effect of BPA did not strongly persist into adulthood. Several factors could account for this difference, the most obvious of which is a species effect. For example, mice exhibit marked strain differences in anxiety levels [62] and there are numerous important neuroendocrine differences between mice and rats which manifest as species-specific behavioral attributes [63]. Dose is also likely a factor as it varies considerably across studies. Use of more than one dose was beyond the scope of the present study, but future work will be necessary to identify the effective dose range for BPA-related effects on affective behavior. More likely, however is that the minimization of perinatal stress, a

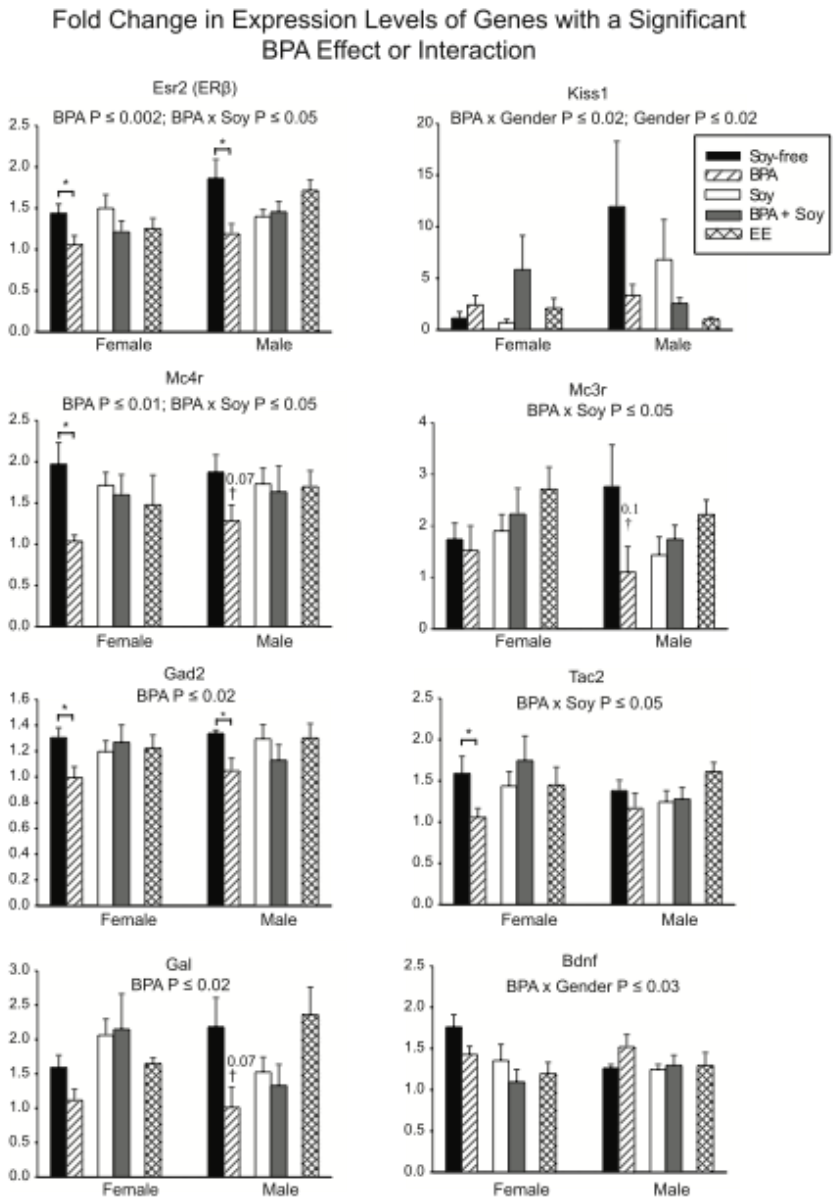

Figure 3. Fold change in juvenile amygdalar gene expression levels. Of the 48 genes examined, a significant main effect of, or interaction with, BPA was found for the 8 genes shown here. The genes depicted met the threshold criteria established for biological significance. For each gene, the raw *P*-values obtained for the main and interaction effects of BPA are indicated. Significant group differences within each sex, identified by post-hoc t-tests, are also shown. *Esr2* (ERβ) and *Gad2* expression were significantly down-regulated by BPA in both sexes compared to Soy-free controls. *Tac2* and *Mc4r* were significantly down-regulated by BPA exposure in females. Among the soy-fed animals, no significant effect of BPA was identified

for any gene, indicating a protective influence of soy. Graphs depict mean fold change in expression levels ± SEM. *P≤0.05, †P≤0.1 compared to soy-free controls.
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well-recognized confound of adult affective behavior, accounts for the less robust effects of BPA on adult anxiety [58,61,64]. By using drinking water and diet as the route of administration for BPA and soy phytoestrogens, we did not have to disturb the dams or their pups to dose the animals. Thus, this study is unique because it is the first to orally administer BPA at a dose sufficient to produce human-relevant serum levels [12,65] while simultaneously minimizing the pronounced confound of perinatal stress. With the adverse impacts of early life stress eliminated, the resulting behavioral changes reported here are more clearly attributable to BPA exposure.

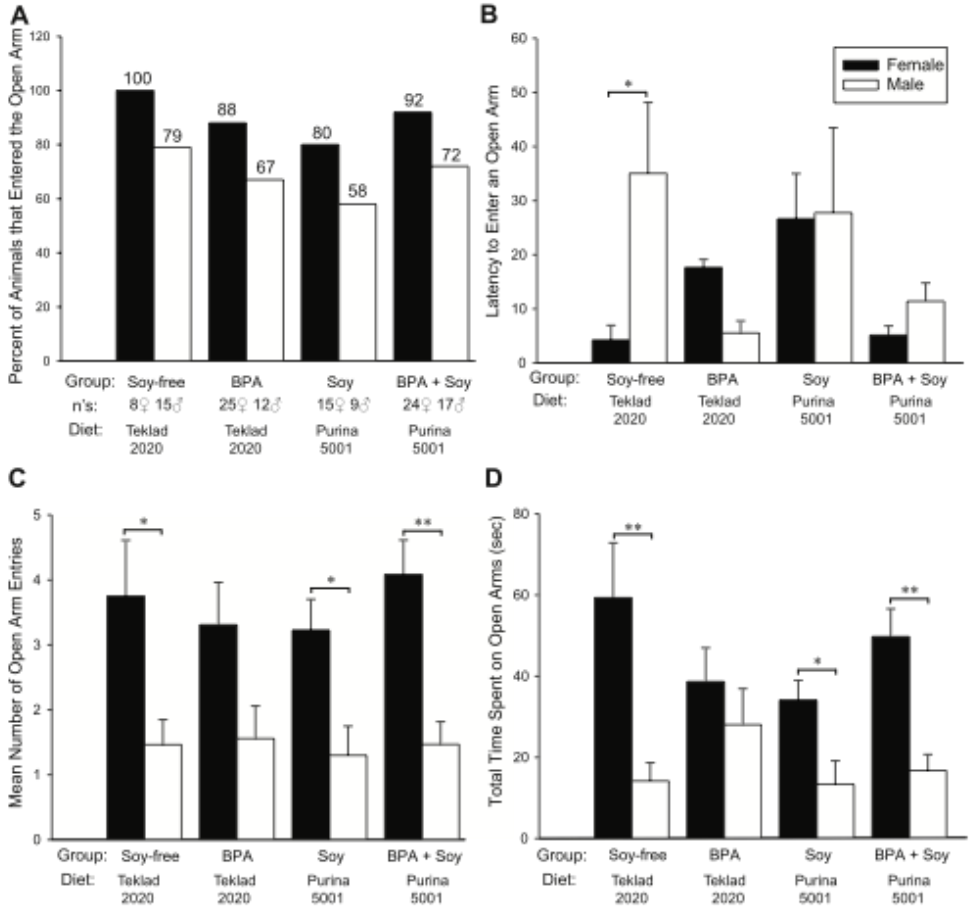

Figure 4. BPA, EE and dietary effects on adult Elevated Plus Maze (EPM) activity. As expected, a significant gender effect was found for all measurements except latency to enter an open arm. In general, BPA exposure eliminated these sex differences. (A) A higher percent of females entered the open arms than males regardless of exposure group. (B) Soy-free males took significantly longer to enter an open arm than females maintained on the same diet. BPA exposure eliminated this sex difference, with a tendency for reversal. Soy diet also eliminated this behavioral sex difference. (C) Females made significantly more open arm entries than males in all groups except those on the soy-free diet and exposed to BPA (BPA). There was no overall effect of BPA or diet on this behavior. (D) Similarly, in all groups except those on the soy-free diet and exposed to BPA, females spent significantly more time on the open arms than males. Graphs depict mean ± SEM. *$P \leq 0.05$, ** $P \leq 0.001$.
doi:10.1371/journal.pone.0043890.g004

Concomitant administration of a soy-based diet with BPA resulted in attenuation of the BPA effects. In both the Light/Dark box and the EPM, soy largely abrogated the anxiogenic impact of BPA on the percent of rats engaging in the "risky" behavior. Sex differences in adult behavior were maintained except in the case of latency to enter an open arm, where females displayed a longer latency. But again, co-administration of soy and BPA rescued the reversed sex difference induced by BPA exposure. These data are the first to suggest that adverse behavioral outcomes resulting from early life exposure to BPA may be mitigated, at least to some degree, by dietary or other factors. Presumably, this mitigating activity is attributable to GEN, or one (or more) of the numerous other isoflavone phytoestrogens aside from GEN that are commonly found in soy-based foods [16,26]. Although identification of the specific dietary compounds capable of minimizing the adverse neurobehavioral impacts of BPA and other EDCs is potentially advantageous to pregnant women, it is important to recognize that GEN and other phytoestrogens can also act as EDCs during development. Thus, the critical windows in which they produce effects, and their mechanism(s) of action and interaction on neural systems that mediate behavior must be more clearly elucidated.

The mechanisms by which BPA alters affective behavior remain poorly understood, but the amygdalar gene expression data obtained here identified 8 genes associated with BPA-induced anxiety including *Esr2* (ERβ), *Mc4r*, *Gal*, *Gad2* and *Kiss1*. These genes have previously been associated with sociosexual and affective behaviors. Interestingly, a recent paper showed transgenerational, epigenetic effects of vinclozolin (a fungicide) on the male F3 generation's responses to stress during adolescence [66]. The male F3 vinclozolin-lineage descendants differed from vehicle-lineage counterparts in how adolescent stress affected subsequent performance on tests of anxiety/stress, and that these same animals differed in expression of genes in hippocampus and amygdala, including *Mc3r* and *Mc4r*, similar to results in BPA-exposed F1 rats in the current study.

