The effects of bisphenol A exposure and calbindin knockout on behavior, gene expression, and sexual differentiation of the brain

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Abstract

Bisphenol A (BPA) is an endocrine disrupting compound used in the production of polycarbonate plastics. Recent data have indicated health risks associated with exposure to BPA, particularly in children exposed during gestation. Gestational BPA exposure has been proposed as a risk factor for the development of neurobehavioral disorders, such as autism spectrum disorder. To address the behavioral impact of developmental exposure to BPA, I tested offspring of mice exposed to a daily low dose of BPA during pregnancy. I also asked if preconception exposure of the sire affected behaviors in offspring. Juvenile offspring exposed to BPA maternally, but not paternally demonstrated increased anxiety-like behavior. However, neither parental exposure group differed significantly from controls in the social recognition task. I also assessed the behaviors of maternally exposed offspring in two novel tasks: ultrasonic vocalizations (USVs) in pups and operant reversal learning in adults. Maternal BPA exposure increased the duration and median frequency of USVs emitted by pups during maternal separation. In the reversal learning task, females responded more accurately and earned more rewards than males and control females received more rewards than BPA females during the acquisition phase of the task. Previous research from our lab demonstrated that gestational BPA exposure not only affects offspring directly exposed *in utero* but also offspring three generations removed from the BPA exposure. We have previously reported that transgenerational exposure to BPA affects estrogen receptor alpha (ERα) in two sexually dimorphic brain areas. In a follow-up study, I determined the effects of ancestral BPA exposure in three sexually dimorphic brain regions: the anteroventral periventricular nucleus (AVPV), sexually dimorphic nucleus of the preoptic area (SDN-POA), and the cerebellum. In adults from F3 control and BPA lineages, I found that females had more tyrosine hydroxylase positive dopaminergic cells than males in the AVPV, particularly in cells that also had nuclei positive for ERα. Contrastingly, the volume of calbindin immunoreactivity in the SDN-POA was greater in males than females. In juvenile cerebellum, however, I found no effect of sex on the amount of calbindin protein. All three areas lacked a significant impact of transgenerational exposure to BPA. Calbindin, a high-affinity

calcium binding protein, is associated with a number of neurobehavioral diseases, many of which are sexually dimorphic in incidence. In the final study, I investigated novel aspects of calbindin function on social behavior, anxiety-like behavior, and fear conditioning in adult mice of both sexes by comparing wildtype (WT) to littermate calbindin knockout (KO) mice. I also examined gene expression in the amygdala and prefrontal cortex (PFC), two areas of the brain intimately connected with control of the behaviors tested, in response to sex and genotype. I found that fear memory and social behavior was altered in male knockout mice and Calb-KO mice of both sexes show less anxiety-like behavior. Moreover, gene expression studies of the amygdala and PFC revealed several significant genotype and sex effects in genes related to brain-derived neurotrophic factor (BDNF) signaling, hormone receptors, histone deacetylases, and GABA signaling. The results reported in each of the three studies demonstrate the important role of both genetic and environmental factors in shaping the development of the brain and behavior in mice.

Acknowledgements

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Chapter I

Introduction

General introduction

Hormones function in the human body at exceptionally low concentrations: on the order of parts-per-*trillion*. Put in perspective, this would be roughly equivalent to one inch in 1.6 million miles, or one second in 32,000 years. A system this sensitive is, unfortunately, also incredibly vulnerable. The sensitivity of human brain development to hormones during critical periods increases the potential risk of permanent dysregulation of neurodevelopment by environmental factors.

The developmental origins of health and disease (DOHaD) hypothesis posits that events during early development impact an organism's risk for development of future disease. This was first proposed by Dr. David Barker, who showed that low birth weight, a surrogate for poor fetal growth, was significantly associated with increased risk of heart disease, obesity and insulin resistance in adulthood (Barker, 1999). The theory stresses the importance of sensitive or critical periods in development when an environmental stimulus can have life-long, potentially irreversible effects on development. This is particularly true for early-life programming actions of hormones, which have been suggested as a risk factor for neurodevelopmental disorders like autism spectrum disorder (ASD), schizophrenia, attention-deficit-hyperactivity disorder (ADHD), and affective disorders (Bale et al., 2010). ASD prevalence, in particular, has a strong male bias, which suggests a significant contribution of sex-specific genetic and hormonal factors.

Autism spectrum disorder describes a collection of heterogeneous neurodevelopmental disorders characterized by varying degrees of social deficits, communication problems, and perseverative/stereotyped behaviors. The incidence of ASD is approximately 1 in 68 children, however it is almost 5 times more common in boys (1 in 42 for boys, 1 in 168 for girls) (ADDM, 2014). Although there is strong evidence for a genetic link in ASD, it is thought that environmental factors, such as endocrine disrupting compounds, could also contribute to the exponential increase in diagnosis of this disorder and the inherent diversity of behaviors associated with ASD (T. Schug et al., 2015). Endocrine disrupting compounds (EDCs) are chemicals that interfere with

the synthesis, secretion, transport, binding, action, or elimination of natural hormones that are responsible for development, behavior, fertility, and maintenance of homeostasis (Gore et al., 2015) (see Figure 1). Appropriate brain development depends on a balance of hormones during critical periods. It is therefore crucial to understand how endocrine disruptors like BPA can disturb this process.

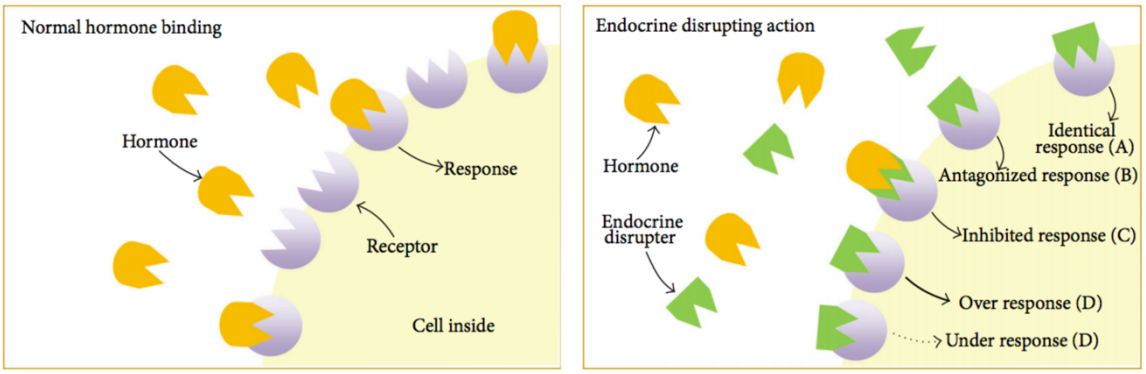

Figure 1: Receptor actions of endocrine disrupting chemicals (EDCs)
By binding to hormone receptors (purple), EDCs (green) can (A) mimic the response of endogenous hormones (yellow), (B) antagonize their action, (C) induce a stronger response than the endogenous hormone, or (D) dampen the response of the endogenous hormone. Adapted from (Sosa-Ferrera et al., 2013)

BPA

Bisphenol A (BPA) is a man-made endocrine disrupting chemical used to manufacture polycarbonate plastics and epoxy resins. It was first synthesized by a Russian chemist named Alexander Dianin in 1891. Charles Dodds later tested BPA as a potential synthetic estrogen for medical use in the 1930s, but concluded that its estrogenic activity was too low. Twenty years later, scientists at Bayer and General Electric discovered a practical use for BPA in the manufacturing of polycarbonate plastics. Since the FDA's approval of the use of BPA in consumer products in the 1960s, BPA has become one of the world's mostly highly manufactured chemicals. Over 3.8 million tons are produced each year, a third of which is manufactured in the United States (Flint et al., 2012). BPA is present in many highly utilized products such as plastic water bottles, storage containers, CDs and DVDs, eyeglass lenses, thermal receipt paper, dental sealants, and the lining

of food and beverage containers (Michałowicz, 2014). Canada was the first country to ban the use of BPA in baby bottles in 2010. Since then, many other countries have followed suit, including the United States (Metz, 2016). Prolonged usage or exposure of these items to high temperatures gradually increases the amount of BPA that can leach from the polycarbonate (Carwile et al., 2009; Le et al., 2008). Therefore, the greatest source of exposure in humans comes from ingestion of food and water in contact with BPA-containing materials. Humans are also exposed to BPA through respiration (dust) and dermal contact (paper) (Liao and Kannan, 2011), although these routes of administration need to be more thoroughly examined.

Human exposure and BPA metabolism

Human exposure to BPA is widespread: the CDC reports detectable levels of BPA in over 93% of human urine samples (CDC (Centers for Disease Control and Prevention, 2009). BPA is also detected in serum, amniotic fluid, umbilical cord blood, and breast milk (Cao et al., 2015; Genuis et al., 2012; Ikezuki et al., 2002). Typical levels of BPA range from 0.3 ng/mL to 18.9 ng/mL in human maternal plasma, 0.2 to 9.2 ng/mL in fetal plasma, and 1.0 to 104.9 ng/mL in placenta tissue (Schönfelder et al., 2002). The lowest observed adverse effect level (LOAEL) for humans as stated by the United States Environmental Protection Agency (EPA) is 50 mg/kg/day (USEPA, 2010). To determine the maximum concentration believed to be safe, the EPA divides this dose by a 1,000-fold-safety factor (50 µg/kg/day). Animal and human studies have demonstrated BPA can cross the placenta and circulate in the fetal system (Balakrishnan et al., 2010; Corbel et al., 2013; Ikezuki et al., 2002; Nishikawa et al., 2010; Schönfelder et al., 2002). Normally, expression of alpha-fetoprotein in the liver and yolk sac during fetal development buffers the fetus from excess maternal estrogens by binding and preventing their passage through the placenta. However, xenoestrogens, like BPA, do not bind to alpha-fetoprotein and are therefore free to enter the fetal environment (Milligan et al., 1998).

The liver enzyme UDP-glucuronosyltransferase (UGTB1) metabolizes BPA in the body. The metabolites are cleared from the blood and excreted in urine and sweat with an overall half-life of about 6 hours in humans (Genuis et al., 2012; Völkel et al., 2002). However, UGTB1 is not expressed in the developing fetal liver (Coughtrie et al., 1988; Matsumoto et al., 2002). Glucuronidated BPA (metabolized by UGTB1) can be de-conjugated by placental β-glucuronidase in humans and rodents (Edlow et al., 2012). Since the developing organism does not express UGTB1, unconjugated BPA gets trapped in the fetal-placental compartment causing high fetal exposure (Corbel et al., 2013; Nishikawa et al., 2010). Additionally, a study in rats reported that maternal activity of UGTB1 decreases by about fifty percent during pregnancy (Matsumoto et al., 2002). Moreover, urinary BPA concentrations in premature infants undergoing intensive medical treatment are significantly higher than the general population, potentially due to prolonged exposure to medical devices containing BPA and their relatively smaller body size (Calafat et al., 2009). Taken together, this suggests a significant susceptibility to developmental reprogramming by BPA during fetal and neonatal stages.

Effects on Human Behavior

Considering the vulnerability of developing organisms to the endocrine disrupting effects of BPA *in utero*, recent research has focused on the potential developmental consequences of gestational BPA exposure. Epidemiological studies in human populations have begun to reveal alarming associations between early-life BPA exposure and neurobehavioral development in children (reviewed in Mustieles et al., 2015). Research in human populations typically uses urinary concentrations of BPA as an indicator of the degree of exposure. Ideally, researchers collect urine at several time points, as a single urine sample may not reflect an individual's long-term exposure to BPA (Mahalingaiah et al., 2008). In a study population of 249 mothers and their children, BPA measured in urine samples collected the mothers at several time points during pregnancy positively associated with anxiety and depressive symptoms in their children, specifically in girls (Braun et

al., 2009; J. M. Braun et al., 2011). Urine samples collected early in the second trimester showed a stronger association with externalizing behavior in girls at 2 years of age than measurements collected after 17 weeks gestation (Braun et al., 2009). In the follow up study, each 10-fold increase in gestational BPA concentration was associated with more anxious and depressed behavior and poorer emotional control in girls at three years of age. These behaviors were not correlated with the urinary BPA levels of the children themselves, indicating the importance of developmental exposure (J. M. Braun et al., 2011). However, the effects of BPA in this study population appear to be age-specific because urinary BPA did not predict scores on the neonatal intensive care unit (NICU) Network Neurobehavioral Scale (NNNS), a measure of early infant behaviors (Yolton et al., 2011). Furthermore, the same children were assessed on the Social Responsiveness Scale (SRS) at 4-5 years of age, but there was no statistically significant association between SRS scores and gestational BPA exposure (Braun et al., 2014).

Other studies have found sex-specific effects of prenatal BPA exposure. In boys, maternal urinary BPA was positively correlated with symptoms of anxiety and depression, but the opposite was true in girls (Evans et al., 2014; Harley et al., 2013; Perera et al., 2016, 2012; Roen et al., 2015). BPA is also associated with an increased risk of hyperactivity and inattention symptoms characteristic of attention deficit hyperactivity disorder (ADHD) in boys and a reduced risk of inattention symptoms in girls (Casas et al., 2015). However, another group found no associations urinary BPA concentrations in the third trimester and scores on the SRS in 137 mother-child pairs at ages 7-9 in a multi-ethnic study population. One limitation of this study, however, was that only one urine sample was measured at the end of the pregnancy (Miodovnik et al., 2014).

Exposure to BPA during childhood (as opposed to gestation) has been associated with worse behavioral scores on the Child Behavior Checklist (Rocio et al., 2016), poor performance on the Learning Disability Evaluation Scale (Hong et al., 2013), as well as increased hyperactivity in girls and decreased social behavior in boys (Findlay and Kohen, 2015). Interestingly, two studies found that urinary concentrations of BPA in children with ASD are higher than in typically

developing children (Kardas et al., 2015; Stein et al., 2015). Despite some inconsistencies in the sex of children primarily affected by BPA, taken together, these studies support a connection between early-life BPA exposure and alterations of human behavior.

Molecular Targets of BPA

Estrogen receptors

Many of the effects of BPA seem to be mediated by its actions on estrogenic signaling. As such, a great deal of research has focused on the effects of BPA on sex hormone-dependent physiology, like reproductive function, fertility, puberty, and urogenital birth defects. BPA has been linked to various anomalies in reproductive physiology: premature puberty onset (Howdeshell et al., 2007), altered mammary gland development (Vandenberg et al., 2007b), prostate cell growth (Prins et al., 2014), changes in female genital tract morphology (Markey et al., 2005). However, the research exploring mechanisms by which BPA affects estrogenic activities has produced variable results.

Estradiol binds to both nuclear estrogen receptors, ERα and ERβ, which are encoded by different genes (*Esr1* and *Esr2,* respectively). Upon estradiol binding to intracellular ER, conformational changes cause the receptor to form a dimer, either a homodimer or a heterodimer consisting of ERα and ERβ (Cowley et al., 1997). This dimer binds to several kinds of response elements in the DNA sequence, including estrogen response elements (EREs), to affect the downstream gene transcription (Klinge et al., 2004). It is thought that BPA-bound ERs induce different conformational changes than the endogenous ligand and therefore alter downstream effects (Gould et al., 1998). Luciferase reporter assays in cell culture studies reveal that BPA evokes about 50% of the activity of estradiol when bound to the receptor (Gould et al., 1998; Kurosawa et al., 2002). BPA binds to ERα at a very low affinity, about 10-100,000 times less than estradiol, its endogenous ligand (Barkhem et al., 1998). BPA binds ERα and ERβ at similar affinities (Kuiper

et al., 1998) and the actions of BPA may be contingent on the receptor subtype and cell type. One study shows that BPA acts as an estrogen agonist at ERβ receptors in the presence of estradiol, but either an agonist or antagonist at ERα receptors depending on the cell line and presence of estradiol (Kurosawa et al., 2002).

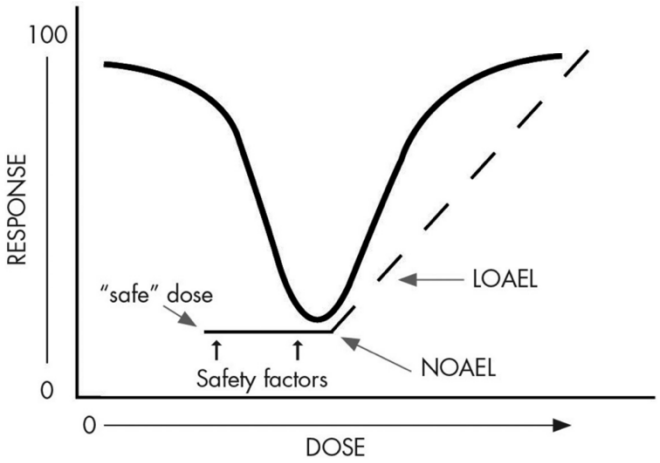

Figure 2: Non-monotonic dose responses
Toxicological risk assessments typically assume a linear dose-response relationship (dashed line). The lowest observed adverse effect level (LOAEL) and the no observed adverse effect level (NOAEL) are experimentally determined and the "safe" reference dose is established after accounting for "safety factors". However, hormones and EDCs sometimes have a non-monotonic response (solid curved line) – meaning that doses lower than the reference dose may also result in a response. Adapted from (Vandenberg et al., 2012).

Recent studies have highlighted the importance of the non-monotonic dose response curve of BPA (Vandenberg, 2014): a distinct pattern commonly exhibited by endocrine disrupters (see Figure 2). For example, females exposed to BPA at low doses have decreased ERα expression in the hypothalamus compared to control, whereas a higher dose of BPA is not significantly different from control. The pattern is opposite for males: BPA increased ERα at low doses, but there was no difference at high doses (Kundakovic et al., 2013). Hormone-sensitive targets could exhibit different responses to the same hormone or EDC based on concentration for a variety of reasons. Hormones bind with different affinities to various receptors. Perhaps activation of one receptor triggers the upregulation of another, which can then respond independently to a higher dose of the hormone or EDC. Several hormones can also influence their own production or release via complex positive and negative feedback systems (Vandenberg, 2014). In the case of BPA and ERα, perhaps at lower concentrations BPA binds to a receptor other than ERα to induce an intracellular response that

increases ERα expression, but at higher doses BPA binds directly to ERα, causing a negative feedback response and decreasing ERα expression. The results of the animal studies reflect patterns seen in epidemiological data as well (Alyea and Watson, 2009; Rubin and Soto, 2009). Non-monotonic responses of BPA exposure may explain some of the conflicting results at different doses in animal studies. However, weak activity at classical estrogen receptors does not fully explain the behavioral and physiological phenotypes reported in rodent models of BPA exposure, especially at the low doses being tested.

In addition to nuclear receptors, BPA also binds with relatively high affinity to the membrane-bound estrogen receptor GPER. BPA binds GPER with higher affinity than nuclear ERs and leads to rapid activation of adenylyl cyclase *in vitro* (Thomas and Dong, 2006). Activation of this receptor can also cause changes in intracellular signaling cascades such as the phosphoinositide 3-kinase (PI3K)-Akt pathway (Revankar et al., 2005) and the mitogen-activated protein kinase pathway (MAPK) (Jang et al., 2012; Xu et al., 2013a). GPER activation of the MAPK pathway mediates phosphorylation of N-methyl-D-aspartate (NMDA) receptor subunits and morphogenesis of hippocampal neurogenesis induced by BPA in male mice (Xu et al., 2013b). Activation of a membrane-bound ER may explain some of the estrogen-related effects of BPA that are not mediated by classical ERs. For example, GPER expression is increased in embryonic mouse brain in males exposed to BPA during gestation, whereas ERβ expression is unaffected (Wolstenholme et al., 2012). However, it is still unclear whether GPER-related signaling events could contribute to the long-term changes caused by BPA in the brain.

ERRγ

BPA binds with high affinity to estrogen-related receptor gamma (ERRγ), an orphan receptor that shares a sequence homology with ERα and ERβ but is not activated by estradiol (Liu et al., 2010; Matsushima et al., 2007; H. Okada et al., 2008; Takayanagi et al., 2006). The IC50 of

BPA for ERRγ (13.1 nM) is 80 to 100-times lower than the IC50 values of BPA for ERα (IC50= 1040 nM) or ERβ (IC50 = 1320 nM) (Takayanagi et al., 2006). BPA binds to ERRγ to preserve its constitutive activity. ERRγ does not bind estradiol; it is constitutively active in the nucleus where it binds to estrogen response elements (EREs) and ERR-response elements (ERREs), among many other regulator elements, in the absence of a ligand (Razzaque et al., 2004). ERRγ can also interact with other ERRs to regulate transcription (Kumar and Mendelson, 2011). ERRγ and ERRα can exist as a heterodimer, but the activities of both receptors are inhibited by this interaction (Huppunen and Aarnisalo, 2004). Peroxisome proliferator-activated receptor gamma (PGC-1a) is an important co-activator and regulator of transactivation for ERRγ (Liu et al., 2005; Zhang and Teng, 2007), whereas small heterodimer partner (SHP) acts as a transcriptional repressor for ERRγ (Zhang and Wang, 2011). This might serve to explain why the effects of BPA sometimes do not match the response of positive estrogen controls for estrogenic activity (diethylstilbestrol (DES) or ethinyl-estradiol (EE)) in some studies (Greathouse et al., 2012; Kubo et al., 2003).

Interestingly, the placenta expresses extremely high levels of ERRγ and its high affinity for BPA causes an accumulation of BPA in placental tissues (Takeda et al., 2009). Within the placenta, ERRγ activity leads to increased expression of aromatase, the enzyme that converts testosterone to estradiol, thereby regulating placental estradiol levels (Kumar and Mendelson, 2011). High doses of BPA during pregnancy in mice can cause changes in placental morphology and ultimately result in abortion (Tachibana et al., 2007). Exposure to BPA at a level commonly detected in human placentas leads to cell death and is associated with adverse pregnancy outcomes (Benachour and Aris, 2009). These results suggest that the placenta is a particularly susceptible target of the effects of BPA, which may be partially mediated by ERRγ.

ERRγ is highly expressed in the developing brain, as well as in the adult brain in specific areas (Lorke et al., 2000). The expression pattern of ERRγ is distinct from estrogen-related receptors alpha and beta, pointing to an important functional difference during development (Hong

et al., 1999). BPA administered at a range of low doses during gestation reversed sexually dimorphic expression of ERRγ in juvenile prefrontal cortex and hypothalamus of BALB/c mice. Male ERRγ mRNA was higher than female in both brain areas, but exposure to 2 or 20 μg/kg BPA reversed this sex difference (Kundakovic et al., 2013). In embryonic C57 mouse whole brain, gestational BPA exposure significantly increased ERRγ gene expression in males (Wolstenholme et al., 2012).

In zebrafish, the synthetic ERRγ agonist GSK4716 can mimic the effects of perinatal exposure to low doses of BPA on social behavior, circadian rhythms, hyperactivity, and learning (Saili et al., 2012; Weber et al., 2015). These data establish the sufficiency of ERRγ to induce similar effects as BPA in a zebrafish model. Even more compelling is evidence that the enhancing effects of BPA on spinogenesis in the adult rat hippocampus can be blocked by an ERRγ antagonist, but not antagonists of ERα or ERβ (N. Tanabe et al., 2012). These studies strongly implicate ERRγ as a mediating factor in the neurodevelopmental effects of BPA.

Androgens

BPA also exerts it effects on other steroid hormones important during development. Testosterone is secreted from the testes in males, the ovaries in females, and from the adrenal gland in small amounts in both sexes. XY males possess the *Sry* gene, which signals undifferentiated embryonic gonads to become testes, which then begin to release testosterone. Testosterone and its metabolite dihydrotestosterone (DHT) bind to the intracellular androgen receptor (AR) to masculinize internal and external genitalia. Testosterone is metabolized by 5a -reductase, which catalyzes its conversion to DHT, a non-aromatizable androgen that binds to AR with high affinity (Azzouni et al., 2012). Testosterone diffuses into the brain where the enzyme aromatase can convert it to estradiol, which can then bind to estrogen receptors. Therefore, testosterone can exert

endocrine regulation via androgenic and/or estrogenic signaling. Aromatization of testosterone to estradiol in the brain during development is critical in establishing normal behaviors in males.

BPA can inhibit the actions of testosterone through multiple pathways. Several studies have demonstrated antagonistic effects of BPA on the androgen receptor (AR) and its expression (Bonefeld-Jørgensen et al., 2007; Kass et al., 2014; Pellegrini et al., 2014; Qiu et al., 2013; Xu et al., 2005)**.** BPA potently inhibits AR transcriptional activation induced by one of its ligands, DHT. Disrupted androgen signaling caused by BPA resulted in spermatogenesis failure in rats (Qiu et al., 2013)**,** as well as dysregulation of steroid receptor co-regulators (Salian et al., 2009)**.** Additionally**,** human epidemiological data reveal an association between parental BPA exposure and a shortening of anogenital distance in boys (AGD) (Miao et al., 2011). AGD is a sexually dimorphic biomarker of prenatal androgen exposure, where increased androgen exposure is associated with a longer anogenital distance.

In rats, BPA suppresses the testosterone surge (normally 2.5 times higher after birth) in males on the day of birth. BPA did not, however, affect the concentrations of testosterone in male fetuses prior to birth (Tanaka et al., 2006). At the level of the pituitary gland, BPA decreases luteinizing hormone (LH), which triggers Leydig cells in the testes to release testosterone. However, BPA also decreases expression of steroidogenic enzymes in Leydig cells, which further decreases testosterone synthesis (Akingbemi et al., 2004). Additionally, BPA exposure in pregnant and nursing dams decreased testosterone levels in interstitial fluid of the testes in adult offspring, suggesting that androgen disruption during critical periods can produce long-term changes in hormone levels (Tanaka et al., 2006).

BPA can also affect sex hormone signaling by disrupting aromatase (Akingbemi et al., 2004; Bonefeld-Jørgensen et al., 2007; Huang and Leung, 2009). One study showed that BPA inhibits aromatase by upregulating the expression of peroxisome proliferator-activated receptor gamma (PPARγ) (Kwintkiewicz et al., 2010), which is known to inhibit expression and function of aromatase (Lebovic et al., 2013). In contrast, BPA *stimulates* aromatase in human granulosa

cells, potentially indicating tissue-specific actions (Kwintkiewicz et al., 2010). ERα can interact with ERRγ in the presence of BPA to increase aromatase expression in cultured human trophoblast cells (Kumar and Mendelson, 2011). BPA, or ERRγ agonist GSK4716 alone, increases brain-specific aromatase in zebrafish (Chung et al., 2011), which is associated with accelerated neurogenesis (Kinch et al., 2015). See figure 3 for a summary of the molecular effects of BPA.