Notably, *Esr2* and *Mc4r*, changes to which were, in the present study, detected in the amygdala, play crucial roles in the regulation of oxytocin and vasopressin secretion [67,68,69]. These two neuropeptides are well-established to be essential for mediating social interactions and affiliation in rodents and primates, including humans [70,71]. For example, Esr2 agonism has been shown to be anxiolytic [69,72], and in the paraventricular nucleus (PVN), Esr2 is required to drive estrogen-dependent oxytocin and vasopressin production [73,74]. α-Melanocyte stimulating hormone (α-MSH), acting through Mc4r, induces dendritic release of oxytocin from magnocellular PVN neurons [68]. Central actions of oxytocin and vasopressin are implicated in a suite of sociosexual behaviors [71,75] and confer anxiolytic effects [76]. Collectively, these observations suggest that disrupted ontogeny of oxytocin and vasopressin signaling pathways may underlie the observed changes in juvenile affective behavior. In further support of this hypothesis, a recent study using mice found that prenatal BPA exposure had significantly lower whole brain levels of oxytocin and vasopressin just prior to birth compared to unexposed controls [77]. Dysregulation of these neuropeptide signaling pathways has been implicated in a range of childhood affective disorders, including autism [78,79]. Although oxytocin and vasopressin gene expression levels were not significantly altered by exposure or diet in the present study (Table 2), the amygdala may not be the most salient region to assess expression levels of these neuropeptides because they are generated primarily in the PVN and supraoptic nucleus (SON). Thus, subsequent investigation of oxytocin and vasopressin production in the PVN and SON will be necessary to confirm the hypothesis that these neuropeptides may be altered by early life BPA exposure.

Notably, BPA significantly impacted the expression of *Kiss1*, a gene only recently discovered to be expressed in the amygdala [80]. This gene codes for the peptide kisspeptin, expression of which in the hypothalamus is now recognized to play a critical role in the timing of sexual maturation, female ovulation, and feedback mechanisms on the hypothalamic-pituitary-gonadal (HPG) axis [81]. Kisspeptin neurons are sparse in number and primarily confined to the medial amygdala, with males having more than females. In the present study, this sex difference in amygdalar *Kiss1* expression was also observed, but, unexpectedly, EE did not masculinize expression in females. Instead, it feminized expression in males (Figure 3), an effect which is unusual in the rodent brain [82]. BPA also reduced *Kiss1* expression in males, an effect which was enhanced in animals maintained on the soy diet, suggesting that this specific effect may be estrogenic. Although adult expression is readily identifiable in the medial amygdala [80], ongoing, concurrent studies in our laboratory indicate that expression is not detectable in pre-weanlings suggesting that a mature HPG axis may be required for maximal expression of *Kiss1*. The functional role of these neurons remains to be delineated. Prior studies have shown that central, but not peripheral, kisspeptin administration elicits oxytocin secretion in females and vasopressin secretion in males, suggesting a role for amygdalar kisspeptin neurons in the modulation of affective behavior [83]. Further anatomical studies are needed to more comprehensively characterize the ontogeny of this neuronal population, their sensitivity to endocrine disruption, and their functional role in sociosexual behaviors.

The hypothesis that early life exposure to EDCs such as BPA and soy phytoestrogens can alter behavior is further supported by parallel work in rodents and humans showing that early life epigenetic changes within the hypothalamic-pituitary-adrenal (HPA) axis, arising from environmental influences, can permanently alter stress sensitivity and coping strategies [84,85]. It also implicates an epigenetic mechanism of action. Here, EE exposure did not completely recapitulate the behavioral or transcriptional effects of BPA, soy, or the combination of the two, demonstrating that BPA and soy are not simply acting as estrogens. Prior work has established that BPA can induce DNA methylation changes that are blocked by concurrent administration of the soy isoflavone GEN [16,86], raising the possibility that a similar interaction may have occurred here, resulting in the dietary-dependent behavioral outcomes. One possibility is that differential methylation of the ERβ promoter resulted in decreased expression, and subsequently the decreased expression of *Mc4r*, *Mc3r*, *Gal* and other genes regulated by *Esr2* activity, but more extensive work is needed to test this hypothesis. Understanding the specific cellular and molecular mechanisms by which early life BPA exposure alters behavior is critical for determining if effects observed in animal models have implications for human health.

Finally, it is important to highlight that the dosing method used here produced serum BPA levels at all phases of the project that were equivalent to, or below, those reported in humans [26,48]. Because BPA was administered in drinking water, exposure was likely low but continuous throughout the day, a pattern that more closely models that of humans than gavage or other methods of bolus administration. Although trace levels of free BPA were observed in some unexposed controls, suggesting an alternative and uncontrolled source of exposure, it may be an artifact of the analysis because the levels were so close to the limit of detection. The most likely source is diet, as we routinely monitor our caging leachate and water to ensure that they are BPA-free. Soy

phytoestrogen exposure was monitored by assessing serum GEN and its metabolites. Serum levels were well below those seen in infants exclusively fed soy-based infant formula [87]. It has long been hypothesized that GEN, BPA and other EDCs are not readily metabolized in neonates, and the absence of the glucoronidated form in PND 12 serum is consistent with this view. Serum levels reported here are high enough to induce physiological effects in rat models [26]. Exposure to BPA and GEN was likely lowest during lactation because, although placental transfer of both compounds have been established, neither is known to lactationally transfer readily [26,49,88].

Conclusions

Affective disorders in adults and children have well recognized sex differences in etiology. Boys are at higher risk of autism spectrum disorders, ADHD, and early onset schizophrenia [89,90] while women disproportionately suffer from anxiety, major depression, panic and eating disorders [91]. Notably, male-biased disorders appear to have their origins in development while female-biased disorders are generally post-pubertal in onset implying that the windows of sensitivity to environmental exposure may be sexually dimorphic with males being more sensitive during development and females later in life [92,93]. The data shown here reveal the potential for BPA and other EDCs to alter behaviors associated with anxiety, but also the potential for those alterations to be modified/mitigated by a soy-rich diet. These observations are a critical reminder that not all EDCs are synthetic and that plant compounds also have the potential to interact with hormone-sensitive mammalian systems, including the brain. Gene expression data from the amygdala imply a role for Esr2, melanocortins, and oxytocin/vasopressin signaling pathways in the manifestation of these behavioral changes, but future work will be needed to confirm this assertion. Collectively, these data highlight the plasticity of complex behaviors, their sex differences, and their potential for alteration by chemicals in the environment.

Finally, while humans and rodents perceive and express stress and anxiety differently, important core elements of the genetic and neurobiological basis of anxiety phenotypes are evolutionarily conserved across species, particularly in the amygdala and hypothalamus [31,94,95,96,97,98]. Thus the rat is an appropriate animal model for understanding how the interaction of BPA and diet influence sociosexual behaviors, and identifying the neural mechanisms by which these changes are induced.

Supporting Information

Figure S1 Juvenile elevated plus maze (EPM) closed arm activity is indicative of overall locomotor behavior. Because no overall effect of gender was identified the data were collapsed across sex for analysis. (A) Two-way ANOVA revealed a main effect of BPA exposure on the number of closed arm entries ($P \leq 0.03$) indicating that these animals were less active compared to unexposed controls. Post-hoc analysis did not identify significant differences between BPA-exposed animals and controls maintained on the same deit. EE had no impact on this behavior. (B) Soy fed animals spent slightly less time in the closed arms than animals maintained on the soy-free diet. EE had no effect on this behavior. (Graphs depict mean ± SEM).
(DOCX)

Figure S2 Adult EPM closed arm activity. (A) Three-way ANOVA revealed a significant sex by diet by BPA interaction ($P \leq 0.05$) on the number of closed arm entries. Post hoc t-tests within sex identified no sex difference in the number of closed arm entries made by the control animals on the soy-free diet. In all other groups, however, females made significantly more closed arm entries than males ($P \leq 0.05$). This difference was attributable to the combination of elevated activity in the females, and decreased activity in the males. (B) Time spent in the closed arms was sexually dimorphic in all groups except the BPA soy-free diet group. (Graphs depict mean ± SEM, $^{*}P \leq 0.05$, $^{**}P \leq 0.001$).
(DOCX)

Table S1 Free levels of BPA and GEN as well as the glucuronidated form of GEN (GEN-gluc) were assessed. BPA levels were within the human range and near the limit of detection. GEN levels were higher in the dams than the pups, reflecting poor lacational transfer. ND = Not detectable.
(DOCX)

Table S2 The original p-value is listed for each gene obtained by parametric (3-way ANOVA) or non-parametric statistics. For parametric tests, main effects and interactions were analyzed. For non-parametric tests data were collapsed across independent variables and analyzed by a Mann-Whine U or Kruskal-Wallis test to determine p-values for combinations of independent variables. Bold text indicates effects of BPA, both main effects and interactions/combinations ($p<0.05$). One gene, DNA methyltransferase 3l (Dnmt3l) did not amplify well or was not expressed in the amygdala and is not included in the results.
(DOCX)

Supporting Information S1 Supporting methods.
(DOCX)

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Author Contributions

Conceived and designed the experiments: HP AS DW AG. Performed the experiments: AS MR DW HA BW JC BB. Analyzed the data: AB AS DW JC BB AG. Contributed reagents/materials/analysis tools: AG JC BW BB HP. Wrote the paper: HP AS.

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CHAPTER 4:

A novel model for neuroendocrine toxicology: Neurobehavioral effects of BPA exposure in a prosocial species, the prairie vole (*Microtus ochrogaster*)

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Abstract

Impacts on brain and behavior have been reported in laboratory rodents following developmental exposure to bisphenol A (BPA) raising concerns about possible human effects. Epidemiological data suggest links between prenatal BPA exposure and altered affective behaviors in children but potential mechanisms are unclear. Disruption of mesolimbic oxytocin (OT)/vasopression (AVP) pathways have been proposed but supporting evidence is minimal. To address these data gaps, we employed a novel animal model for neuroendocrine toxicology: the prairie vole (*Microtus ochrogaster*) which are prosocial than than lab rats or mice. Male and female prairie vole pups were orally exposed to 5 μg/kg bw/day, 50 μg/kg bw/day, or 50 mg/kg bw/day BPA or vehicle over postnatal days (PNDs) 8-14. Subjects were tested as juveniles in open field and novel social tests and for partner preference as adults. Brains were then collected and assessed for immunoreactive (-ir) tyrosine hydroxylase (TH; a dopamine marker) neurons in the principal nucleus of the bed nucleus of the stria terminalis (pBNST) and TH-ir, OT-ir and AVP-ir neurons in the paraventricular nucleus of the hypothalamus (PVN). Female open field activity indicated hyperactivity at the lowest dose and anxiety at the highest dose. Effects on social interactions were also observed and partner preference formation was inhibited at all dose levels. BPA masculinized pBNST TH-ir neuron numbers in females. Additionally, 50 mg/kg bw BPA exposed females had more AVP-ir neurons in the anterior PVN and fewer OT-ir neurons in the posterior PVN. At the two lowest doses, BPA eliminated sex differences in PVN TH-ir neuron numbers, and sex reversed it at the highest dose. Minimal behavioral effects were observed in BPA-exposed males. These data support the hypothesis that BPA alters affective behaviors, potentially via disruption of OT/AVP pathways.

Introduction

Bisphenol A (BPA) is an endocrine disrupting compound (EDC) first synthesized in 1891, identified as a synthetic estrogen in the 1930's, and subsequently commercialized in the 1950's as a component of epoxy resins and hard polycarbonate plastics (Vogel, 2009). BPA is a high volume production chemical ubiquitous in the environment and thus detectable in human urine, saliva, and blood (reviewed in (Vandenberg et al., 2012)). Concerns have been raised regarding the potential long-term human health effects of environmentally relevant BPA exposure in early life (Richter et al., 2007; Vandenberg et al., 2012), particularly on neural development and behavior (FAO/WHO, 2011). Here we introduce and demonstrate the utility of the prairie vole (*Microtus ochrogaster*), a more prosocial animal model than laboratory rats or mice, for assessing BPA-related impacts on neuroendocrine development and behaviors related to sociality and social investigation.