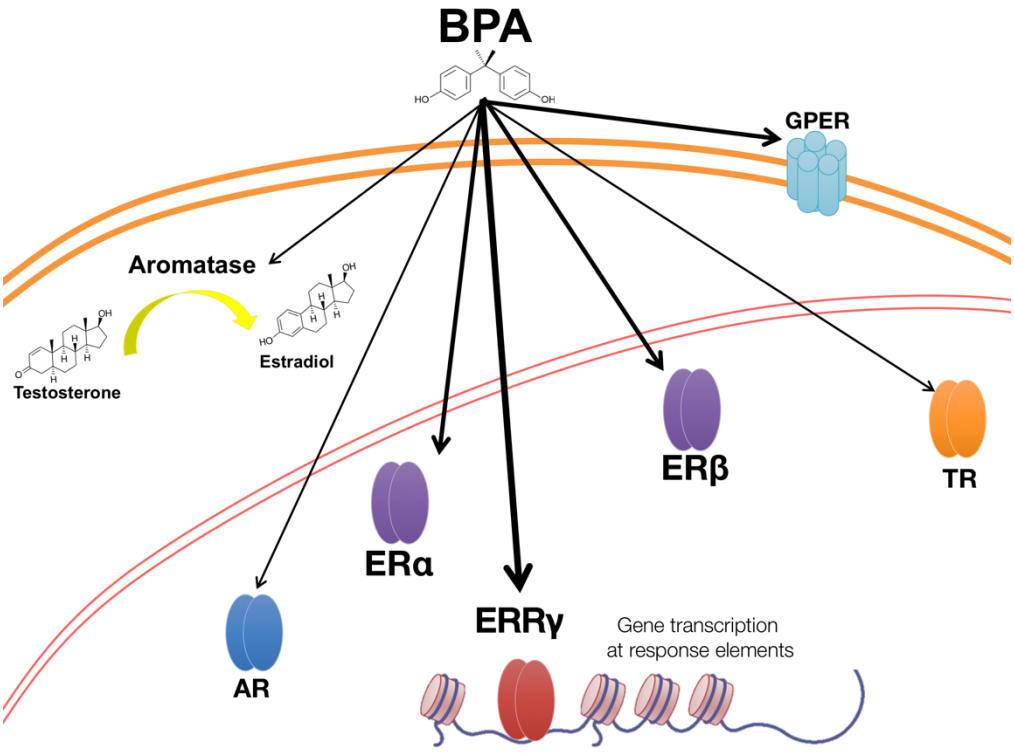

Figure 3: Molecular targets of BPA
BPA acts on targets on the cell membrane (orange lines), in the cytosol, and in the nucleus (red lines). BPA binds the G-protein coupled estrogen receptor (GPER), estrogen receptors alpha (ERα) and beta (ERβ), estrogen related receptor gamma (ERRγ), androgen receptor, thyroid hormone receptor (TR). BPA affects the expression and activity of the steroidogenic enzyme, aromatase.

BPA and the brain

Neurodevelopment

BPA can affect anatomical and functional measures of brain development and sexual differentiation. Estradiol and BPA are both known to affect proliferation and differentiation of

neural stem/progenitors cells (NS/PCs) (Agarwal et al. 2016; Huang et al. 2016; Okada et al. 2008, 2010; Tiwari et al. 2015; Yin et al. 2015). Gestational exposure to BPA disrupts normal neocortical development by accelerating neuronal differentiation/migration (Nakamura et al., 2007a). Prenatal BPA exposure has also been shown to alter neuronal migration/positioning patterns during cortical histogenesis (Ling et al., 2016; Nakamura et al., 2007b). Oral exposure to BPA from gestational day 8.5 to 13.5 is associated with cortical hyperplasia, accelerated cell cycle exit, and decreased numbers of neural stem/progenitor cells in mice (Komada et al., 2012). Interestingly, very low-dose (0.0068 μM) exposure to BPA leads to precocious neurogenesis in the hypothalamus of zebrafish, whereas a moderate dose (1 μM) did not affect hypothalamic neurogenesis (Kinch et al., 2015). BPA reportedly upregulates expression of thyroid hormone receptor alpha in embryonic brain at gestational day 14.5 (Nakamura et al., 2006). Therefore, some of the effects of BPA on neuronal development could potentially reflect disrupted thyroid hormone signaling in the brain.

Thyroid hormone and its receptors are critical for nervous system development and maintenance of adult brain function (Schroeder and Privalsky, 2014). Thyroid hormone (T3) is a peptide hormone that acts by binding the thyroid hormone receptor (TR), which is widely expressed in the developing brain (Thompson, 2000). BPA can bind the TR and decrease TR-mediated transcriptional activity (Moriyama et al., 2002). BPA displaces T3 and inhibits TR by recruiting co-repressors through a non-genomic mechanism (Sheng et al., 2012). Additionally, prenatal exposure to 20ug/kg BPA per day has been shown to disrupt normal neocortical development by accelerating neuronal differentiation and migration (Nakamura et al., 2006). In a mother-child pair study in humans, maternal urinary BPA concentrations across two time points during pregnancy were negatively associated with neonatal thyroid stimulating hormone in males (Chevrier et al., 2013). However, some groups have reported no effects of BPA on thyroid hormone function (Ferguson et al., 2011; Kobayashi et al., 2005). The conflicting results of these experiments indicate a need for more research on BPA's effect on thyroid hormone signaling.

Sexual differentiation of the brain

Sexual differentiation of the hypothalamus is critical because of its role in coordinating the expression of physiological responses and reproductive behaviors with external environmental cues. The high density of steroid hormone receptors within the hypothalamus makes it a sensitive target for the organizational effects sex steroid hormones. The neonatal surge of testosterone in males, which is aromatized to estradiol in the brain, serves to establish sex differences in size, cell number, and expression of steroid hormone receptors in specific nuclei (Davis et al., 1996; Dohler et al., 1982). Within the preoptic area of the hypothalamus, two sexually dimorphic nuclei have been studied as reflection of the hormonal environment during early life: the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the anteroventral periventricular nucleus (AVPV). (See figure 4)

In the late 1970s, researchers identified a group of cells in the medial preoptic area that was substantially larger in male rats, which was designated the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski et al., 1978). They further showed that perinatal exposure to testosterone or estradiol would produce an SDN in females similar in size to males (Gorski, 1985). Neonatal treatment of both male and female rats with tamoxifen, an estrogen receptor antagonist, decreased the volume of SDN-POA (Dohler et al., 1984). Neonatal castration also decreases SDN-POA volume in male rats (Jacobson et al., 1981; Sickel and McCarthy, 2000). Estradiol orchestrates the sexual dimorphism in SDN-POA volume by preventing programmed cell death of neurons in the males during development. Due to the low level of estradiol in the female SDN-POA during development, more neurons die and the nucleus becomes smaller (Tsukahara, 2009). These studies established the importance of the hormonal environment during early life in programming neuronal development.

The SDN may contribute to the regulation of male sexual behaviors and partner preference in rats. Bilateral lesion of the SDN in sexually naïve male rats resulted in decreased male sexual behaviors (ejaculation, mounting, etc.) (De Jonge et al., 1989). Neonatal aromatase inhibitor

treatment decreased SDN-POA volume in male rats, which correlated with demasculinized sexual behaviors and partner preference (choosing to spend time with a female over a male partner) (Brand et al., 1991; Houtsmuller et al., 1994). One study found that the volumes of SDN-POA and AVPV appeared to be predictors of masculine sexual behavior in male rats, as measured by ejaculation. Male rats that were not sexually active had a smaller SDN-POA volume and a larger AVPV volume compared to sexually active males, suggesting incomplete masculinization of these brain areas (Rhees et al., 1999). However, this does not point to an indispensable role of the SDN in expression of mating and sexual behaviors in male rats, as this study is only correlational in nature.

In addition to overall volume of the nucleus, neuronal expression of calbindin is also sexually dimorphic in the SDN of rats and some strains of mice (Edelmann et al., 2007; Orikasa and Sakuma, 2010; Sickel and McCarthy, 2000). Calbindin has also been shown to have neuroprotective effects (Sun et al., 2011; H.-H. Yuan et al., 2013), potentially adding to the neuroprotective actions of estradiol in the male SDN. Similar to overall SDN volume, neonatal castration decreased the volume of calbindin immunoreactivity in the SDN (Calb-SDN) relative to intact males in both rats and mice (Orikasa and Sakuma, 2010; Sickel and McCarthy, 2000). In female rats and C57 mice, neonatal treatment with testosterone or estradiol, but not DHT, can masculinize (increase) the volume of Calb-SDN relative to vehicle treated females (Orikasa and Sakuma, 2010; Sickel and McCarthy, 2000).

Female rats given an antisense oligodeoxynucleotide directed against estrogen receptors prior to administering testosterone prevented masculinizing effects of neonatal testosterone on SDN volume, female sex behavior, and estrogen-induced increases in motor activity (McCarthy et al., 1993). However, a study in C57 mice found that the number of calbindin cells in the SDN in females treated with DHT at birth was not significantly different from untreated females nor untreated males, indicating a slight masculinization (Bodo and Rissman, 2008). Another study also suggested actions of androgen signaling on Calb-SDN cells. In male mice lacking a functioning AR (testicular feminization, Tfm) there were significantly fewer Calb-SDN cells compared to

wildtype males (Edelmann et al., 2007). Taken together, some studies in mice suggest that AR, in addition to estrogen receptor signaling, may be partially responsible for masculinization of Calb-SDN. The preferential investigation of a female stimulus mouse (or female-soiled bedding) over a male stimulus is disrupted in Tfm males and in females injected with DHT at birth, suggesting that AR is important for the differentiation of partner preference behaviors (Bodo and Rissman, 2008, 2007). Neuronal activation in the mPOA as measured by *c-fos* immunoreactivity was also significantly reduced in Tfm males and wildtype females, although it is unclear whether this activation was specific to calbindin neurons (Bodo and Rissman, 2007).

In contrast to the SDN, the female AVPV is nearly twice as large in volume as the male AVPV (Davis et al., 1996). In mice and rats, the female AVPV also contains nearly three times as many neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme involved in the synthesis of dopamine (R.B. Simerly et al., 1985; Simerly et al., 1997). Dopamine neurons in the AVPV are important in regulating the release of pre-ovulatory luteinizing hormone (LH) (Le et al., 2001; Orikasa et al., 2002; R.B. Simerly et al., 1985). Gonadotropin releasing hormone (GnRH) neurons also receive dopaminergic input from the AVPV (Gu and Simerly, 1997; Liu and Herbison, 2013). LH stimulates the ovaries to produce estrogen and progesterone. Estrogen acts in the brain to increase the release of LH, creating a positive feedback loop. Estrogen antagonists infused into the AVPV of female rats blocks the release of LH, resulting in a disrupted estrous cycle, which demonstrates the importance of the AVPV in regulating reproductive functions in females (Wiegand et al., 1978).

The dopaminergic cell population within the AVPV is sensitive to developmental actions of sex steroid hormones. Neonatal testosterone treatment of female rats masculinizes the number of TH neurons in AVPV and abolishes female-typical LH release (Richard B Simerly et al., 1985). Male ERαKO mice have feminized numbers of TH cells in AVPV, whereas males lacking a functional androgen receptor do not differ from wildtype males in AVPV TH neurons (Simerly et al., 1997). In aromatase knockout males (ArKO) the volume and number of neurons in AVPV is

feminized, while ArKO and WT females do not differ from each other (Kanaya et al., 2014). Males lacking ERβ also have increased numbers of TH cells in AVPV and neonatal treatment with receptor-specific agonists suggests that ERα and ERβ both contribute to the sexual differentiation of the AVPV in mice (Bodo et al., 2006). In contrast to the SDN, estradiol controls the number of TH neurons in the AVPV by promoting cell death (Tsukahara, 2009; Waters and Simerly, 2009). These studies demonstrate the importance of estradiol signaling in masculinizing the AVPV to produce a sexually dimorphic nucleus capable of properly regulating reproductive functions.

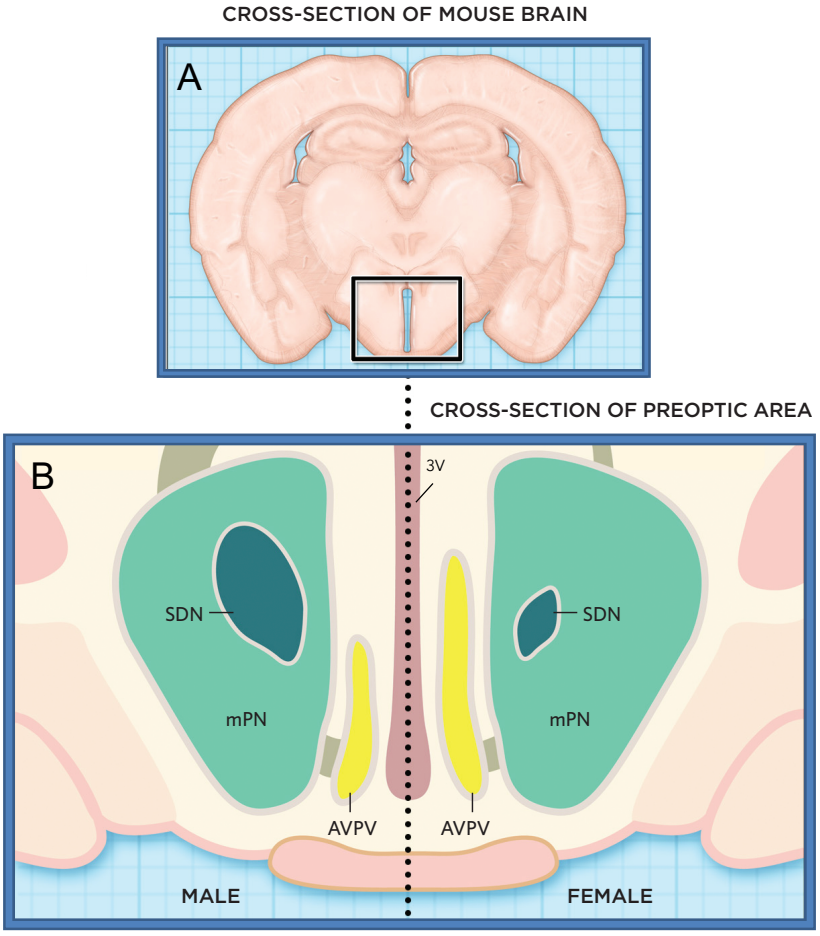

Figure 4: Sex differences in the mouse preoptic area
A) coronal section of the mouse brain, B) The preoptic area surrounding the 3rd ventricle (3V, dark pink) enlarged. On the left side, the male SDN is larger than the female SDN. On the right side, the female AVPV is larger than the male AVPV. [SDN: sexually dimorphic nucleus (dark green), AVPV: anteroventral periventricular nucleus (yellow), mPN: medial preoptic nucleus (light green)] Adapted from (McCarthy, 2015)

Cerebellum development

Sex hormones also influence the development of the cerebellum. The final cytoarchitecture of the cerebellum is achieved approximately 15 days after birth in mice (Chizhikov and Millen, 2003). The expansion of external granule cell layer (EGL) primarily occurs postnatally, which results in cerebellar foliation (Millen et al., 1994). Estrogen receptors α and β reach their peak expression during this time (Belcher, 1999; Ikeda and Nagai, 2006) and they likely contribute to differentiation and maturation of neurons in the cerebellum (Jakab et al., 2001). Locally synthesized estradiol is important for circuit formation, neural growth, and spinogenesis (Hoffman et al., 2016). Purkinje cells, the sole output cells of the cerebellum, express aromatase and synthesize estradiol *de novo* during postnatal cerebellar development (Sakamoto et al., 2003).

In aromatase knockout mice (ArKO), or in mice treated with tamoxifen, purkinje neurons exhibit less dendritic growth and spinogenesis, which is mediated by BDNF (Haraguchi et al., 2012; Sasahara et al., 2007). Sex differences in electrophysiological properties of purkinje neurons also emerge during this critical period and require local synthesis of estradiol (Dean et al., 2012). ERβ is the primary ER in adult cerebellum and is responsible for the rapid effects of estradiol on synaptic plasticity and motor learning in adults (Andreescu et al., 2007). The cerebellum also expresses AR and its levels are regulated by testosterone in males (Perez-Pouchoulen et al., 2016).

BPA and animal behavior

Anxiety

Human epidemiological data supports a role of early-life BPA exposure in the modulation of various behaviors. Although several human studies have shown effects of BPA effects on anxiety-like and depressive behaviors, the results in animal models are somewhat variable. Researchers use well-characterized behavioral tests to assess the anxiety-like behavior of rodents. Most assays are based on a conflict between a rodent's natural inclination to be in a dark, protected

environment or to explore a novel area that is also open, bright, and vulnerable. The elevated plus maze (EPM) consists of two open and two closed arms raised above the floor. The closed arms have high walls, whereas the open arms do not have walls and are therefore unprotected and open to the environment. Similarly, the light-dark box (LDB) has one dark side and one light side. Spending more time in the closed arms or dark side of the box and less time in the open arms or light side of the box classically represents "anxiety-like" behavior. The EPM has been validated as a test of anxiety behavior in mice and rats (Espejo, 1997; Lister, 1987; Pellow et al., 1985). Researchers may also use the open field test to assess whether a rodent in an open arena spends time at the corners and edges or the exposed, center of the arena. Similar to EPM and LDB, rodents that spent more time in the unprotected area of the apparatus (the center) are considered to be less "anxious".

Using these assessment methods, many groups have reported BPA-induced anxiety phenotypes. Developmental exposure to BPA is associated with increased anxiety like behavior in mice (Cox et al., 2010; Gioiosa et al., 2013, 2007; Kundakovic et al., 2013; Matsuda et al., 2012; Ryan and Vandenbergh, 2006; Tian et al., 2010; Xu et al., 2012) and in rats (Kubo et al., 2003; Patisaul et al., 2012; Patisaul and Bateman, 2008; Poimenova et al., 2010; Zhou et al., 2015). Contrastingly, two studies report that BPA decreases anxiety-like behavior (Chen et al., 2015; Tian et al., 2010). The studies vary based on BPA dose, exposure timing, sex of animals, and behavioral assay.

Social behavior

Neurobehavioral analysis is commonly used to evaluate mouse models of ASD, as well as other neurodevelopmental disorders. Researchers can utilize behavioral tasks in mice to approximate core human symptoms of ASD: social deficits, communication problems, and perseverative/stereotyped behaviors. Social behaviors are of particular interest considering BPA's connection to disrupted social behaviors in children. Models of social behaviors in rodents are

effective because mice are a highly social species (e.g., investigating an unfamiliar conspecific, making communal nests, exhibiting maternal care of pups, and showing play behaviors) (Crawley, 2007).

The social behavior circuit depends on brain area-specific expression of the neuropeptides oxytocin (OT) and vasopressin (AVP). OT and AVP are primarily synthesized in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. These neurohormones are either released into the general circulation through the posterior pituitary gland, or signal to other brain areas as a neurotransmitter. In general, oxytocin controls social motivation and social approach, whereas vasopressin is involved in "male-typical" social behaviors like scent marking and aggression (Lim and Young, 2006). Social stimuli are sensed by smell, which activates the olfactory bulb, sending signals to the piriform cortex, and relaying though the medial amygdala to the preoptic area of the hypothalamus (POA) and bed nucleus of the stria terminalis (BNST) (Wacker and Ludwig, 2012). Although social behavior is a complex construct, several simple behavioral tasks can encompass many different aspects of social behaviors. There are several components: motivation to approach and interact with another individual, the ability to identify social signals and cues in order to recognize that individual, and finally, to form a social memory (Lim and Young, 2006). Three behavioral assays are described below: (1) social preference, (2) social recognition, and (3) social interactions.

The *social preference* test consists of two phases. In the "sociability" phase, the test animal must choose between a novel object and a social stimulus (an unfamiliar conspecific). In "discrimination" phase, the animal has to distinguish between a now familiar stimulus mouse and a novel stimulus mouse. The *social recognition* task is somewhat similar to social preference, but is a more sensitive test of social memory. The task consists of two phases: habituation and dishabituation. In the habituation phase, the test animal is repeatedly exposed to the same stimulus animal for several trials and the test animal gradually decreases its investigation of the stimulus animal. However, in the dishabituation phase, a novel stimulus animal is introduced and the test

mouse increases its investigation, indicating that the test animal recognizes that this is a new stimulus animal. Functioning oxytocin and vasopressin signaling is a molecular prerequisite for this test. Oxytocin knockout mice exhibit "social amnesia" and do not show habituation or dishabituation in a social recognition task. The effect can be rescued by infusing oxytocin into the ventricles of the brain, showing that oxytocin is necessary for social recognition (Ferguson et al., 2000). Similarly, vasopressin receptors in the lateral septum are essential for this behavior. V1aR transgenic knockout mice are deficient in the test, but can be rescued by expression of V1aR by viral vector in the lateral septum (Bielsky et al., 2005). This demonstrates the necessity and sufficiency of the receptor in mediating this aspect of social behavior.

The *social interaction* task is an open-ended, dyadic behavioral assessment. Two individuals of the same treatment group, age, and sex spend the trial together in a neutral cage. The researcher scores each of their social interactions and non-social behaviors during the trial. Functioning oxytocin and vasopressin signaling is a molecular prerequisite for this test. Oxytocin receptor (OTR) and vasopressin receptor (V1aR) expression in the amygdala are regulated by ERα and associated with increased social interactions in mice (Murakami et al., 2011). Oxytocin and vasopressin mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) are regulated by ERβ and also positively correlated with social interactions (Murakami et al., 2011). Additionally, extra-hypothalamic AVP projections are androgen dependent in mice (Tejada and Rissman, 2012). Therefore, differences in early-life sex steroid hormone levels can shape the sex-specific social behaviors.

BPA can dysregulate these normal social functions, potentially by altering sex hormone signaling (Porrini et al., 2005). Early-life exposure to BPA has also been shown to affect social behaviors in rats (Dessì-Fulgheri et al., 2002; Porrini et al., 2005), mice (Kundakovic et al., 2013; Wolstenholme et al., 2013, 2012, 2011a), zebrafish (Weber et al., 2015), prairie voles (Sullivan et al., 2014), and monkeys (Negishi et al., 2014). Prenatal exposure to 200 µg BPA/kg bodyweight in

BALB/c mice reversed sex differences in time spent sniffing during the social interaction task in postnatal day 70 offspring (Kundakovic et al., 2013). Low doses of BPA are also associated with altered social interactions in rats (Dessì-Fulgheri et al., 2002). Offspring of either high or low doses of gestational BPA were tested on post-natal day 35, 45, and 55. The researchers measured behaviors from a comprehensive list of seven non-social and 18 social behaviors, which were then grouped into eight different factors for analysis. Early-life BPA exposure attenuated sex differences in non-sexual social behaviors or "social interest". Control males engaged in more "sociosexual exploration" than control females (ex. genital sniffing). Low-dose and high-dose BPA males showed significantly fewer sociosexual behaviors than male controls, whereas low-dose BPA females displayed a slight increase in this group of behaviors compared to control females. This indicates a sex and dose-dependent effect of BPA exposure in juvenile rats: a demasculinizing effect in males and a slight masculinizing effect in females (Dessì-Fulgheri et al., 2002).

The Rissman lab has published several studies reporting significant effects of gestational BPA on various social behaviors in juvenile offspring. Gestational BPA exposure affected several social interaction behaviors such as self-grooming, nose-to-nose sniffing, social approach, and side-by-side interactions (Wolstenholme et al., 2011b). We have also reported significant decreases in side-by-side interaction and anogenital investigations as well as increased side-by-side sitting and frequency of play solicitations in BPA males and females compared to controls (Wolstenholme et al., 2012). In the social preference task, when given a choice between interacting with a novel conspecific or investigating a novel object, males spent more time investigating the stimulus animal, whereas females spent less time. There was a clear sex difference in this task, but no diet effects (Wolstenholme et al., 2011b). However, in a separate study, we detected significant effects on social preference behaviors due to gestational BPA exposure at a higher dose (Cox et al., 2010). Interestingly, in the social recognition task BPA-exposed juveniles fail to show a rapid habituation to the familiar animal and spend significantly more time sniffing in trials 1-8 than controls.

However, BPA males and females do dishabituate to the novel stimulus female on the ninth trial (Wolstenholme et al., 2013; and unpublished results).

Data from our lab show significant gene expression changes in whole brains of exposed offspring on embryonic day 18.5. *Esr1* expression is significantly lower in F1 BPA brains of both sexes. There is an accompanying decrease in *Avp* expression and a trend for a sex by diet interaction in *Oxt* expression in BPA F1 animals. We have also shown a sex-specific decrease in oxytocin receptor (Oxtr) expression in whole brain associated with a lower dose of BPA (Wolstenholme et al., 2011b). In a dose-response study, BPA dose-specific changes in expression of *Esr1* and *Esr2* in the prefrontal cortex and hypothalamus of exposed mice, which were also accompanied by significant alterations to social behavior (Kundakovic et al., 2013). Contrastingly, gestational exposure to 2.5, 250, and 25000 µg/kg BPA increased *Esr1* expression in neonatal hypothalamus specifically in female rats. *Oxt* expression was also increased in 2.5 and 250 µg exposure groups in neonatal female hypothalamus and 25 µg BPA exposure in neonatal male hypothalamus (Arambula et al., 2016). Taken together, these data indicate that gestational BPA exposure is associated with dose and species-specific expression changes in genes related to regulation of social behavior.

Maternal behavior

Several studies have indicated that BPA exposure during can affect maternal behavior. Female rats exposed to 40 µg BPA/kg bodyweight during pregnancy and lactation decreased arched-pack nursing ("active" nursing) postures as well as licking and grooming behaviors of their pups (Della Seta et al., 2005). CD-1 female mice exposed to 10 µg BPA/kg bodyweight during pregnancy spent less time nursing their pups and more time engaged in non-maternal behaviors out of the nest than control dams. BPA also affected these maternal behaviors in F1 offspring who were exposed *in utero*. However, maternal behavior was unaffected in females exposed to BPA gestationally as well as during their own pregnancies (Palanza et al., 2008, 2002). In BALB/c mice exposed to a range of doses during pregnancy, the highest dose (200 µg/kg) *increased* licking,

grooming, and arched-back nursing, indicated that these behaviors may be dose- and strain-dependent (Kundakovic et al., 2013). One study in non-human primates described altered sexually dimorphic mother-infant interactions associated with BPA exposure, such as decreased clinging behavior and increased "outward-looking" behaviors in male infants with their mothers (Nakagami et al., 2009). It is hypothesized that some of the maternally transmitted molecular and behavioral effects of BPA are due to changes in maternal behavior, which then secondarily affect the behavior of the offspring (Cummings et al., 2010). Natural variations in licking and grooming behavior in rat dams are associated with higher licking and grooming behavior of their own female offspring as well as significant differences in stress reactivity in offspring (Champagne et al., 2003; Meaney, 2001). This maternal transmission of behaviors appears to be mediated by epigenetic regulation, such as modifications in DNA methylation (Champagne and Curley, 2009). In fact, the study by Kundakovic and colleagues reported that maternal care did mediate changes in dominance behaviors in a social interaction task associated with BPA exposure (Kundakovic et al., 2013).

Transgenerational effects

Epigenetic regulation refers to heritable changes that alter gene expression without changing the DNA sequence. These alterations occur through regulation of the shape and conformation, not to the sequence of nucleotides, thereby changing the accessibility of DNA to gene transcription machinery. Modifications can be made to the properties of the DNA strand itself (i.e. methylation of cytosines) or to proteins that interact with DNA, such as post-translational modifications of histones (Crews, 2011). Environmental factors can affect epigenetic modulations, thereby creating an interface between the environment and control of gene expression (Skinner et al., 2011).

This interaction represents an intriguing mechanism by which environmental endocrine disrupters can affect behaviors and gene expression across generations. Researchers are now asking whether the effects of early-life exposure to EDCs can persist across generations. In addition to the

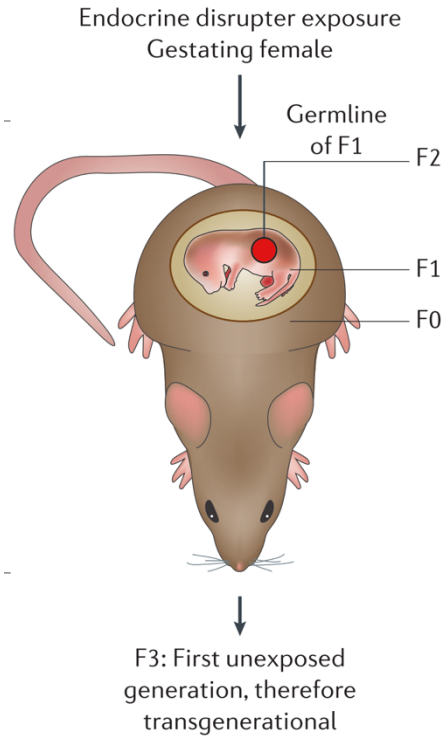

Figure 5: Transgenerational inheritance The F0 generation female is exposed to an EDC during gestation, thereby exposing the F1 (embryos), and F2 (developing germ cells) generations as well. The F3 generation has had no direct exposure to the EDC. From (Skinner, 2015)

first-generation offspring directly exposed to BPA *in utero* (F1 generation), the developing germ cells that eventually produce the second generation are also exposed (F2). However, the third generation removed from the initial exposure (F3) has had no contact with the exposure and therefore represents a transgenerational effect. (See Figure 5)

We have previously observed significant behavioral effects of transgenerational exposure to BPA. F2 and F4 generation mice descended from a BPA lineage engaged in more social behaviors and spent less time exploring alone during the social interaction task (Wolstenholme et al., 2012) (See Figure 6). We also found a sex-specific effect of ancestral BPA exposure on social recognition. In F3 mice, the altered habituation pattern of observed in F1 generation offspring is sustained, but the F3 BPA offspring also fail to recognize the novel female in trial 9. An odor discrimination test during adulthood revealed normal olfactory functioning and therefore does not explain these deficits in social memory (Wolstenholme et al., 2013).