Elevated gestational urinary concentrations of BPA have been correlated with adverse behavioral outcomes in children, including hyperactivity, anxiety, and executive function deficits (Braun et al., 2011; Braun et al., 2014; Braun et al., 2009; Harley et al., 2013) suggesting the possibility that early life exposure to BPA could impact behavioral development and the respective neural correlates. Although numerous animal studies have reported effects consistent with the available human data and repeatedly shown that early life exposure to low levels of BPA results in heightened anxiety or activity and decreased social and cognitive abilities (Kundakovic et al., 2013a; Williams et al., 2013; Wolstenholme et al., 2013a), the data are inconsistent, and the mechanisms by which these BPA-related behavioral changes manifest remain poorly understood (Wolstenholme et al., 2011). Novel to toxicology, but a well-established animal model in the behavioral neuroendocrinology and biomedical studies of social deficit disorders, prairie voles are advantageous because they display affiliative behaviors more typical of humans, such as social monogamy and alloparental care, they form family units, and display relatively low levels of aggression (Young et al., 2011). Additionally, transformative work, spanning decades and involving several vole species with varying degrees of sociality, has linked prosocial traits to the oxytocin/vasopressin (OT/AVP) system and its interactions with the mesolimbic dopamine

pathways, governed by estrogen (Young et al., 2011). Furthermore the translational importance of the vole model for elucidating the role of these neuropeptides in sociality has now been demonstrated in humans (reviewed in (Modi and Young, 2012)). For example, intranasal OT administration is presently being explored as a potential therapy for autism, and manipulation of OT and AVP is being considered therapeutically for depression. Only one study to date has used voles to explore the impact of environmental EDC exposure on brain and behavior. Female pine voles (*M. pinetorum*), which are socially monogamous like prairie voles, born to dams orally exposed to 2 mg/kg/day of the estrogenic pesticide methoxychlor (Gray and Ostby, 1998; Gray et al., 1999) during gestation and lactation displayed reduced physical contact during the partner preference test, indicating impaired sociality (Engell et al., 2006). Exposure to methoxychlor also resulted in a reduction in oxytocin receptor binding in the cingulate cortex. These data support our rational that the vole is a potentially valuable, yet underutilized, animal model for testing the hypothesis that early life exposure to BPA (and other EDCs) alters social behavior and related, coordinating, pathways.

Information regarding BPA-related impacts on neuropeptide pathways is limited, but available data support the hypothesis that developmental exposure to BPA alters the organization and function of OT and AVP pathways (Adewale et al., 2011; Patisaul et al., 2012), an outcome which may not be surprising as both OT and AVP expression and effects are steroid dependent (Cushing and Kramer, 2005b). Effects may persist across generations (Wolstenholme et al., 2012; Wolstenholme et al., 2013a). Early life exposure to BPA has also been shown to affect the dopamine system in varying manners (reviewed in (Masuo and Ishido, 2011)). For example, it has recently been shown in non-human primates that gestational exposure to human-relevant exposure levels of BPA decreases midbrain dopamine neuron numbers (Elsworth et al., 2013). To date, three rodent studies have linked BPA-related disruption in the dopamine system with hyperactivity (Ishido et al., 2004; Kundakovic et al., 2013a; Masuo et al., 2004) further supporting the overarching hypothesis that BPA exposure may have an organizational effect on the mesolimbic dopaminergic system and elevate the risk of hyper-investigative and anxiety related behaviors.

BPA is primarily thought to induce behavioral effects by perturbing estrogen action, although other modes of action, including epigenetic changes and organizational effects on other mechanisms, are plausible (Kundakovic et al., 2013a; Wolstenholme et al., 2011). Because OT/AVP and dopaminergic pathways are heavily influenced by sex steroids across the lifespan (Cavanaugh and Lonstein, 2010; Cushing and Kramer, 2005a; Kabelik et al., 2011; Lonstein et al., 2005; Simerly et al., 1985), the ontogeny of OT/AVP and dopaminergic pathways may be particularly vulnerable to EDCs like BPA. In prairie voles, neonatal manipulation of estradiol or testosterone alters affiliative behaviors later in life, and estradiol administration during adulthood affects estrus and locomotor activity (Kramer et al., 2009; Lonstein et al., 2005; Roberts et al., 1997). For example, males castrated on the day of birth fail to form a pair bond after AVP administration (Cushing et al., 2003) and neonatal castration is one of the few ways to disrupt the expression of male alloparental behavior (Northcutt and Lonstein, 2009). Manipulation of OT/AVP levels as a result of direct exposures to exogenous hormones, agonists, or by altering the social environment can modify the number of OT, AVP, and TH neurons in the paraventricular nucleus of the hypothalamus (PVN), thereby resulting in behavioral outcomes such as anxiety-like behavior and alterations of prototypical male and female sociosexual behavior (Ahern and Young, 2009; Curtis et al., 2003; Lieberwirth et al., 2012; Martin et al., 2012; Yamamoto et al., 2004).

A related brain region of interest is the principal bed nucleus of the stria terminalis (pBNST), a posterior division of the bed nucleus of the stria terminals (BNST) that is an interconnection site for brain areas integral for social behaviors related to reproduction and defense including locomotor activity and motivation (Dong and Swanson, 2004; Northcutt and Lonstein, 2011; Northcutt et al., 2007). The pBNST also plays a role in the mediation of the stress response by relaying limbic information to the amygdala and corticotropin releasing hormone (CRH) expressing neurons in the PVN (Been and Petrulis, 2011; Herman et al., 1994). Dopaminergic pathways in this area are thought to regulate CRH-dependent affective states (Meloni et al., 2006). Male prairie voles have significantly more dopaminergic neurons (identified by tyrosine hydroxylase (TH) immunoreactivity (-ir)) in the

pBNST than female prairie voles and other rodent species that do not exhibit the prosocial monogamous behaviors (Young et al., 2011). This sex- and species-specific difference in TH-ir neuron number in the pBNST is important for sex specific prosocial behavior. Moreover, TH-ir neuron number in the pBNST is sensitive to sex steroid hormones (Northcutt and Lonstein, 2008, 2009, 2011; Northcutt et al., 2007), thus making the system potentially vulnerable to endocrine disruption. For the present studies, we tested the hypothesis that alteration in OT-, AVP-, and TH-ir neuron numbers in the PVN and sex specific TH-ir in the pBNST may contribute to the expression of social behavior, defensive behavior, and locomotor activity effects associated with BPA exposure.

Materials and Methods

Subjects

Husbandry

The animals used in this study were laboratory-reared prairie voles that originated from wild stock from Urbana, IL. The prairie vole is a well-established rodent model for examining prosocial behaviors in the field and laboratory (reviewed in (Young et al., 2011)) but has housing, diet and husbandry requirements that differ from conventional laboratory rodents. (For details on basic ethology and laboratory housing needs refer to (Carter et al., 1980; Carter et al., 1988; Cushing et al., 2001; Solomon and Crist, 2008; Solomon et al., 2009)). Animals were maintained on a 14:10 light-dark cycle in thoroughly washed polysulfone cages and provided with Purina high fiber rabbit chow (Purina, St. Louis, Missouri) and water ad libitum at the NEOMED in an AAALAC approved facility affiliated with the Cushing lab at U Akron. Rabbit chow is the established diet for laboratory prairie voles because it is more similar to their natural diet than rat chow and has a high fiber content, which is required to keep their rootless teeth trimmed. This diet contains phytoestrogens from alfalfa and other ingredients vital for vole health and reproductive success in captive environments (Cushing et al., 2001). Thus, although use of a phytoestrogen-free diet is typically preferable for studies evaluating the impacts of EDCs like BPA in animals (Thigpen et al., 1999a; Thigpen et al., 2013), this is not feasible for voles. On the day of birth, animals were sexed and marked for identification via toe clip. Litters

were weaned at 21 days of age and housed in same-sex sibling pairs in 12 x 18 x 28 cm cages.

Exposure

Male and female prairie vole pups were orally exposed across postnatal days 8-14, which has been identified as the crucial sociosexual developmental window in this species and akin to the neonatal period in rats/mice (Kramer et al., 2009). On postnatal day (PND) 8 litters were removed from their home cage and placed in a clean cage on cotton bedding. All individuals were randomly assigned to exposure groups and weighed. Animals received an oral dose of one of three doses of BPA 5 μg/kg bw, 50 μg/kg bw (established reference dose) and 50 mg/kg bw (lowest observed adverse effect level) (Geens et al., 2012). Doses were based upon average weight of pups on PND 8 and dissolved in 2.7g of Hydroxypropyl Beta CD – Pharm Grade in 10ml 0.9% NaCl. A fourth group received vehicle only. For all groups, delivery was 25 μl orally to the pups by micropipette (as described previously for mice (Palanza et al., 2002b)). This oral route was chosen because it is less stressful than orogastric gavage, a procedure that has recently been found to affect hypothalamic gene expression (Cao et al., 2013b). All litters contained at least one control and no more than one exposed animal per exposure per sex per litter. Pups were returned to the parents as a group following exposure. Dosing was repeated daily on PNDs 9-14. Specific pharmacokinetics regarding BPA uptake and metabolism are not available for the vole but presumed to be similar to rats, mice and rhesus monkeys (Yang et al., 2013). A detailed pharmacological assessment was beyond the scope of the present study. An estrogen-exposed group was not included because prior work in our lab has shown that BPA effects on non-reproductive behavior are not consistent with those induced by estrogen (Patisaul et al., 2012), Thus, estrogen was not assumed to be an appropriate positive control for effect. Additionally, it is unclear what the appropriate dose would be in a prairie vole because information about how early-life estrogen exposure influences neuroendocrine development and behavior in this species remains limited. Housing and all procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were

preapproved by the NEOMED Institutional Animal Care and Use Committee. Although testing materials were polycarbonate, they were not considered a significant source of BPA contamination in the laboratory (Thigpen et al., 2013).

Behavior

Open Field Test

One week after weaning (PND 28) subjects participated in an open field test, developed to ascertain anxiety-related and locomotor activities (Palanza et al., 2002a). Animals were placed in the center of a 40 cm^2 Plexiglas test area with blackened walls. The subjects were allowed to move freely about the arena for 10 min. The tests were video recorded from above on a DVR and then TopScan (Clever Sys Inc) software was used to analyze time spent in the center, perimeter, and corners; frequency of transition between sections; distance traveled by section; and total distance traveled. Time spent in a freezing position was hand scored by an observer blind to exposure groups. Final animal numbers were as follows: Females: control n = 23; 5 μg n= 25; 50 μg n = 15; 50 mg n = 16. Males: control n = 12; 5 μg n = 16; 50 μg n = 12; 50 mg n = 16.