Our data also showed that BPA can affect neuronal gene expression transgenerationally. Similar to F1 exposure, *Avp* and *Oxt* remained significantly decreased in embryonic whole brain from F4 BPA mice compared to controls (Wolstenholme et al., 2012). However, unlike the F1 generation, we found no significant changes in estrogen receptor expression in the F4 generation embryos. Since F4 generation mice had never been exposed to BPA, neither *in* utero nor as germ

cells, we hypothesized that the behavioral and molecular changes observed could be transmitted through a transgenerational epigenetic mechanism.

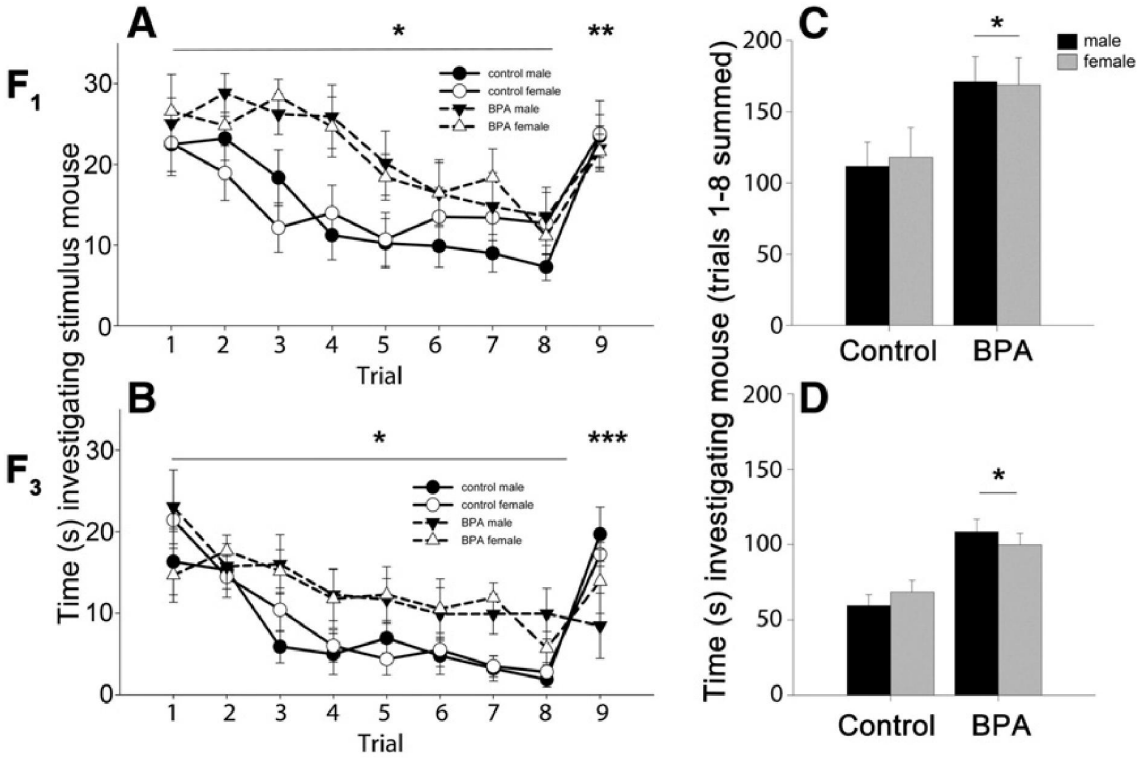

Figure 6: Transgenerational effects of BPA on social recognition in mice
Panels A and B: Mean±SEM time in seconds investigating the OVX stimulus female. A) F1 generation juveniles directly exposed to BPA, B) F3 generation juveniles from control or BPA lineages. Panels C and D: Mean±SEM time in seconds investigating stimulus mouse summed across trials 1-8. C) F1 generation mice, D) F3 generation mice. *Significant main effect of BPA exposure, **Significant difference between trial 8 (familiar mouse) and 9 (novel mouse), ***Significant effect of BPA lineage, only controls significantly increase investigation between trials 8 and 9.
Figure from (Wolstenholme et al., 2013)

BPA has been implicated in epigenetic regulation of gene expression. In the viable yellow agouti mouse (A^{vy}), BPA diet given during pregnancy skewed the coat color distribution of offspring towards yellow by decreasing CpG (cytosine-guanine dinucleotide) methylation in a contra-oriented intracisternal A particle (IAP) retrotransposon upstream of the Agouti gene, which is typically highly methylated (Dolinoy et al., 2007a). However, methyl donors like genistein (a phytoestrogen) or folic acid fed during pregnancy negated the hypomethylation effect of BPA and

shifted the coat color distribution towards pseudo-agouti (Dolinoy et al., 2007b). Others have also reported hypomethylating effects of BPA (Kundakovic et al., 2013; Monje et al., 2007; Yaoi et al., 2008; Yeo et al., 2013). Some studies have shown that DNA methylation mediates the effects of endocrine disrupting compounds like BPA in the first generation (Kitraki et al., 2015; Kundakovic et al., 2013) and across multiple generations (Jang et al., 2012). Other groups have also reported transgenerational effects of BPA exposure on behavior and physiology, but did not test whether DNA methylation was involved (Bhandari et al., 2015; Ziv-Gal et al., 2015). However, both proposed that epigenetic mechanisms were likely at play.

In conclusion, the endocrine disrupting compound BPA has widespread effects on behavior, physiology, and the brain. Epidemiological studies have established that statistically significant associations exist between early-life exposure to BPA and human behaviors related to neurodevelopmental disorders. Transgenerational studies in animal models have also demonstrated some of these effects can be transmitted across generations, impacting individuals that were never directly exposed to BPA. It remains uncertain which molecular targets of BPA are the most important and relevant in the changes observed. However, genetic and epigenetic factors clearly affect the outcomes of brain development and result in permanent alterations of behavior and physiology in late life. The studies that follow attempt to address the gaps in our current understanding of the relationship between BPA, behavior, and the brain as well as the function of calbindin, a gene important for regulating neuronal functioning.

Chapter II

Effects of maternal or paternal bisphenol A exposure on offspring behavior

Introduction

Endocrine disrupting chemicals (EDCs) interfere with the synthesis, secretion, transport, binding, and/or action of endogenous hormones (Gore et al., 2015). Bisphenol A (BPA) is a an EDC commonly used in the production of polycarbonate plastics and epoxy resins (Michałowicz, 2014). Human exposure to BPA is widespread: the CDC reports detectable levels of BPA in over 93% of human urine samples (CDC (Centers for Disease Control and Prevention, 2009). BPA is also detected in serum, amniotic fluid, umbilical cord blood, and breast milk (Cao et al., 2015; Ikezuki et al., 2002). BPA is primarily considered a xenoestrogen, capable of binding to the receptors of endogenous estrogens (Kurosawa et al., 2002; Matthews et al., 2001). However, BPA can also bind to various other receptors and proteins to disrupt the functions of thyroid hormone (Chevrier et al., 2013; Moriyama et al., 2002), testosterone (Tanaka et al., 2006; Xu et al., 2005), and glucocorticoids (Poimenova et al., 2010). Hormones play an important role in shaping the developing brain, so it is crucial to understand how gestational exposure to EDCs, like BPA, can affect neurodevelopment and behavior in later life.

Studies in humans and animal models have demonstrated associations between gestational exposure to BPA and adverse neurobehavioral outcomes (Mustieles et al., 2015; Palanza et al., 2016). Higher concentrations of BPA measured in urine during pregnancy have been correlated with sex-specific alterations in anxiety, aggression, hyperactivity, and externalizing behaviors in humans (Braun et al., 2009; J. M. Braun et al., 2011; Harley et al., 2013; Perera et al., 2012). Environmental factors, such as EDC exposure, have been implicated in the pathogenesis of neurodevelopmental disorders, like autism spectrum disorder (ASD) (Thaddeus T Schug et al., 2015). Two studies found that urinary concentrations of BPA metabolites in children with ASD are higher than in typically developing children (Kardas et al., 2015; Stein et al., 2015).

Similar outcomes have been reported in models of developmental BPA exposure in animals. Several groups have described differences in anxiety-like behavior (Chen et al., 2015; Gioiosa et al., 2013; Xu et al., 2015; Zhou et al., 2015), and motor activity (Anderson et al., 2013;

Komada et al., 2014; Zhou et al., 2011), in response to developmental BPA exposure. Some studies report impairments in learning and memory (Kumar and Thakur, 2014; Tian et al., 2010; Xu et al., 2013a), while others do not find any effect of BPA on spatial learning (Sadowski et al., 2014). Other types of learning, such as acquisition and reversal in an operant learning task have not been explored. Early-life exposure to BPA has also been shown to affect social behaviors in rats (Dessì-Fulgheri et al., 2002; Porrini et al., 2005), mice (Kundakovic et al., 2013; Wolstenholme et al., 2013, 2012, 2011a), prairie voles (Sullivan et al., 2014), and monkeys (Negishi et al., 2014). In our previous studies, we found significant effects of gestational exposure to BPA on social interactions, social preference, and social recognition behaviors in juvenile mice compared to controls (Wolstenholme et al., 2013, 2012, 2011b).

These studies focused on the effects of BPA during gestation or during early postnatal life. Growing evidence supports the notion that sires can transmit the effects of environmental exposures to their offspring, likely via sperm (Kundakovic and Champagne, 2015). For example, paternal exposure to chronic stress affects stress physiology and behavior in offspring (Rodgers et al., 2013), and these effects appear to be mediated by epigenetic mechanisms (Rodgers et al., 2015). Additionally, paternal exposure to BPA in zebrafish disrupts cardiac development in offspring (Lombó et al., 2015). While the negative reproductive consequences of adult exposure to BPA in males have been fairly well-studied (decreased spermatogenesis, lower sperm counts, and reduction in pregnancy rates) (Dobrzyńska et al., 2015; Jin et al., 2013; Liu et al., 2013; Qiu et al., 2013), it remains unclear how preconception exposure to BPA via the sire affects offspring behavior. Only one study has reported behavioral effects of preconception paternal BPA exposure: offspring had impaired spatial memory in the Morris water maze test (Fan et al., 2013).

In the current study, we expanded on previous studies by including a paternal exposure group. We administered BPA to the parents via daily treats, which avoids the stress related to oral gavage. Paternally exposed and maternally exposed offspring were tested for social recognition and anxiety-like behavior on the elevated plus maze as juveniles. In maternally exposed mice only, we

examined two previously unreported behavioral endpoints in offspring: ultrasonic vocalizations (USVs) in pups and associative learning/perseverative behaviors in an operant reversal learning task (Heyser et al., 2000). The behavioral tests in this study were chosen to address the three core symptoms in ASD: communication difficulties, impaired social interactions, and perseverative behaviors (Crawley, 2007).

Materials and Methods

Animals

The mice were generated in our C57BL/6J breeding colony at the Biological Resources Facility at NC State University (Raleigh, NC, USA). The progenitor mice were purchased from Jackson Labs (Bar Harbor, ME). All animals were maintained on a 12:12 light/dark cycle (lights off at 1200) and provided food (soy-free Teklad 2020X; Madison, WI) and water ad libitum. The Institutional Animal Care and Use Committee at NC State University approved all procedures.

Exposure of Breeders to BPA

Mice received BPA daily on a small chocolate-flavored treat that weighed approximately 0.5 g (Bio-serv chocolate treats F05472; Flemington, NJ). BPA (>99% purity, Sigma-Aldrich; St. Louis, MO) was initially dissolved in 100% ethanol, and then diluted in ethanol to a concentration of 2 mg/mL. Ten μL of this solution was pipetted onto the treat for a final dose of 20 μg BPA per day. For control treats, the ethanol solution contained no BPA. The ethanol was allowed to evaporate from the treats overnight. This dose was chosen based on the amount of BPA consumed per day using a custom diet with BPA incorporated into the chow (5 mg/kg diet BPA, Teklad 09386). Free BPA levels in the plasma of pregnant dams consuming the BPA diet averaged 3.9 ng/ml (Wolstenholme et al., 2012), which is within the range (0.3–4.0 ng/ml) reported in pregnant women (Schönfelder et al., 2002). Males and females were approximately 3 months of age when dosing

began and had no prior breeding experience. For 5 days prior to dosing, all mice received a plain treat to acclimate them to the novel food. By the end of this period, all mice immediately approached and consumed the entire treat.

Maternal exposure

For maternal BPA exposure, singly housed females were randomly assigned to the control group (n=15) or BPA group (n=18). Dosing began one week prior to mating; treats were given one hour after lights out each day. Each female was paired with a naïve male (no exposure to treats) for six days. Males were briefly removed from the cage each day when the treat was presented to ensure the female consumed the entire treat (about 10 minutes). Daily dosing of the dam continued through gestation. Cages were checked for litters, and dosing ended on the day of birth.

Paternal exposure

To expose sires to BPA, males were randomly assigned to the control group (n=8) or the BPA group (n=12) and received a daily treat for 50 days. We chose this length of exposure in order to cover one cycle of spermatogenesis. Beginning on day 51, each male was paired with a naïve female (no exposure to treats). Females were checked daily for the presence of a mating plug. Males remained with females for up to seven days, or until a plug was observed, at which time the male was weighed, euthanized using CO_2, and the testes, seminal vesicles, and epididymis were weighed. The caudal epididymis was reserved for sperm collection (procedure below).