Novel Social Test

On PND 30, 2 days after open field testing, subjects were tested in the novel social arena. The test arena consisted of two 12 x 18 x 28 cm Plexiglas cages connected by a Plexiglas tube to test for exposure group effects on social interactions. Food and water were provided in both cages of the arena. A novel unrelated stimulus animal (same sex, size and age matched, and from an untreated litter) was gently tethered via a lose-fitting collar connected via a leader to a steel rod that runs the length of the cage, permitting it to freely move about within its cage but not to enter the other. This is a standard test (with variations in duration and other factors) that has been used in vole preference tests for over a decade (Insel et al., 1995), and the specific procedure used for the present studies is routinely used in the Cushing lab (Cushing and Carter, 2000). The test animal was then placed in the other cage and permitted to move about freely for 1 hr. Latency to enter the stimulus animal cage, frequency to movement between cages, number of contact bouts, duration in the stimulus

animal cage, duration of exploratory behavior, and time spent in side-by-side physical contact were analyzed using TopScan software (Clever Sys Inc.). Exploratory behavior was scored by an algorithm based upon orientation of the test animal to the stimulus animal, distance (< 20 pixels) and time between movements (< 4 seconds). A random visual sampling of 50 records indicated that this primarily (> 90%) represented sniffing by the test animal toward the stimulus animal. Side-by-side contact was based on an algorithm using distance < 20 pixels between the test and stimulus animal and period of immobility. Endpoints were also scored by a exposure-blinded observer to better determine the type of contact. All investigatory data was collected by hand. Prairie voles display very low levels of aggression; thus, injury to the stimulus animal is low or absent. As a precautionary measure, however, an observer remained in the room throughout the test period to monitor signs of aggression (not observed in any tests.). Final animal numbers were as follows: Females: control n = 24; 5 μg n= 24; 50 μg n = 15; 50 mg n = 17; Males: control n = 16; 5 μg n = 13; 50 μg n = 11; 50 mg n = 14.

Partner Preference Test

Adult (PND 60-75) animals were tested with an opposite sex conspecific for the effects of exposure on social interaction and formation of partner preference, using a standard, well-established paradigm detailed previously (Cushing and Carter, 2000). Briefly, test subjects were cohabitated for 3 hours with an unrelated, sexually naïve "partner" a length of time sufficient to induce a pair bond even though mating typically does not occur (Carter et al., 1987). No mating was observed and females were in anestrus because, unlike rats and mice, female prairie voles do not undergo spontaneous estrus, and estrogen levels remain low unless a female is exposed to a male for an extended period of time (up to 24 hr). All tests were videotaped.

Immediately following cohabitation, test animals participated in the preference test as described previously (Young et al., 2011). The stimulus animals, a "partner" and a "stranger," were loosely tethered for the duration of the test (see Novel Social test above) in their respective chambers. These stimulus animals were opposite in sex to the test animal, sexually naïve, similar in age and weight to the test animal, not exposed to BPA, and

unfamiliar and unrelated to each other. The experimental animal was placed in the neutral chamber and its movements and interactions with the stimulus animals recorded for three hours on a DVR. TopScan software (Clever Sys Inc) was used to score the test. Time spent in side-by-side contact (huddling) with each animal was scored and analyzed. By definition a partner preference is formed if a test animal spends significantly more time in physical contact with the partner than the stranger (Young et al., 2011). Final animal numbers were as follows: Females: control n = 21; 5 μg n= 17; 50 μg n = 13; 50 mg n = 17; Males: control n = 15; 5 μg n = 16; 50 μg n = 10; 50 mg n = 16.

Immunohistochemistry

Animals were sacrificed over PNDs 60-90. Subjects were given 0.05 ml buprenorphine IP and then deeply anesthetized 15 min later with 0.05 ml of a ketamine-xylazine (at a concentration of 67.7 mg/kg and 13.33 mg/kg) mixture administered subcutaneously. Brains were removed and immersion fixed in 4% paraformaldehyde for 24 hrs at 4°C and transferred to fresh solution at 2 and 4 hrs. The brains were then cryoprotected in 30% buffered sucrose with 0.1% sodium azide and shipped to the Patisaul lab, where they were stored in fresh cryoprotectant overnight at 4°C then flash frozen, and stored at -80°C. Brains were coronally sectioned at 35 μm on a frozen sliding microtome. Then sections for each individual corresponding to the regions of interest (ROIs) were collected and processed for immunohistochemistry.

PVN OT and AVP

For each individual, eight sequential sections of the PVN were collected and processed for immunohistochemical staining of OT and AVP using routine procedures described previously (Adewale et al., 2011). Sections were washed in cold KPBS and preincubated for 24 hours in 0.02M KPBS, 0.3% Triton-X, and 2% normal goat serum at 4°C. Sections were then incubated in a primary antibody cocktail consisting of, 1:12,000 monoclonal mouse anti-OT (Cat # MAB5296, Millipore, Temecula, CA) and polyclonal rabbit anti-AVP (Cat # 20069, Immunostar, Hudson, WI) for 72 hrs on a shaker at 4°C. Sections were then washed and incubated in a cocktail of Alexa Fluor 568 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit secondary antibodies (both at 1:200) for 120 min. After a final wash in cold

KPBS, sections were mounted on Fisher super frost plus glass slides, coverslipped with a glycerol mountant and stored at -20°C.

PVN and pBNST TH

For each individual, three consecutive caudal PVN and pBNST sections were collected and processed for the immunohistochemical staining of TH using routine lab procedures (Adewale et al., 2011) , and counterstained with Hoescht. Following a 24 hour preincubation, in cold 0.02 MKPBS with 0.3% Triton-X, and 2% normal donkey serum, the sections were then incubated with the polyclonal rabbit anti-TH (Cat # AB152, Millipore, Temecula, CA) primary antibody at 1:4000 for 72 hrs on a shaker at 4°C. Sections were then washed in cold KPBS and incubated with the Alexa Fluor 488 donkey anti-rabbit secondary antibody at 1:200 for 120 min, wash and counterstained with Hoechst (Cat # H3569, Invitrogen Life Technologies, Grand Island, NY) via a 45 sec incubation. After a final wash in cold KPBS, sections were mounted on Fisher super frost plus glass slides, coverslipped with a glycerol mountant and stored at -20°C.

Quantification and analysis

A prairie vole brain atlas is not available; thus the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007) was used to identify the PVN and its surrounding landmarks (as described in (Gobrogge et al., 2007)) along with available studies identifying anatomical subregions of the vole PVN (Ross et al., 2009; Wang et al., 1996). Although relatively little is known about the functional distribution of specific neuronal phenotypes in the vole PVN, most significantly for the present study, the majority of anterior and medial OT/AVP neurons in the prairie vole PVN have been putatively characterized as magnocellular neurohypophysial neurons while the posterior PVN is known to contain a population projecting to the hindbrain and spinal cord; features indicative of parvocellular neurons (Ross et al., 2009).

With these subdivisions in mind we subdivided the PVN into anterior, medial, and posterior regions based on results and figures published by Ross and colleagues (Ross et al., 2009). Anterior sections correspond with Paxinos and Watson plates 38-41, medial sections correspond with plates 42-47, and posterior sections correspond with plates 48-51. From

these 14 consecutive sections, 2 anterior, 4 medial, and 2 posterior sections from the middle of each subregion (anterior, medial and posterior) were selected. All OT- and AVP-ir neurons were then bilaterally counted and averaged for each individual using a fluorescent Leica DM5000 scope. Two consecutive sections from the most caudal (posterior) PVN region were assessed for TH-ir, where we found bilateral populations, round in shape, located lateral and dorsal to the third ventricle (3V), corresponding to the paraventricular hypothalamic nucleus, posterior part (PaPo) and A13 dopamine cells (A13) in the depicted in plates 51-52 in Paxinos and Watson. The TH-ir cells confined to this region were manually counted and averaged for each animal. The pBNST of each animal was imaged at 200x using a Leica DM5500 confocal microscope. We then used Image J to quantify the TH-ir cells found in a dense cluster within three consecutive sections corresponding to plates 21-23 in the Swanson Atlas (Swanson, 1998) as described in (Northcutt et al., 2007) but, most specifically plate 21 where the most densely populated area was found.

Statistical Analysis

Behavior

All open field and novel social behavior results were analyzed by a two-way ANOVA with exposure and sex as factors. A one-way ANOVA was then performed within sex because the behaviors analyzed are known to be sexually dimorphic (Young et al., 2011). If overall exposure effects were significant ($P \leq 0.05$) then a protected Fisher's least significant differences (PLSD) post-hoc test was performed to evaluate pair-wise differences ($P \leq 0.05$). All analyses were performed using Prism 6 and outliers were identified using the ROUT method. All results were considered significant if $P \leq 0.05$.

Time spent with the partner versus the stranger was analyzed using a paired t-test for each exposure and sex. A partner preference was considered to have occurred if time in side-by-side contact with the partner was greater than the stranger with $P \leq 0.05$.

Neuroanatomy

To detect sex and exposure group differences in numbers of OT-ir , AVP-ir, and TH-ir cells, average cell numbers per region (anterior, medial, and posterior PVN, pBNST) were

analyzed by a two-way ANOVA and followed up with a one-way and PLSD as described for behavior analysis.

Results

Behavior

Open Field

A significant exposure by sex interaction was detected by a two-way ANOVA for total overall bouts (all entries made into any area of the arena) made in the OF arena ($F_{(3,114)}$=2.866, $P \leq 0.05$; Figure 1A) as well as total distance traveled ($F_{(3, 115)}$=3.883, $P \leq 0.01$; Figure 1B). Within females, main effects of BPA exposure were identified for total overall bouts ($F_{(3,69)}$=6.496, $P \leq 0.001$; Figure 1A), center bouts ($F_{(3,70)}$=3.120, $P \leq 0.03$; Figure 1C), corner bouts ($F_{(3,70)}$=4.441, $P \leq 0.01$; Figure 1E), and perimeter bouts ($F_{(3,70)}$=5.564, $P \leq 0.002$; Figure 1G). The 5 µg BPA group made significantly more bouts than controls overall ($P \leq 0.005$) and into the center ($P \leq 0.05$) and perimeter ($P \leq 0.01$), while the 50 mg BPA female group made significantly fewer bouts into the corners than the controls ($P \leq 0.05$). No significant differences in duration of time spent in any portion of the OF arena were found (Figure 1 D, F, H). No significant effects of BPA were identified in the males.

One-way ANOVA revealed that freezing behavior was significantly elevated in the female 50mg BPA group compared to controls (($F_{(3,49)}$=2.854, $P \leq 0.05$); Figure 1I). This group had high variation so ROUT was employed to identify outliers and six were identified. If these six outliers were removed from the dataset, the significant difference was lost. No significant exposure effects on freezing behavior were found for males.

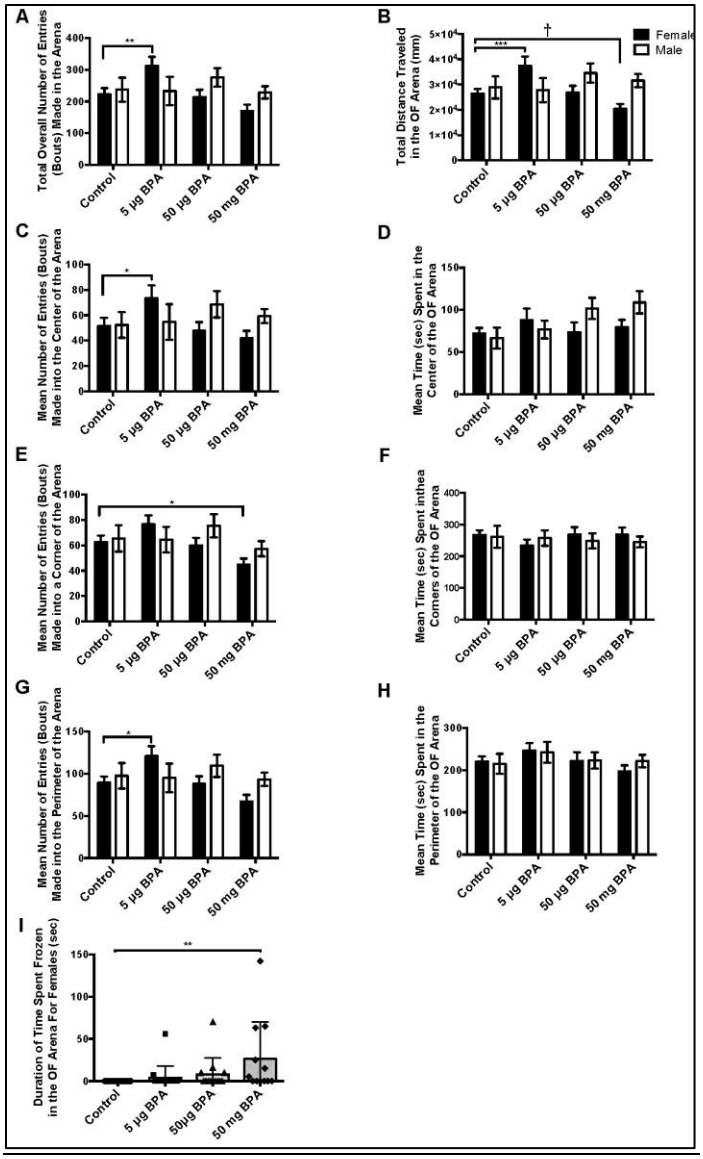

Figure 1. Postnatal BPA exposure caused a dose-specific effect on activity levels in female prairie voles. (A) 5 µg BPA group made significantly more entries into the subregions and (B) traveled significantly more, while females exposed to 50 mg BPA traveled less, than control females. (C) 5 µg BPA group made significantly more entries into the center than control females but (D) duration of time spent in the center was unchanged. (E) Females exposed to 50 mg BPA made fewer entries into the corners compared to control conspecifics but (F) Duration of time spent in the corners was unchanged. (G) 5 µg BPA group made significantly more entries into the perimeter compared to controls but (H) duration of time spent in the perimeter was unchanged. (I) Females exposed to 50 mg BPA froze significantly more than controls. Data are shown as the mean ± SE. Differences from same sex controls indicated by *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; †, $P \leq 0.1$.

Novel Social

No significant effects or interactions were found for latency to enter the stimulus animal chamber (Figure 2A), latency to contact the stimulus animal (Figure 2B), or number of investigations (sniffing) of the stimulus animal (Figure 2C). Two-way ANOVA revealed a significant interaction between sex and exposure for time spent investigating the stimulus animal (($F_{(3,95)}$=3.894, $P \leq 0.01$); Figure 2D). Control males spent significantly more time investigating the stimulus animal than did the control females ($P \leq 0.02$), 50 μg exposed males ($P \leq 0.03$) males, 50 mg BPA exposed males ($P \leq 0.01$). Females exposed to 50 mg BPA spent significantly more time investigating than did the control females ($P \leq 0.05$) and males exposed to 50 mg BPA ($P \leq 0.02$). Thus, the sex difference observed in the control animals was reversed in the 50 mg BPA exposure group (Figure 2D).

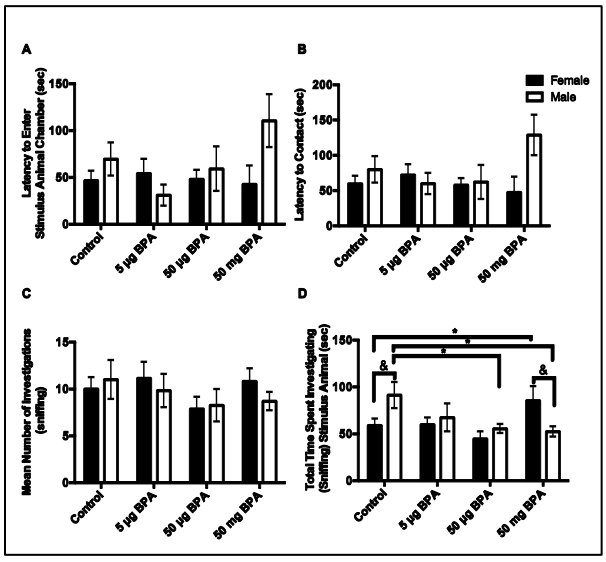

Figure 2. Postnatal BPA caused a sex and dose-specific response in duration of time spent sniffing a novel stimulus animal. (A) No significant differences were found in the latency to enter the stimulus animal's chamber, (B) latency to contact the stimulus animal, or (C) number of investigations of a stimulus animal. (D) Control males spent significantly more time investigating the stimulus animal than control females. Exposure to 50 μg and 50 mg BPA significantly decreased the time males spent investigating, and exposure to 50 mg BPA increased the amount of time females spent investigating the stimulus animal. Data are shown as the mean ± SE. A significant difference from control indicated by *, $P \leq 0.05$.

Partner Preference

The control females formed a partner preference, spending significantly more time in side-by-side contact with the partner versus the stranger (t_{20}=2.165, $P \leq 0.05$, Fig. 3A). In contrast females treated with any dose of BPA failed to form a "partner" preference as there was no significant difference in the duration of side-by-side contact between the partner and the stranger (5µg (t_{16}=1.642, ns), 50µg (t_{12}=1.971, ns), and 50mg (t_{16}=1.427, ns)). None of the male exposure groups, including controls, spent significantly more time in side by side contact with either the partner or the stranger (Fig. 3B).

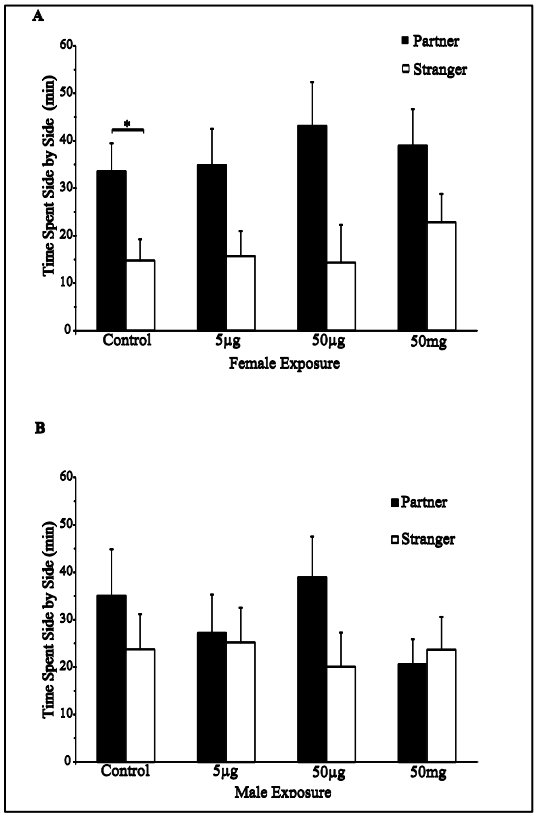

Figure 3. Postnatal BPA exposure resulted in a loss of statistical significance in the partner preference test. (A) Control females spent significantly more time huddling with the stranger versus the partner. In the BPA exposed groups, females still spent more time beside their partner, but the statistical significance in time spent with the partner versus a stranger was lost. (B) None of the male groups showed a significant partner preference. Data are shown as the mean ± SE. A significant difference from control indicated by *, $P \leq 0.05$.

Number of OT, AVP, and TH immunoreactive neurons in the PVN

OT

No significant interaction between sex and exposure was found by two-way ANOVA but a one-way ANOVA within sex indicated a significant main effect of exposure on female OT-ir ($F_{(3,43)} = 3.010$, $P \leq 0.04$) neuron numbers in the anterior PVN. Females exposed to 50 mg BPA had significantly more OT-ir neurons than the 5 μg ($P \leq 0.01$) and 50 μg ($P \leq 0.05$) groups (Fig. 4A) but did not statistically differ from controls. Differences in medial PVN OT-ir neuron numbers did not reach statistical significance (Fig. 4B). There was a significant main effect of exposure on OT-ir ($F_{(3, 42)} = 2.754$, $P \leq 0.054$; Figure 4C) neuron numbers in the posterior PVN. The 50 mg BPA group had significantly fewer OT-ir neurons compared to controls ($P \leq 0.02$; Figure 4C and Figure 5E-F) and 5 μg exposed females ($P \leq 0.05$).

A significant main effect of exposure was found for male OT-ir ($F_{(3, 42} = 3.341$, $P \leq 0.03$; Figure 4A) neuron numbers in the anterior PVN. Males exposed to 50 mg BPA had significantly more OT-ir cells than 5 μg BPA ($P \leq 0.01$) and 50 μg BPA ($P \leq 0.05$). No significant effect of exposure was found for OT-ir neuron number in the medial or posterior PVN (Figure 4C-B).

AVP

No significant interaction was found by a two-way ANOVA, but one-way ANOVA within sex indicated a significant main effect of exposure on female AVP-ir ($F_{(3,42)} = 3.406$, $P \leq 0.03$) neuron numbers in the anterior PVN. Females exposed to 50 mg BPA had significantly more AVP-ir neurons than all other groups (controls ($P \leq 0.01$; Figure 4D, Figure 5A-B); 5 μg ($P \leq 0.01$) and 50 μg ($P \leq 0.05$)). No significant effects were found for female medial (Figure 4B) or posterior PVN AVP-ir neuron numbers (Figure 4C).

In males, no effects of exposure were found for male AVP-ir neuron number in the anterior PVN (Figure 4D), the medial PVN (Figure 4E), or posterior PVN (Figure 4F).

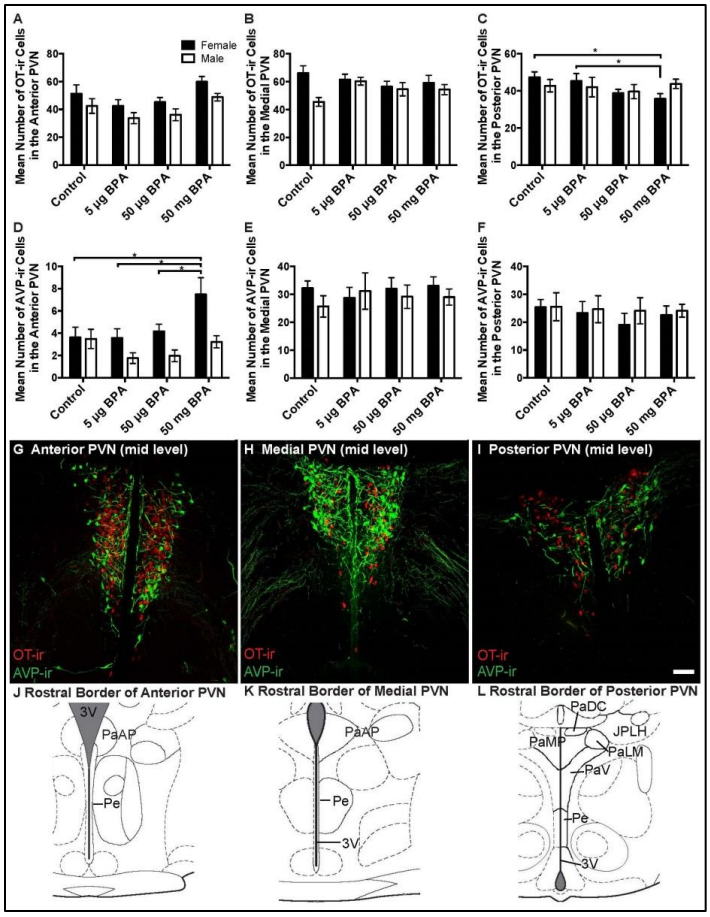

Figure 4. Postnatal exposure to 50 mg/kg BPA resulted in a significant decrease in OT-ir cells and a significant increase in the AVP-ir cell in the female prairie vole PVN. (A-B), BPA exposure did not significantly alter OT-ir cell numbers in the anterior or medial PVN of either sex but females exposed to 50 mg BPA had significantly fewer OT-ir cells in the posterior PVN (C) and significantly more AVP-ir cells in the anterior PVN (D). (E-F) Exposure to BPA had no significant effect on AVP-ir cell numbers in the medial and posterior PVN of either sex. (G-I) Representative images of a mid-level immuolabeled section for each of the regions of interest in the PVN and (J-L) corresponding illustrations adapted from the Paxinos and Watson Rat Brain Atlas used to identify the rostral border of each subregion: Anterior (Plates 38-41), Medial (Plates 42-47), or Posterior (Plates 48-51). Scale bar in (I) is 100, µm and applies to all panels. PaAP paraventricular hypothalamus anterior parvicellular, PaDC paraventricular hypothalamic nucleus dorsal cap, PalM paraventricular hypothamus lateral magnocellular, PaMP paraventricular hypothalamus medial parvicellular, PaPo paraventricular hypothalamic nucleus, posterior, PaV paraventricular hypothalamic nucleus ventral, Pe periventricular hypothalamic nucleus, ZI zona incerta, Data are shown as the mean ± SE. A significant difference from control indicated by *, $P \leq 0.05$.