For both maternal and paternal exposure studies, all litters were culled to 6 pups with a balanced sex ratio on the day after birth (postnatal day 1, P1). Litters were weaned on P21 and housed in same-sex, same-treatment groups. No more than one mouse of each sex per litter was used in each behavior test.

F0 male sperm collection and counting

Sperm was collected from males used in the paternal exposure study, on the day the mating plug was detected. No mating plug was detected for one control male and two BPA males and their

sperm counts were removed from the analysis. Briefly, the caudal epididymis was removed, placed in a petri dish containing 500 μL of warmed PBS and minced. The dish containing the minced sample was incubated at 37°C for 15 minutes to allow sperm to swim out of the epididymis. After incubation, the remaining sperm was gently extruded from the caudal epididymis. The solution was aspirated from the dish with a wide bore 200 μL pipette tip and placed in a 1.5 mL centrifuge tube. The dish was rinsed with an additional 500 μL of warm PBS, which was then recovered and added to the same tube. 10 μL of the sperm solution was loaded into a hemocytometer and counted. All cell counts were performed in triplicate and the average was used for statistics (Wang, 2003).

Social recognition

Each juvenile (postnatal day 28) was acclimated to a test cage (37 x 19 x 13 cm) for 20 minutes under red lights one hour after lights off. An empty cylindrical metal holding cell (10 cm diameter X 14 cm tall) was placed in the test cage for the last 10 minutes of the acclimation period. The social recognition test consisted of two phases: habituation and dishabituation, as previously described (Wolstenholme et al., 2013). During each one-minute trial, the time the juvenile spent investigating the stimulus mouse was measured. Investigation was defined as the nose of the test mouse within 1 cm of the head or body of the stimulus animal or directly touching the bars of the cylinder. Investigation was scored live during the test by an investigator blind to the treatment and sex of the mice. We tested 24 control mice (6 males and 6 females from the maternal exposure study and 7 males and 5 females from the paternal exposure study). We tested 12 maternally exposed BPA mice (6 males and 6 females) and 18 paternally exposed mice (9 males and 9 females).

Elevated plus maze

Juvenile (P28-32) mice were habituated to the dark testing room for one hour under red lights. Each mouse was gently placed in the center of the elevated plus maze (Columbus Instruments,

Columbus, OH; wall height: 15.25 cm, arm length: 30 cm, arm width: 5 cm, height above ground: 32 cm) facing an open arm and recorded for 5 minutes. A trained observer blind to treatment groups scored the time spent in each area of the maze (closed arms, open arms, center area) and the number of crosses between each area (defined as all four paws within the area) the video using Noldus Observer (Leesburg, VA). We tested 28 control mice (7 males and 7 females from maternal exposure study and 7 males and 7 females from paternal exposure study). We tested 18 maternally exposed BPA mice (7 males and 11 females) and 16 paternally exposed BPA mice (8 males and 8 females).

Ultrasonic vocalizations

On P8, dams and their litters were moved to the testing room 30 minutes after lights-off and habituated there for one hour. After habituation, we tested two randomly selected pups (one of each sex) from each litter. Individual pups were placed in a small cup below an ultrasonic microphone (Avisoft-Bioacoustics CM16/CMPA; Glienicke, Germany) in a sound-attenuating chamber (Med Associates ENV-022S; Fairfax, VT). Ultrasonic vocalizations were recorded for 5 minutes. The tail of each pup was marked with a sharpie, to differentiate which pup had been recorded, and returned to the rest of the litter. Five minutes after the first recording, the pup was removed again recorded for an additional five minutes. This paradigm is referred to as maternal potentiation (Scattoni et al., 2009). After the second recording, the pup was anesthetized with isoflurane then euthanized by decapitation. We recorded vocalizations from 8 control pups (5 males and 3 females) and 10 maternally exposed pups (5 males and 5 females).

To analyze ultrasonic vocalizations we recorded USVs up to 200 KHz and analyzed using published methods (Young et al., 2010). The raw signal was cleaned by first filtering with a finite impulse response filter then performing spectral subtraction. In spectral subtraction the average of the noise in each frequency band is subtracted from the sound (R. C. Liu et al., 2003). A sound envelope calculated using this "cleaned" sound was then passed through a thresholder to detect

putative mouse calls. The sound files were thus segmented into two categories: putative calls and regions where no call was detected. A trained experimenter used a custom-made interface to ensure that all the putative calls were correctly identified and none were missed by the thresholding algorithm. We assessed for the number of calls, call duration, and call median frequency. Call median frequency was calculated by examining the distribution of frequencies contained in a call. For each call, all frequencies in each time segment were counted in the frequency distribution if the power in that frequency and time bin exceeded 3 standard deviations above the mean noise level. The median of this distribution was then defined as the median frequency of the call. A total of 6,582 calls were analyzed. We also analyzed the number of bursts, which refers to a group of calls that are separated from another group of calls by an interval in time that is statistically longer the mean time between individual calls. Bursts were detected by defining a threshold on the inter-call intervals.

Operant reversal learning

At 15 weeks of age, one male and one female from each litter of maternally exposed offspring were weighed and paired with a same-sex, same-exposure partner from a different litter based on body weight. These matched pairs were housed together. To motivate responding in the operant task, animals were food-restricted to 85% of their initial body weight. Animals were weighed daily and each pair was given a measured amount of food to maintain the desired weights. To habituate to the reward pellets, mice were given reward pellets (Dustless precision pellets, 14 mg each [F05684]; Bioserv) in their cages for several days before the beginning of the training.

The 5-nose poke hole operant conditioning apparatus (Med Associates MED-NP5M-B1) was housed inside a ventilated, sound-attenuating chamber. Each session in the testing chamber lasted fifteen minutes and took place during the dark cycle. The testing schedule consisted of five consecutive days of training sessions followed by one day off.

During the habituation phase, none of the nose-poke holes were illuminated and no rewards could be earned. Habituation trials continued for three days, or until the mouse poked fewer than 10 times in any one hole, in order to ensure low baseline levels of nose poke activity. During the training phase, two of the five holes were illuminated. One illuminated hole was designated the "active" hole and the other illuminated hole was the "inactive" hole. Only a nose poke in the active hole triggered the release of a pellet into the hopper. The location of the active hole remained the same for each mouse across training sessions. A fixed-ratio schedule determined the number of active hole responses required for a reward pellet. For fixed-ratio 1 (FR1), one active hole response elicited one reward. For fixed-ratio 3 (FR3), three active hole responses were required for one reward, etc.

All animals were trained on an FR1 schedule for 7 days. FR3, FR5, and FR10 sessions each lasted for five days and FR15 was 10 days. Finally, during the reversal phase, the positions of the active and inactive holes for each mouse were reversed at an FR10 schedule for 8 sessions. During reversal, responding in the previously active hole resulted in no rewards, whereas 10 responses in the previously inactive hole was rewarded. Nose-pokes in the hopper and all holes (active, inactive, and unlit) were recorded during each session. The percent accuracy was calculated for each session: active responses / total (active responses + inactive responses) x 100% (Heyser et al., 2000). We tested 12 control mice (6 males and 6 females) and 12 maternal BPA mice (6 males and 6 females).

Statistics

All data were analyzed using NCSS software. Pairwise interactions were evaluated by Bonferroni-corrected multiple comparisons tests. The control groups from the maternal and paternal exposure studies were combined for social recognition and elevated plus maze behavior, as there were no statistically significant differences between the two control groups. For social recognition, we analyzed the habituation (trials 1-8) and dishabituation (trials 8 and 9) phases

separately by three-way repeated measures ANOVA. Elevated plus maze data were analyzed by two-way ANOVA with sex and BPA exposure as the two factors.

For ultrasonic vocalizations, data were collapsed across trial and sex as there were no significant differences between trials or sexes. Operant reversal learning data were analyzed by three-way repeated measures ANOVA and separated according to training schedule (FR1 versus reversal). Body weight was used as covariate in the analysis of reproductive organ weights in F0 males. Sperm counts and body weights were analyzed by general linear model.

Results

Social recognition

BPA exposure primarily affected investigation time during the habituation phase of the social recognition task. Investigation of the stimulus mouse decreased significantly across the habituation trials (1-8) ($F(7,432)=56.8$, $p<0.0001$; Figure 1). Exposure group also affected the time spent investigating the stimulus mouse ($F(7,432)=3.3$, $p<0.05$; Figure 1A). Maternally exposed offspring spent more time investigating the stimulus mouse than paternally exposed offspring during the habituation phase. However, neither BPA exposure group was significantly different from the controls. An interaction between sex and trial ($F(7,432)=4.1$, $p<0.001$) revealed that females spent less time investigating a stranger than the males did on trial 1 ($p<0.05$; Figure 1B). A three-way interaction between exposure group, sex, and trial demonstrated that the difference between maternal and paternal BPA exposure was primarily caused by behavior of females, specifically on trial 1 ($F(14,432)=1.81$, $p<0.05$). In the dishabituation trials, social investigation increased in response to the novel stimulus female (trial 9 compared to trial 8) ($F(1,108)=87.8$, $p<0.0001$). However, we noted no other significant effects.

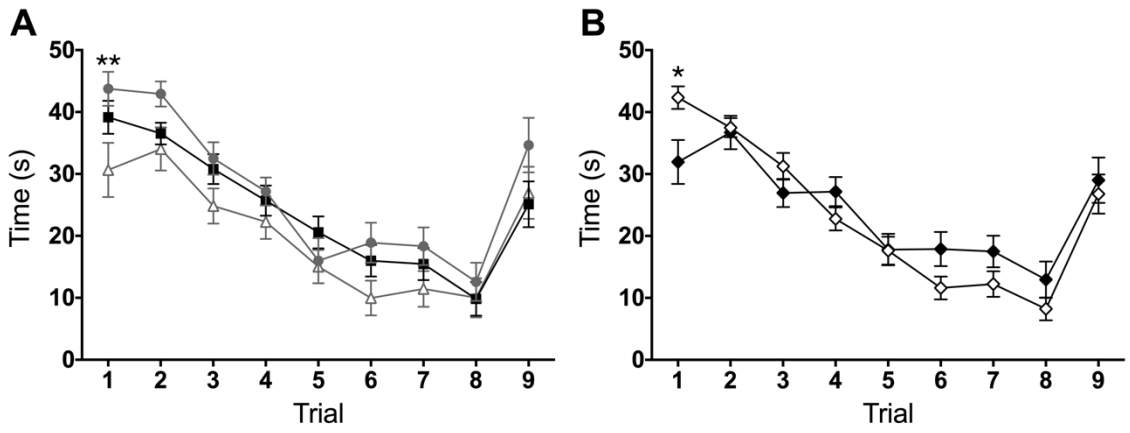

Figure 1: Social recognition
Mean ± SEM time (sec) spent investigating the stimulus mouse A) grouped by exposure. Black squares represent control males and females, gray circles denote maternal BPA males and females, unfilled gray triangles represent paternal BPA males and females. B) grouped by sex. Black filled diamonds denote females, black unfilled diamonds represent males.
** Maternal BPA group is significantly different from paternal BPA group on trial 1, $p<0.05$.
* Significant sex difference on trial 1, $p<0.05$.
Control Males n=13, Control Females n=11, Maternal BPA Males n=6, Maternal BPA Females n=6, Paternal BPA Males n=9, Paternal BPA Females n=9

Elevated Plus Maze

Maternal, but not paternal BPA exposure, increased anxiety-like behavior on the elevated plus maze (EPM). We noted an overall effect of BPA exposure on time spent in the open arms of the EPM ($F(2,61)=6.1$, $p<0.01$; Figure 2A). Juveniles from BPA-exposed dams spent less time in the open arms than control juveniles ($p<0.05$). BPA exposure also affected time spent in the closed arms of the EPM ($F(2,61)=4.1$, $p<0.05$; Figure 2B), and this effect was caused by the difference between maternal and paternal exposure. BPA exposure did not affect time spent in the center of the maze, nor the number of crosses between regions of the EPM ($F(2,61)=3.0, 1.24$; $p>0.05$; Figure 2C and 2D). Sex did not affect any the time spent in any region (open, closed, or center) nor the total number of crosses ($F(1,61)= 1.5, 2.7, 2.1, 0.8$; $p>0.05$).

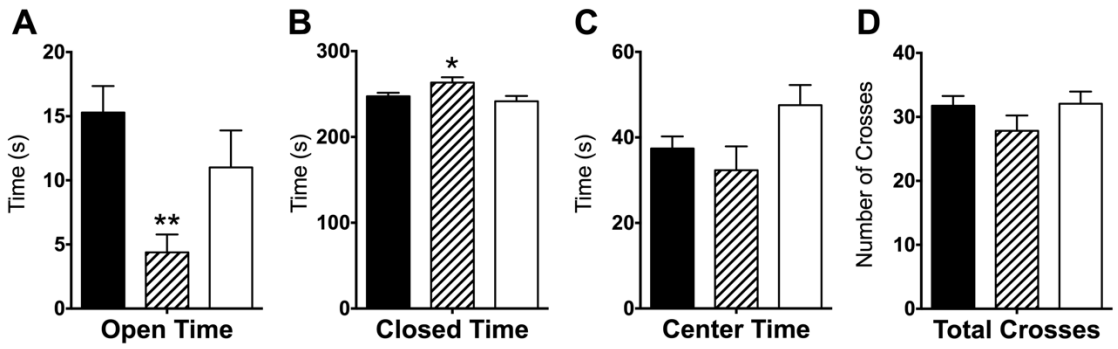

Figure 2: Elevated plus maze
Mean ± SEM of time (sec) spent in A) the open arms B) closed arms C) center portion of the maze and D) total number of crosses through the middle of the EPM.
Bars from left to right: Control (Black), Maternal BPA (White, Diagonal stripes), Paternal BPA (White)
** Maternal BPA exposure group spend less time in the open arms than control mice, $p<0.01$.
* Maternal BPA exposure group spend more time in closed arms than paternal BPA exposure group, $p<0.05$.
Control Males n=14, Control Female n=14, Maternal BPA Male n=7, Maternal BPA Females n=11, Paternal BPA Males n=8, Paternal BPA Females n=8

Ultrasonic vocalizations

We noted no significant increase in the number of calls between the first and second recordings, thus the two recordings for each pup were combined. Gestational BPA exposure significantly increased the median frequency and average duration of ultrasonic calls on postnatal day 8. The distribution of the median frequency (kHz) of calls emitted by BPA-exposed pups differed significantly from the median frequency distribution of control pups (interaction between frequency and exposure: $F(22,414)=1.85$, $p<0.01$; Figure 3A). This was not due to an increase in overall number of calls, as BPA exposure alone did not significantly affect the number of calls ($F(1, 414)=2.68$, $p>0.05$). BPA exposure also shifted the distribution of call durations towards longer durations ($F(10,198)=2.17$, $p<0.05$; Figure 3B). The percentage of call durations at 0.01 seconds (a short call duration) was significantly higher for control pups as compared to BPA pups ($p<0.05$). Pups exposed to BPA *in utero* also tended to have more call "bursts" than control pups [Control: 44.0 ± 11.1, n=8; BPA: 87.2 ± 19.1, n=10] ($F(1,18)=3.5$, $p=0.08$).