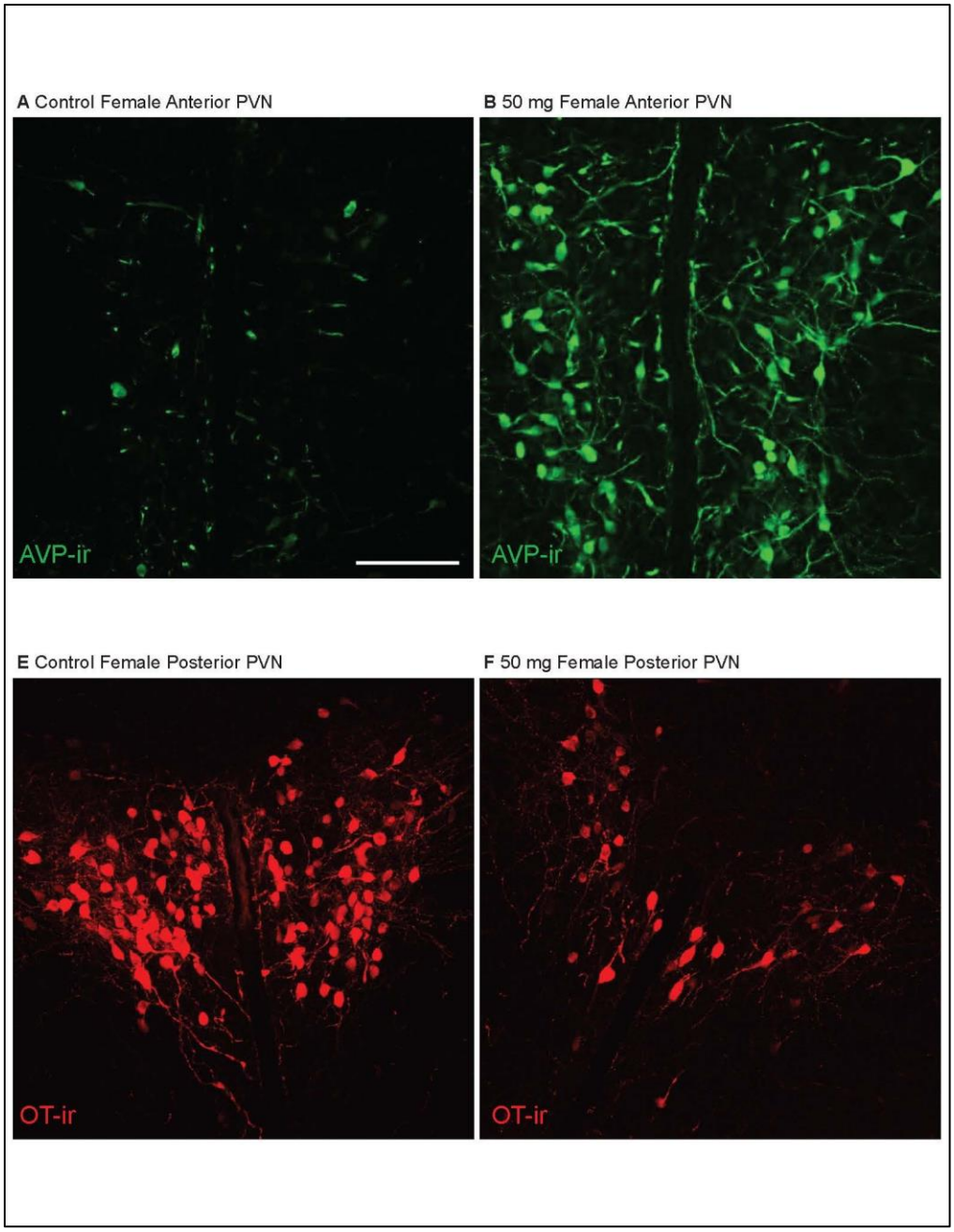

Figure 5. Representative images depicting increased AVP-ir cell numbers in the female anterior PVN (A-B) and decreased OT-ir cell numbers in the posterior PVN of 50 mg/kg BPA exposed females compared to control conspecifics (C-D). Scale bar in (B) is 100, μm and applies to all panels.

TH

Number of TH immunoreactive neurons in the PVN

A two-way ANOVA revealed a significant exposure by sex interaction ($F_{(3, 39)} = 19.61$, $P \leq 0.0001$). Male controls had significantly more TH-ir cells than the female controls ($P \leq 0.0001$; Figure 6A, D-E). Exposure to 5 μg, 50 μg, or 50 mg BPA caused a significant decrease in TH-ir cell number in males ($P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$ respectively) and a significant increase in cell number in females ($P \leq 0.0001$, $P \leq 0.001$, $P \leq 0.0001$ respectively Figure 6A, D-G). The sex difference in TH-ir cells was lost in the 5 μg BPA group and reversed in the 50 μg and 50 mg BPA exposure groups compared to unexposed controls.

Number of TH immunoreactive neurons in the pBNST

A two-way ANOVA indicated main effects of exposure ($F_{(3,51)}=2.708$, $P \leq 0.05$), and sex ($F_{(1,51)}=30.67$, $P \leq 0.0001$) but no significant interaction. Males had significantly more TH-ir cells than females in the control ($P \leq 0.005$), 50μg BPA ($P \leq 0.005$) and 50 mg BPA ($P \leq 0.01$) exposure groups (Figure 7A, D-F). Postnatal exposure to 5 μg BPA caused a significant increase ($P \leq 0.05$) in TH-ir cell number in the female pBNST, thus attenuating the sex difference.

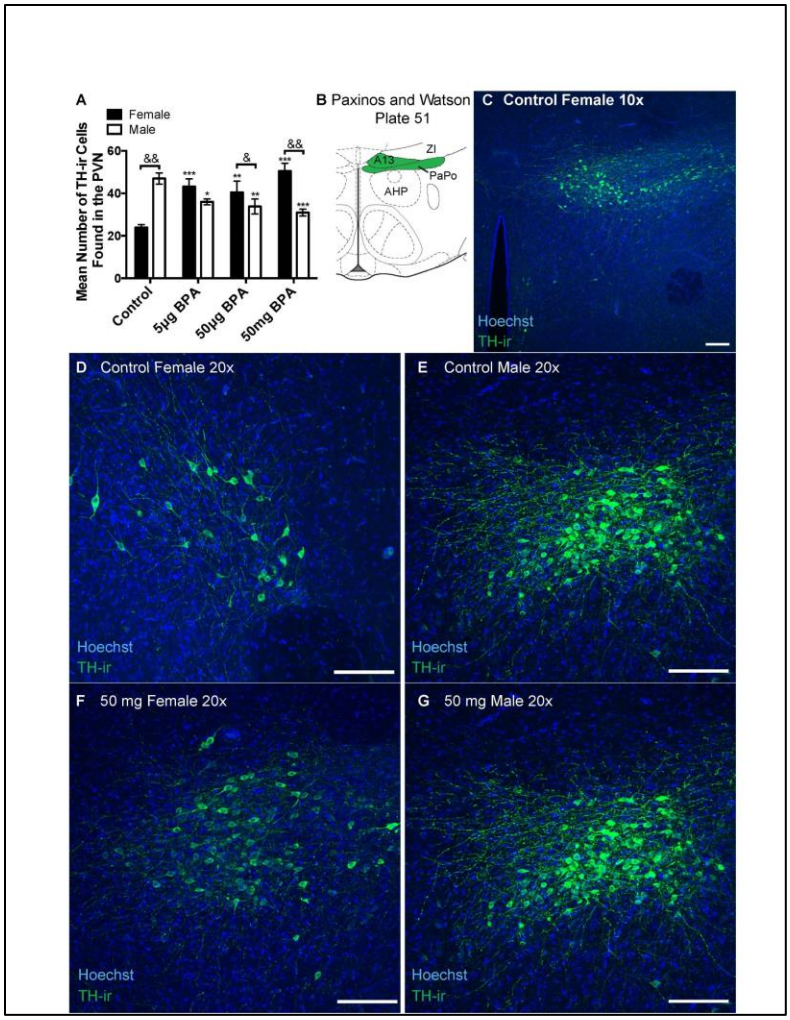

Figure 6. In the posterior PVN, postnatal BPA exposure significantly increased the number of TH-ir cells in females and significantly decreased their numbers in males. (A) Control males had significantly more TH-ir cells than control females in the posterior PVN area (B) The shaded portion of the diagram adapted from Paxinos and Watson plate 51 depicting the region of interest and (C) a representative image depicting the rounded, dense cluster of TH-ir cells found in the A13 region quantified. (D-E) Representative images depicting that TH-ir density is sexually dimorphic with females (D) having significantly fewer than males (E). (F-G) Representative images revealing that 50 mg/kg BPA reverses the sexual dimorphism in posterior PVN TH-ir density. Scale bar in each panel corresponds to 100 μm. Data are shown as the mean ± SE. A significant difference from control indicated by *, $P \leq 0.01$; **, $P \leq 0.001$; ***, $P \leq 0.0001$ within sex. A significant difference between sexes within exposure indicated by &, $P \leq 0.01$; &&, $P \leq 0.0001$. A13 A13 dopamine cells, AHP anterior hypothalamic area, posterior, DA dorsal hypothalamic area, PaPo paraventricular hypothalamic nucleus, posterior, Pe periventricular hypothalamic nucleus, ZI zona incerta.

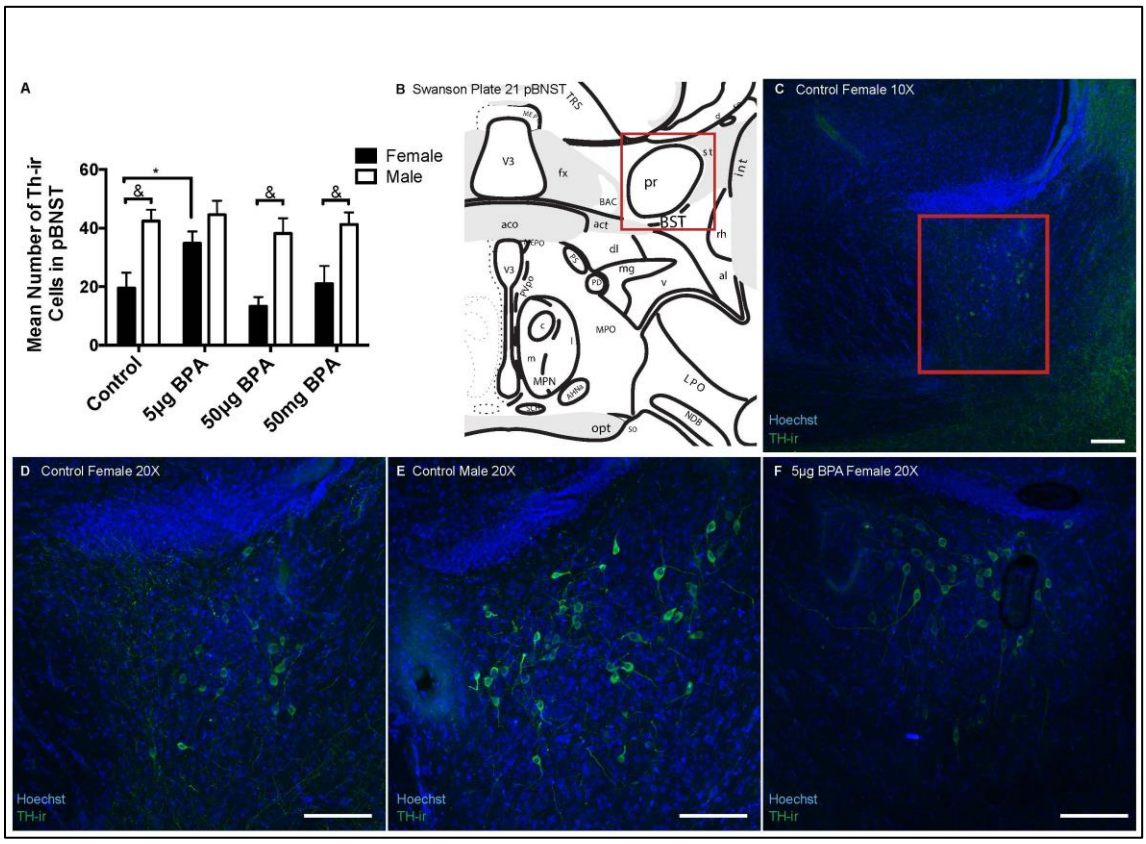

Figure 7. Postnatal exposure to 5 µg/kg BPA caused a significant increase in female TH-ir cell number in the pBNST. (A) BNST TH-ir was significantly elevated in the 5 µg/kg BPA females compared to unexposed conspecifics but unaltered in the other exposure groups. (B) An illustration adapted from the Swanson Rat Brain Atlas plate 21 depicting the region of interest (boxed) and (C) a low magnification image of the population of TH-ir cells quantified in this region. (D-F) Representative images showing the pronounced sex difference in BNST TH-ir and the significant increase in TH-ir cell numbers in the female 5 µg/kg bw BPA exposure group. Scale bar in each panel corresponds to 100 µm. Data are shown as the mean ± SE. A significant difference from control indicated by *, $P \leq 0.05$. A significant difference between sexes within the same exposure group indicated by &, $P \leq 0.01$. pr principal bed nucleus stria terminalis.

Discussion

Consistent with what has been reported in other species, BPA altered the expression of behaviors associated with anxiety, activity, and sociality in prarie voles, with the majority of effects occurring in females. In females, BPA exposure affected exploratory activity and behavior and inhibited the formation of partner preferences. Behavioral outcomes were accompanied by dose- and sex-dependent changes in TH-ir, in the pBNST and TH-ir, OT-ir, and AVP-ir neuron numbers in the PVN resulting in the loss of region-specific sex differences at some doses. Collectively these studies provide further evidence that developmental exposure to BPA can significantly impact the brain and behavior, and highlight the utility of the prairie vole model when seeking to explore the effects of exposure to BPA or other EDCs on prosocial behaviors and the neuroendocrine systems that coordinate them.

Female prairie voles exposed to the lowest dose (5 μg/kg bw) of BPA demonstrated heightened exploratory activity in the open field test, suggestive of hyperactivity. This observation is consistent with what has been reported in mice (Anderson et al., 2013; Kundakovic et al., 2013a; Williams et al., 2013; Wolstenholme et al., 2013a), rats (Ishido et al., 2004; Ishido et al., 2011; Ishido et al., 2007; Masuo et al., 2004) zebrafish (Saili et al., 2012), and young children (Braun et al., 2011; Braun et al., 2009; Harley et al., 2013). Similarly, the results from the novel social test and the partner preference test indicate that BPA alters the time course/development of prosocial behavior in a sex-specific manner. In females there was a significant effect of BPA on the exploratory investigation of novel individuals. Control males investigated novel individuals significantly more than females and treatment with BPA either eliminated or reversed this effect, with females exposed to the highest dose of BPA investigating novel individuals more than exposed males. In the two lower dose groups, there was no difference between males and females in investigation of novel conspecifics. These findings suggest that BPA, while not altering total time spent with novel conspecifics alters how individuals interact socially.

The formation of a partner preference by both males and females is the initial and critical process in the establishment of long-term pair bonds, an essential aspect of social monogamy

(Young et al., 2011). While the effects on partner preference formation may have been subtle, they were statistically significant. BPA-exposed females failed to form a partner preference, while control females formed the predicted partner preference. The fact that there still appears to be a strong tendency (Fig 3a) to spend more time in contact with the partner suggests that BPA, instead of disrupting the formation of a possible preference, increased behavioral variability. One possible explanation is that BPA exposure increases the total amount of time it takes female prairie voles to form a preference. If this is correct, then increasing the period of cohabitation would be predicted to lead to the formation of partner preferences and, conversely, a shorter period of cohabitation would be predicted to increase variability. Partner preference findings are thus consistent with the response in the novel social test where BPA exposure altered how females investigate a novel individual; an effect which likely impacts the required time for specific individuals to form specific preferences. In contrast to females, there was no effect of BPA on partner preference in males, which is not surprising given that control males did not form a partner preference. The lack of formation of a male partner preference is consistent with previous studies in voles which have concluded that, unlike females, mating is required for males to form a partner preference without central administration of oxytocin or vasopressin (Young et al., 2011). A logical follow up to the current study would be to determine if early exposure to BPA alters the response to centrally administered neuropeptides, as occurs in neonatally castrated males (Cushing et al., 2003).

Sex specific behavioral effects and the loss of behavioral sex differences have been reported in other species following oral developmental BPA exposure in the low dose range. For example, gestational and lactational exposure to dietary BPA (50 mg of BPA/kg feed weight; resulting in approx. 150µg BPA daily exposure) caused a loss in sexually dimorphic exploratory behaviors in *Peromyscus californicus* (another socially monogamous rodent), and reduced territorial marking in exposed males (Williams et al., 2013). Loss of sex differences in emotional responses and exploration have also been observed in adult mice perinatally exposed to 10 µg/kg BPA (Gioiosa et al., 2007) and juvenile mice reared on a diet containing 50 mg of BPA/kg feed weight (Cox et al., 2010). The neural mechanisms

underlying the behavioral changes in this collective set of studies were not investigated but sex specific neurochemical effects reported here are consistent with the hypothesis that BPA-related effects on brain and behavior may be sex dependent.

Numerous prior studies have reported BPA-related effects on anxiety in rodents (Wolstenholme et al., 2013a; Wolstenholme et al., 2011) and, in the present study, enhanced freezing behavior in the open field test provides further evidence that BPA may induce a high anxiety phenotype. For most rodents, freezing behavior is typically considered to be a predator avoidance tactic that is associated with heightened anxiety. The proportion of females engaging in this behavior was highest in the 50 mg BPA group. Variability in the expression of this behavior increased with dose, a phenomenon which suggests that some individuals are more predisposed to respond to an environmental stressor with this type of activity than others. Essentially, within the 50 mg BPA group, there were "responders" and "non-responders" and the significant difference in freezing behavior was lost if the six most robust data points were removed as "outliers." Our interpretation of this behavioral variability is that the "responders" may represent individuals within a population that are more sensitive to a change in the environment. Limited information is available regarding genetic and other differences within a population contributing to variation in coping styles (Koolhaas et al., 2011; Koolhaas et al., 2010) but work in this area is critically needed as the concept of "adaptation" (or "resilience") is emerging as a pivotal but controversial concept in endocrine disruption toxicology (Andersen et al., 2005).

Use of elevated plus maze and other "traditional" tests of rodent anxiety to further assess the potential impacts of BPA on anxiety-like behaviors may not be appropriate because voles have a very different life history than rats and mice. This may also account for why no impacts of BPA were observed on other aspects of open field behavior in either sex. For example, in other laboratory rodent species, avoidance of the center of the open field is considered a hallmark measure of anxiety, but this may not be an appropriate measure of vole "anxiety" because they typically move about in grass runways where they are openly exposed to predators. And thus, prairie voles may display a differential response to "open" environments than traditional rodent behavioral models. Use of voles in toxicity testing will

require design of more ethologically relevant tests, specific to the prairie vole pro-social life history.

Prior work investigating the behavioral consequences of early-life low dose BPA exposure has also generated data implicating disruption of the OT/AVP system as an underlying mechanism. Perinatal (gestational through adolescent) exposure to BPA via drinking water (1 mg/L), resulting in serum levels approximately equivalent to humans (FAO/WHO, 2011), elevated anxiety-related behaviors in juvenile rats and decreased Esr2 (ERβ) and Mc4r expression levels in the amygdala (Patisaul et al., 2012). These genes play crucial roles in regulating the production and release of OT and AVP in the PVN. Specifically, agonism of Mc4R in magnocellular neurons induces dendritic secretion of OT (Sabatier et al., 2007), an effect which is anxiolytic (Insel, 2010; McCarthy and Altemus, 1997). In female rats, neonatal BPA exposure (50 mg/kg bw or 50 µg/kg bw by subcutaneous injection) significantly increased OT-ir neuron numbers in the anterior PVN of female rats (Adewale et al., 2011), a result which was interpreted to potentially indicate sequestration of OT and reduced release from nerve terminals. Mice reared on a diet delivering approximately 170 µg/kg bw BPA (to the dams) during gestation displayed transgenerational changes in sociality that coincided with a decrease in AVP and OT mRNA expression levels in whole embryonic brains. A subsequent study revealed hyperactivity in the F3 generation accompanied by increased investigative sniffing of a novel conspecific (Wolstenholme et al., 2012; Wolstenholme et al., 2013a). Collectively, these findings support the hypothesis that BPA exposure may disrupt the organization of OT/AVP pathways arising in the PVN, thereby impacting related behaviors.