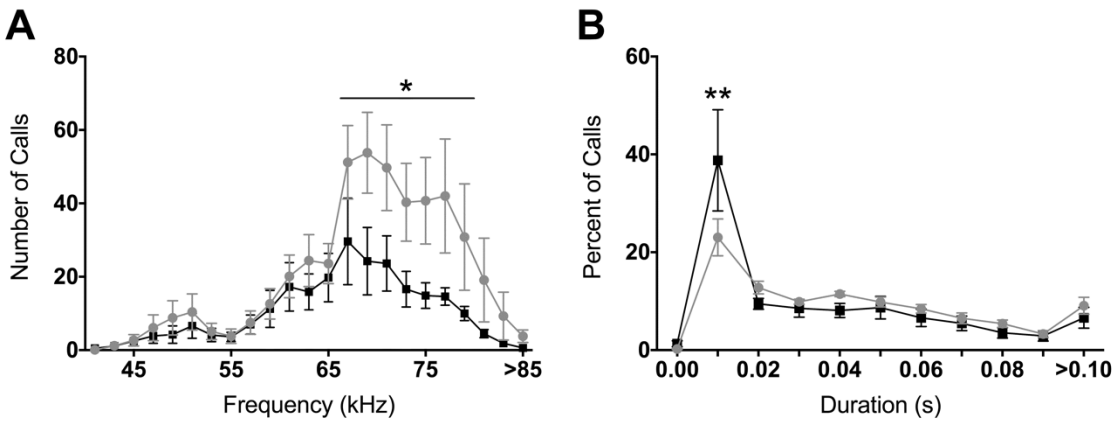

Figure 3: Median frequency distribution and call durations of ultrasonic vocalizations
A) Distribution of individual call median frequencies: Mean ± SEM number of calls per frequency bin (kHz)
B) Relative distribution of call durations, mean ± SEM percent of calls per duration bin.
Black squares represent control group, gray circles represent BPA group.
* Significant interaction between median frequency and BPA exposure, $p<0.01$.
** Control pups display a significantly higher percentage of calls in the 0.01 duration bin than BPA-exposed pups, $p<0.05$.
Control: n=8 (5 males, 3 females), BPA: n=10 (5 males, 5 females)

Reversal learning

There were two primary measures of performance in the reversal learning task: accuracy, measured by the percent of correct responses, and number of rewards received. We noted substantial sex differences in the accuracy of responding (percent correct) and number of rewards throughout the operant reversal learning task. Females were significantly more accurate (Figure 4A) and received more rewards (Figure 4B) than males ($F(1,928)=10.7, 31.8$; $p<0.01, 0.0001$; respectively). Over the first twelve days of training (FR1 and FR3), the percent of correct responses and the number of rewards earned significantly increased ($F(11,288)=16.7, 34.0$; $p<0.0001$, respectively; Figure 5A and 5B). Females responded more accurately ($F(1,288)=7.0$, $p<0.05$) than males. An interaction between sex and trial ($F(11,288)=3.07$, $p<0.001$; Figure 5A) was noted. Females received more rewards than males in FR1 and FR3 ($F(1,288)=68.5$, $p<0.0001$) and again we found an interaction: ($F(11,288)=13.1$, $p<0.0001$; Figure 5B). Interestingly, the number of rewards per session was also affected by BPA exposure ($F(1,288)=4.95$, $p<0.05$) and an interaction ($F(6,288)=1.82$, $p=0.052$). BPA mice earned fewer rewards than controls in FR1 and FR3, and

post-tests revealed that this effect was limited to females ($p<0.05$). Despite earning fewer rewards, there was no effect of BPA exposure on the accuracy of responses in FR1 and FR3 ($F(6,288)=1.22$, $p>0.05$).

During the reversal phase, accuracy and number of rewards received increased over the 8 days of sessions ($F(7,192)=174.4$, 66.81 respectively; $p<0.0001$). Females continued to be more accurate (main effect of sex: $F(1,192)=4.76$, $p<0.05$; Figure 5C) and receive more rewards than males during reversal (main effect of sex: $F(1,192)=11.29$, $p<0.01$; interaction of sex by trial: $F(7,192)=2.27$, $p<0.05$; Figure 5D). However, there were no effects of BPA on accuracy or rewards during reversal (exposure by trial interaction: $F(7,192)=0.35$, 0.97 respectively; $p>0.05$).

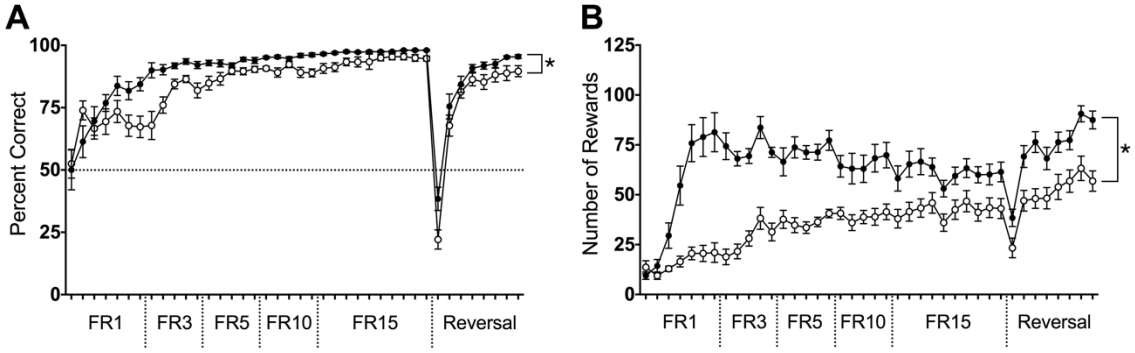

Figure 4: Sex differences in reversal learning
Mean ± SEM of A) Percent correct responses in the active hole B) Number of rewards earned per session. Filled circles represent the females and open circles represent the males. FR1 = fixed ratio 1, etc.
*Significant sex difference across all training sessions, $p<0.001$.
Male n=12 (6 control, 6 BPA); Female n=12 (6 control, 6 BPA)

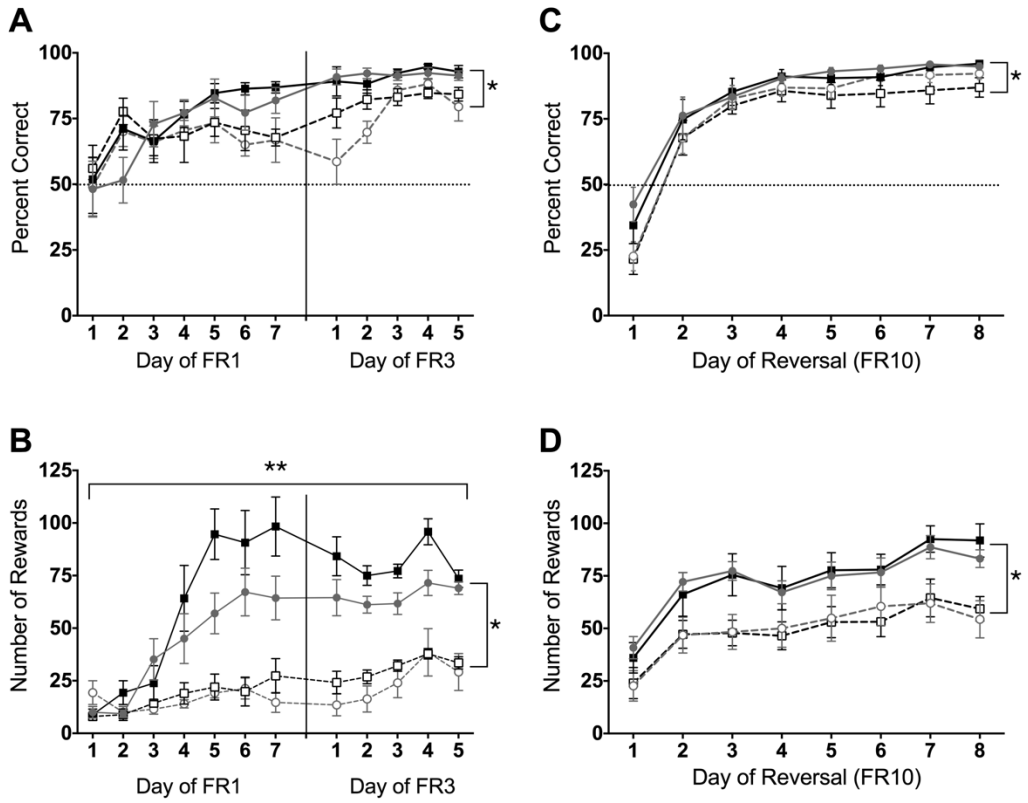

Figure 5: Percent correct and rewards earned during Fixed Ratio (FR) 1 and 3 and reversal sessions
Mean ± SEM of A) percent correct responses in active hole during FR1 and FR3 B) number of rewards earned per session in FR1 and FR3 C) percent correct responses in active hole during reversal D) number of rewards earned per session during reversal
Black squares represent control groups, gray circles represent BPA groups. Filled symbols with solid lines are females; unfilled symbols with dashed lines are males.
Dotted line at 50% in A and C represents chance responding.
* Significant sex difference, $p<0.01$.
** Significant effect of BPA exposure on rewards earned during FR1 and FR3 training, $p<0.05$.
Control Males n=6, Control Females n=6, Maternal BPA Males n=6, Maternal BPA Females n=6

F0 Males

BPA exposure in adult males did not affect reproductive outcomes, but significantly impact body weight. We detected mating plugs in 7 of 8 females paired with control males, but all 8 females paired with control males delivered litters. We found mating plugs in 10 of the 12 females paired with BPA exposed males, and 11 females paired with BPA exposed males delivered litters. Adult males consuming BPA for 50 days weighed significantly less than control males at the time of sacrifice ($F(1,20)=14.9$, $p<0.001$; Table 1). Sperm counts were not significantly affected by BPA

exposure (F(1,17)=0.03, p>0.05; Table 1). After adjusting for body weight as a covariate in the analysis, we found no effect of BPA exposure on the weights of seminal vesicles, testes, or epididymis (F(1,20)=0.68, 0.24, 0.23 respectively; p>0.05, Table 1).

	Control Males			**BPA Males**		
	Mean	±*SEM*	*n*	*Mean*	±*SEM*	*n*
Body Weight (g) *	32.16	0.9	8	27.35	0.82	12
Sperm Counts	2.98E+06	1.84E+05	7	2.94E+06	1.31E+05	10
Seminal Vesicles (mg)	297.7	19.8	8	294.42	13.15	12
Testes (mg)	220.3	5.7	8	214.43	5.44	12
Epididymis (mg)	100.56	15.34	8	88.89	6.88	12

Table 1: F0 male body weights and reproductive organ weights
***** significant effect of BPA exposure on body weight at the time of sacrifice, p<0.001

Discussion

Mouse pups exposed to BPA throughout gestation differed from controls in several behavioral measures reported here. Preconception exposure of the sires to BPA did not change behavior in social recognition or EPM. In comparing the effects of paternal and maternal exposure to BPA, we reported that neither exposure had strong effects on juvenile social recognition. Juvenile mice maternally exposed to BPA spent less time in open arms of the EPM compared to controls, indicating increased anxiety-like behavior. Pups exposed to BPA *in utero* emitted USVs with a higher median frequency distribution and longer duration. We reported a significant sex difference in an operant learning paradigm, and a decrease in the number of rewards earned per session by BPA females compared to control females during training.

Ultrasonic vocalizations

To our knowledge, this is the first report of *in utero* BPA exposure affecting USVs in pups. USVs in rodent pups have been proposed as sensitive behavioral measure in animal models of neurodevelopmental disorders (Scattoni et al., 2009). Mouse pups emit high frequency calls when isolated from the dam and nest as distress signals intended to elicit maternal approach and retrieval (Dirks et al., 2002). Multiple studies in genetic mouse models of neurodevelopmental disorders (*Fmr1*, *Mecp2*, *Foxp2*, etc.) show differences in the number, duration, and types of ultrasonic calls emitted during maternal separation (Lai et al., 2014; Scattoni et al., 2008; Williams et al., 1998, 1995; Young et al., 2010). Pup vocalizations can also be modified by prenatal manipulations like chronic stress, drug administration, and environmental contaminants (Dirks et al., 2002; Mychasiuk et al., 2011; Trezza et al., 2008; Venerosi et al., 2009). In rat pups, the frequency distribution of calls emitted during isolation is indicative of the pup's affective state; stressful stimuli increased the number of higher frequency calls (Ise and Ohta, 2009). The rightward shift in median frequency distribution of BPA exposed pups suggests a heightened sensitivity to stress. This hypothesis is supported by increased anxiety-like behavior in juveniles exposed to BPA. Rats selectively bred across many generations for high levels of USVs during maternal separation demonstrate significantly more anxiety and depressive-like behaviors in later life (Brunelli and Hofer, 2007; Dichter et al., 1996). Several other studies have also shown positive associations between pup USV calling and anxiety/depressive-like behaviors in adulthood (Barua et al., 2014; Trezza et al., 2008; Veronesi et al., 2017). A recent study reported that prenatal exposure to polychlorinated biphenyls (PCBs) significantly affected USVs produced by rats during affiliative interactions in adolescence and sociosexual interactions in adulthood (Bell et al., 2016). Future studies should address whether gestational BPA exposure alters USVs emitted during juvenile and adult social interactions.