The present data enhance available information about how BPA might be altering PVN organization by revealing that only specific subpopulations of PVN OT, AVP and TH neurons may be vulnerable to endocrine disruption. The high dose of BPA (50 mg/kg bw) significantly increased anterior PVN AVP-ir and decreased posterior PVN OT-ir. BPA exposure also resulted in elevated female TH-ir and decreased male TH-ir neuron numbers in the posterior PVN. Interpreting the functional significance of these changes is hampered by the limited information regarding the subarchitecture of the vole PVN, although some

inferences can be drawn given available data from other rodent species. The rat PVN is subdivided into anatomical and functional regions, with OT/AVP neurons being either magnocellular or parvocellular. In the rat, magnocellular neurons produce only OT and AVP and descend to the posterior pituitary where these neuropeptides are released into general circulation to regulate physiology coordinating osmotic balance and related homeostatic functions (ex. blood pressure, lactation) (Herman et al., 2002; Sawchenko and Swanson, 1983). Parvocellular OT and AVP neurons produce other neuropeptides, and project to hypothalamic and other brain regions as well as the anterior pituitary and play a coordinating role in motivational, reproductive, and affective behaviors (Simmons and Swanson, 2008). Although we hypothesize that the population of OT-ir and AVP-ir neurons impacted by BPA is parvocellular, the possibility that BPA alters the density of magnocellular neurons cannot be ruled out. This would suggest a potential mechanism for homeostatic disruptions associated with BPA exposure including cardiovascular effects and hypertension (Melzer et al., 2010; Shankar and Teppala, 2012). Similarly, in the rat, parvocellular OT and AVP neurons in the affected areas of the PVN, along with coordinating input from the BNST, are involved in the stress response (Herman et al., 1994) supporting the possibility that BPA may influence the hypothalamic-pituitary-adrenal (HPA) axis. The only study to date attempting to identify specific OT/AVPV neuronal populations, and their projections, in the vole PVN found that magnocellular and parvocellular cells are intermixed, and that their projections may not match those of the rat (Ross et al., 2009). Thus, we cannot readily deduce which PVN subpopulations of dopaminergic or OT/AVP neurons were significantly impacted by BPA, nor conclude with certainty that the observed behavioral changes are a consequence of the anatomical differences, but ongoing studies should provide greater resolution.

Actions on estrogen receptors (ERs) may be a primary mechanism by which BPA may induce effects on dopaminergic and OT/AVP pathways. We have shown that developmental BPA exposure can perturb ERα and ERβ expression throughout the rat mesolimbic dopamine system, including the PVN and BNST (Cao et al., 2014; Cao et al., 2012; Cao et al., 2013a; Rebuli et al., 2014) across the lifespan. These data suggest that early life exposure to BPA can fundamentally and permanently alter the density of ERα and ERβ in limbic nuclei,

including those critical to sociality. This altered distribution of ERs may underpin the observed behavioral and structural changes reported herein. OT pathways coordinating social recognition are well known to be regulated by estrogen, with both ERα and ERβ knockout mice showing social deficits (Choleris et al., 2008; Choleris et al., 2003), and ERβ knockout females failing to generate OT or AVP mRNA expression in response to exogenous estrogen administration (Nomura et al., 2002; Patisaul et al., 2003). Critically, the distribution of ERα differs across social and nonsocial species and plays a significant role in regulating species-specific sociality (Cushing and Wynne-Edwards, 2006). Increased ERα in the pBNST reduces male pro-social behaviors, including affiliation (Lei et al., 2010; Young et al., 2011). Although the specific functional role of limbic ERβ remains poorly understood, ERβ in the PVN and associated structures, including the BNST, plays a fundamental role in mediating motivational and anxiety-related behaviors (Kudwa et al., 2014; Lund et al., 2005; Sullivan et al., 2011). Moreover, estradiol administration to female prairie voles has been shown to induce estrous, but also modulates locomotor activities (Cushing and Hite, 1996; Cushing et al., 1995) suggesting a role for ERs in heightened exploration. The pBNST is richly populated with TH-ir cells, some of which are co-localized with ERα. Interestingly, this morphology is a unique feature of male prairie voles as few of these co-localized cells have been detected in the female vole pBNST or the BNST of promiscuous rodent species (in either sex). Sexually dimorphic populations in the pBNST co-expressing TH and ERα were more responsive to estrogen administration in maintaining TH (Young et al., 2011), suggesting that ERα mediates dopamine production. These data suggest that future work should focus on EDC-related impacts on ER levels within vole limbic nuclei, and male social interactions, including aggression.

Although not specifically explored in the present study, an additional possibility is that social and anxiety-related behavioral changes following BPA exposure result from altered distributions of OT and AVP receptors. Notably, higher OT receptor (OTR) binding in the female prairie vole NAcc has been associated with decreased levels of anxiety-like behaviors (Bales and Perkeybile, 2012). Moreover, it is well recognized that species (and, to some extent, individual) differences in pro-social behavior largely depend on differences in OTR

and AVP1aR distribution (reviewed in (Insel, 2010)). For example, monogamous females have a greater density of OTRs in the NAcc, a region that coordinates reward and reinforcement via dopaminergic pathways.

Conclusions

While there are consistencies with the results from rats and mice there are also differences indicating that using a more socially relevant species may be critical for understanding the effects of BPA, as well as, other endocrine disruptors in humans. The increase in activity in females treated with the lowest level of BPA, indicative of hyperactivity, is consistent with available human and mouse data. In contrast the subtle, but significant changes in social interaction seen in the novel social test and formation of partner preferences represent new findings that may not be observable in less social laboratory rodents. Additionally these effects could be magnified in the natural world where time of interaction is critical, such that minor changes could have major consequences, as extending the time or inhibiting initial interactions might disrupt the entire process of partner preference. Behavioral changes were accompanied by altered OT- and AVP-ir cell numbers in subregions of the PVN, and TH-ir cell numbers in the pBNST. While the specific functional significance of these changes needs to be further elaborated, impacts are wide ranging as these brain regions and mechanisms are known to be involved in the regulation of prosocial behaviors as well as responses to stress.

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CHAPTER 5: Conclusions

Overall, the behavioral outcomes reported in my dissertation are indicative of a endocrine disruption in the mesolimbic dopamine system. The medial preoptic area (MPOA) activates the mesolimbic system to regulate the appetitive components of male sexual behavior, which is driven largely in part by estradiol (Stolzenberg and Numan, 2011). Thus it is reasonable to hypothesize that EDC interference with ERβ in turn disrupts the mesolimbic reward pathway because ERβ neurons in the AMYG and PVN can influence dopamine production (Creutz and Kritzer, 2004; Patisaul et al., 2003). For example, ERβ can increase OT production in the PVN while ERα increases the expression of OTR in the hypothalamus (Rissman, 2008) and binding of OTR results in an increase of dopamine release from the amygdala and elsewhere. Consistent with my hypothesis that BPA and other EDCs impact mesolimbic pathways, I observed that ESR2 was downregulated in the AMYG of rats exposed to BPA and that sexually dimorphic TH-ir neuron number in the pBNST wasreversed in BPA exposed prairie voles.

Additional work is needed to more comprehensively establish if these observations are indicative of BPA-related hyperactivity and elucidate the underlying mechanisms by which exploratory and sociosexual behavior occurs. Hyperactivity in rodents has been tied to various alterations in the dopaminergic system. For example rats displaying hyperactivity in a novel environment had increased D1 receptor binding in the NAcc core and the caudate putamen (CP), increased D3 receptor mRNA expression in the NAcc shell, and increased D2 receptor levels in the NAcc core (Brake et al., 2004). Disruption of monoamine activity by BPA resulting in behavioral effects is further supported by recent studies showing that perinatal BPA exposure causes spontaneous and hyperactive outcomes in mice (Anderson et al., 2013; Viberg and Lee, 2012), rats (Ishido et al., 2007; Zhou et al., 2011) and zebrafish (Saili et al., 2012), a subset of which reported associated disruption within the dopaminergic system (Ishido et al., 2007; Zhou et al., 2011) and altered monoamine levels (Matsuda et al., 2012; Matsuda et al., 2010; Nakamura et al., 2010; Zhou et al., 2011). Alterations in D2 receptors and autoreceptors can cause a range of disorders but most relevant here is that these

receptors can cause either a loss in activity/locomotion and reward reinforcement (Ford, 2014) or hyperactivity and increased sensitivity to acute rewards (Anzalone et al., 2012). Recently, a mutation in the human dopamine transporter gene (hDAT) has been associated with autism spectrum disorder (ASD) diagnosis. Expression of the hDAT in DA neurons-lacking *Drosophila melanogaster* DAT conferred hyperlocomotion (Hamilton et al., 2013). Collectively my data suggests that sex steroid receptors and the mesolimbic dopamine system are linked and exposure to BPA and phytoestrogens alter the mesolimbic dopamine system in a sex and age dependent manner.

Now that I have established the prairie vole as a valuable animal model for EDC research, future studies can address more specific questions about impacts on sociosexual behavior. For example, allowing males and females to mate prior to the partner preference test would facilitate analysis of prosocial behavior specific to their mating strategy, time to mate, and strength of the post-copulatory bond. Additionally, other important, associated, prosocial behaviors could be assessed, including alloparental care and aggression. The underlying mechanisms could be characterized by quantifying sex-specific receptor distribution in various regions of interest known to relegate the expression of that particular behavior, including mesolimbic pathways. For example, if BPA-exposed males or females displayed a significant loss in partner preference, we could then flash freeze the brain to perform autoradiography specific for OTR and V1aR and quantify differences, with the expectation of finding a loss of V1aR in male ventral pallidum and OTR in female NAcc.

Because hyperactivity and anxiety have been reported in BPA-exposed animals including humans, it is important to fully characterize these behaviors to better distinguish between behaviors indicative of anxiety versus activity. I believe tracking animal movement in the home cage for a 24hr period would help us determine true baseline activity levels that could then be compared with exploratory activity, sociality (play behavior in subadults and novel-social in adults), and anxiety. It is important to stress to the public that while soy cannot be considered a "rescue" from endocrine disruption effects or from affective disorders, it can be used to better understand the mechanisms behind varying health outcomes and address to the

public that aspects of the diet and environment (including social) are important and should be considered by future scientific studies.

Collectively my data support currently controversial low dose responses for BPA and other EDCs that have been reported previously and attributed to several possible factors including interacting mechanisms of action and divergent cellular responses across the dose range (Kendig et al., 2010; Kundakovic et al., 2013b; Vandenberg et al., 2009). Differential/opposite effects on behavior (Holmes et al., 2002) and physiology (vom Saal et al., 1997) are known to be associated with low versus high doses of estrogen. Intrauterine position (IUP) has been an excellent naturally occurring model for illustrating how prenatal exposure to low levels of endogenous hormones can differentially affect physiology and behavior (Ryan and Vandenbergh, 2002). Regulatory policies on risk assessment must adapt to the growing field of endocrine disruptors, a task difficult to tackle in a high throughput manner.

My dissertation contributes to science in several ways. By using the prosocial prairie vole, I have identified a unique, yet key, rodent species to strengthen toxicological screening. This model is especially important because the underlying neural circuitry of sociality, including mating strategy, is highly plastic, well-characterized, similar to that of humans, and its disruption results in behavioral outcomes similar to those seen in the diagnosis of ASD and other affective disorders. Importantly I showed that diet does matter; rearing on a soy-rich diet resulted in behavioral and gene expression effects. Studies should be controlled and designed to recapitulate natural conditions as closely as possible, including route of exposure, levels of exposure (with the understanding of variance in metabolomics) mixtures, cycles, as well as housing/environmental conditions. Furthermore, my dissertation adds to the body of scientific evidence that endocrine disruption occurs in a non-monotonic manner. While this phenomenon is not novel, toxicology studies appear slow to adopt methods to address it and this dissertation provides findings and better-suited models to expedite those critical assays. Finally, my data indicated a diet by exposure by gene interaction, further supporting the idea that all three factors, as well as individual variation, should be strongly considered. Although I did not execute a study to specifically address individual variation, I addressed this idea in

the discussion of AIM3, Chapter 4, where a subset of female voles froze in the center of the open field arena.

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