Reversal learning

Several studies have noted significant spatial learning and memory deficits in rodents developmentally exposed to BPA (Kumar and Thakur, 2014; Sadowski et al., 2014; Tian et al.,

2010; Xu et al., 2013a) as well as non-human primates (Elsworth et al., 2015). However, no studies have indicated whether BPA may affect non-spatial, operant conditioning. In the current study, we examined how gestational BPA exposure impacted operant reversal learning in both sexes in a non-spatial task. We hypothesized that BPA-exposed mice would be more perseverative in the reversal portion of the test than control mice: BPA mice would continue to poke in the previously active hole despite receiving no rewards. However, the main finding was a significant sex difference in the accuracy and number of rewards earned across all training and reversal sessions. Females were significantly more accurate and received more rewards pellets per session than males, regardless of gestational BPA exposure. The significant difference between BPA and control females for rewards earned in FR1 did not coincide with decreased accuracy of responding, which likely indicates that BPA is affecting general activity rather than the acquisition of the task. However, we did not directly measure motor activity in adult offspring, so this result is difficult to interpret.

Studies in rats have shown that females outperform males in operant learning tasks unrelated to food such as shuttle box avoidance and passive avoidance (Dalla and Shors, 2009; Kokras and Dalla, 2014). Female mice also perform better than males in a more complex operant task, 5-choice serial reaction time (Groves and Burne, 2016). Appetitive operant learning is somewhat confounded by the necessity of food restriction in order to motivate responding. However, sexually dimorphic body weight and food consumption do not explain this behavioral sex difference. In a progressive ratio operant learning task, male and female rats do not differ in their motivation to respond for a food reward, even after food restriction (van Hest et al., 1988). The enhanced performance of females could be partially attributed to higher motor activity of females compared to males (van Haaren et al., 1990).

Anxiety and social behavior

Juvenile mice exposed to BPA during gestation demonstrated increased anxiety-like behavior on the EPM. This finding is in line with multiple studies that also report increased anxiety-

like behaviors in offspring as a result of early life exposure to BPA at various doses. BPA exposure to the sire, however, did not significantly impact behavior on the EPM of juvenile offspring. In our previous studies, females received a phytoestrogen free diet supplemented with 5 mg BPA per kg diet, this produces a daily dose of about 20 µg per day (approximately 500-800 µg/kg bodyweight per day, depending on the weight of the female). The mice in the current study received a daily treat with 20 µg of BPA. We chose this exposure method to more precisely control the timing and dose of BPA without causing stress to the animal. Other researchers have used a similar daily oral dosing method and observed behavioral changes in offspring (Ogi et al., 2013; Palanza et al., 2002; Poimenova et al., 2010). Also, in contrast to our previous studies, pups remained with their biological dam instead of being fostered to a control dam at birth.

Exposure to BPA has been shown to affect maternal behavior in a dose-, timing-, and species/strain-dependent manner (Rosenfeld, 2015). However, results differ as to whether maternal behavior produces behavioral changes associated with BPA exposure (Kundakovic et al., 2013). We have previously shown that BPA exposure and cross-fostering interact to produce differing behavioral outcomes on the elevated plus maze (Cox et al., 2010). In that study, juvenile and adult offspring exposed *in utero* to a higher dose of BPA than we used here (50 mg BPA per kg diet) and raised by their biological dams spent less time in the open arms of the EPM, indicating increased anxiety-like behavior. However, when all mice were fostered to a control dam at birth, gestational BPA exposure did not affect anxiety-like behavior on the EPM in juveniles exposed to two lower BPA doses during gestation (5 mg and 1.25 mg BPA per kg diet) (Wolstenholme et al., 2012, 2011b). Likewise, no effect of BPA on anxiety-like behavior in the open field was found (Wolstenholme et al., 2013). In the current study, we reported a significant decrease in time spent in the open arms of the EPM in maternally, but not paternally, exposed juveniles in this study. This is in agreement with our previous work showing that pups exposed to BPA *in utero* and raised by their biological dams spend less time in the open arms of the EPM than controls. Likewise, studies

that report increased anxiety-like behavior associated with early life BPA exposure (cited previously) did not foster pups to unexposed dams at birth.

In previous studies, we also examined the effect of BPA exposure on social behaviors. Juvenile offspring exposed during gestation to BPA showed significantly different behaviors from controls in a 30-minute social interaction task (Wolstenholme et al., 2012, 2011b). Notably, BPA mice spent less time engaged in side-by-side interactions and anogenital investigations of their test partner. We also found that gestational BPA exposure reversed sex differences in the social preference task. In the social recognition task, a test of social memory, juvenile BPA offspring spent more time investigating a familiar stimulus female than controls during the habituation phase, but the dishabituation response was unaffected (Wolstenholme et al., 2013). In the current study, neither maternal nor paternal exposure to BPA affected the habituation or dishabituation responses in social recognition. While maternal and paternal exposure groups were significantly different from each other, neither group differed from controls. We tested juveniles at an older age in this study because P21 juvenile control mice were less exploratory and did not exhibit the expected dishabituation response. Testing juveniles one week later may have had an impact on social investigation behavior in this test. However, it is more likely that fostering all pups to control dams at birth interacted with gestational BPA exposure to produce the previously reported differences in investigation during the habituation phase of social recognition (Wolstenholme et al., 2013).

Paternal preconception exposure

This is one of the first reports to examine behavioral effect of preconception exposure to BPA. First, we established that BPA exposure in males at this dose did not affect plugging, pregnancy rate, sperm counts, or reproductive organ weights. Effects of BPA on male reproductive outcomes vary based on timing and length of exposure, and strain of mouse or rat. We did not expect to find significant alterations in sperm quality or reproductive capacity at this dose, however, we were surprised to find that males exposed to BPA for 50 days weighed significantly less than

controls. This is contrary to a recent study reporting an increase in body weight and fat mass of C57 male mice after 5 weeks of oral exposure to BPA at doses ranging from 5-5000 μg/kg body weight per day (Yang et al., 2016). However, other studies in C57 mice have found no differences in body weight after long-term exposure to BPA (Moon et al., 2015; Takao et al., 2003). Biomonitoring data from the National Health and Nutrition Examination Survey (NHANES) suggest that BPA exposure in adult humans is associated with metabolic syndromes (Teppala et al., 2012). However, responses to EDCs like BPA are often non-monotonic, so varying doses of BPA may affect metabolic processes differently (Vandenberg et al., 2012). We found no effect of preconception paternal BPA exposure on anxiety-like behavior or social recognition in juveniles in this study. It is possible that BPA exposure at this dose does not lead to molecular changes in sperm robust enough to be maintained throughout the extensive epigenetic reprogramming of germ cells that takes place post-fertilization. Therefore, the question of preconception parental exposure to BPA is still an important one that will require further study.

Conclusions

The results of this study add to the growing literature on BPA-induced increases in anxiety-like behavior in maternally exposed offspring and expand the sparse knowledge concerning behavioral effects of paternal BPA exposure. Most notably, ours is the first study to report effects of BPA exposure during gestation on ultrasonic vocalizations of pups and associative learning in adult offspring.

Chapter III

Transgenerational bisphenol A exposure and brain sexual dimorphism

Introduction

During early development, gonadal steroid hormones organize the brain areas necessary for sex-specific functions in later life, such as those involved in mating and reproduction. Alterations to the hormonal environment during critical periods can impact the sexual dimorphism of important nuclei in the brain and the physiology and behaviors they control (Simerly, 2002). Two such regions are located within the preoptic area of the hypothalamus: the anteroventral periventricular nucleus (AVPV) and sexually dimorphic nucleus of the medial preoptic area (SDN-POA).

The abundance of steroid hormone receptors in these areas account for their sensitivity to hormonal signals during development. The neonatal surge of testosterone in males, which is aromatized to estradiol in the brain, serves to establish sex differences in size, cell number, and expression of steroid hormone receptors within specific nuclei (Davis et al., 1996; Dohler et al., 1982). Failure to properly organize these structures can result in abnormal behaviors. For example, knockout of estrogen receptor alpha (ERα) in male mice demasculinizes the AVPV and also results in significantly fewer male-typical sex and aggressive behaviors (Rissman et al., 1999).

The SDN-POA and is significantly larger in males than females. Based on several studies in rats, the SDN likely serves to regulate male sexual behaviors. For example, bilateral lesion of the SDN in sexually naïve male rats resulted in significantly decreased male sexual behaviors (ejaculation, mounting, intromission, etc.) (De Jonge et al., 1989). The postnatal sexual differentiation of the SDN depends on estrogen as well as androgen receptor signaling (Edelmann et al., 2007; Orikasa and Sakuma, 2010). Expression of calbindin-d28k, a calcium-buffer/sensor protein, has classically been used as a marker to identify the nucleus (Sickel and McCarthy, 2000). Calbindin is expressed across the brain, but most notably in Purkinje cells of the cerebellum. Neonatally castrated males have fewer calbindin-immunoreactive (calb-ir) cells in the SDN than intact males, whereas females given testosterone (Orikasa and Sakuma, 2010) or

DHT at birth have more male-like numbers of calb-ir cells than untreated females (Bodo and Rissman, 2008). DHT-injected females also exhibited a preference for female soiled bedding, unlike untreated females (Bodo and Rissman, 2008).

Contrastingly, the female AVPV is twice as large in volume as the male AVPV (Davis et al., 1996) and contains nearly three times as many neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme involved in the synthesis of dopamine (R.B. Simerly et al., 1985). Dopaminergic neurons in the AVPV are important in regulating the pre-ovulatory release of luteinizing hormone (LH) (Orikasa et al., 2002; R.B. Simerly et al., 1985). Within the AVPV, a select population of TH cells also express ERα (Patisaul et al., 2006). The organization of this region is controlled by estradiol signaling via ERα and ERβ (Bodo et al., 2006; Simerly et al., 1997). Similarly, neonatal sex hormone manipulations result in significant alterations of AVPV size and cell numbers (Bodo et al., 2006; Davis et al., 1996; Kanaya et al., 2014).

The development of sexually dimorphic nuclei can also be altered by endocrine disrupting chemicals (EDCs) like bisphenol A (BPA), a high-volume production chemical commonly used in polycarbonate plastics. The ubiquitous use of BPA in many products results in high levels of human exposure, predominantly via oral ingestion (Vandenberg et al., 2007a). BPA acts as an estrogenic chemical by binding to estrogen receptors in the body (Kurosawa et al., 2002; Matthews et al., 2001). Multiple studies in rodents have examined the effect of developmental BPA exposure on sexual differentiation of the brain (He et al., 2012; Naulé et al., 2014; Patisaul et al., 2007, 2006; Rebuli et al., 2014; Tando et al., 2007). Gestational exposure to BPA at a wide range of doses diminished sex differences in the SDN-POA by decreasing calbindin-immunoreactive volume and cell number in male rats. BPA exposure also decreased the number of TH-ir cells in the AVPV in a dose- and sex-specific manner (McCaffrey et al., 2013). A similar study in mice found that perinatal BPA exposure likewise diminished sex differences in the number of TH neurons in AVPV (Rubin et al., 2006). Interestingly, approximately 31% of ERα-ir cells in the AVPV and 40% in

mPOA colocalize with ERRγ, a nuclear orphan receptor highly responsive to BPA (Takayanagi et al., 2006; Tanida et al., 2017). ERRγ stimulates the expression of MAO-B, the enzyme that metabolizes dopamine, but this effect dampened by ERα and ERβ activation *in vitro* (Zhang et al., 2006). These data represent an intriguing mechanism by which BPA may affect dopamine in the AVPV.

The development of the cerebellum is also sensitive to the effects of sex hormones, particularly estradiol (Hoffman et al., 2016). Interestingly, BPA has been shown to affect the cerebellum in various ways. BPA exposure during development in rats disrupted intracellular signaling cascades in developing cerebellar cells *in vitro* (Le et al., 2008; Zsarnovszky et al., 2005) and repressed calcium uptake into cerebellar granule cells in primary culture (Imamura et al., 2005). BPA decreased thyroid hormone receptor expression in cerebellar cell cultures, but increased receptor expression in the presence of thyroid hormone or estradiol (E2) (Somogyi et al., 2016), suggesting that BPA interferes with the complex relationship between thyroid hormone and E2 during cerebellar development. Postnatal BPA exposure has also been associated with significant pathological changes of cerebellum cytoarchitecture in rats, including reduced size, abnormal morphology, and disorganized location of purkinje neurons (Atif et al., 2015). However, neonatal administration of a large dose of BPA (500 μg per neonate for 3 days) facilitated dendritic outgrowth of calbindin-immunopositive purkinje cells compared to vehicle control, but did not affect cell number, or soma size (Shikimi et al., 2004). These data demonstrate perinatal BPA exposure may act in a dose-dependent manner via several mechanisms to disrupt the development of the cerebellum, which occurs late in gestation and continues into postnatal life.

While several research groups have examined the direct effects of BPA exposure on cells in the AVPV, SDN-POA, and cerebellum it remains unknown whether these effects can be transmitted across multiple generations. We have previously shown that BPA exposure significantly affected social behaviors in gestationally exposed offspring (F1 generation), as well as mice three generations removed from the initial exposure (F3 generation) (Wolstenholme et al.,

2013, 2012). In one study, transgenerational behavioral changes were also accompanied by gene expression differences in embryonic whole brain (Wolstenholme et al., 2012). More recently, our lab showed that BPA affects ERα-ir cells populations sexually dimorphic brain areas in females of both the F1 and F3 generations (Goldsby et al., 2017). Based on these results, we hypothesized that ancestral exposure to BPA in the F3 lineage mice would affect sexual dimorphism the AVPV and SDN-POA. In F3 generation males and females, we measured the colocalization of TH and ERα in the AVPV and the volume of calbindin-ir in the SDN. In addition, we quantified levels of calbindin in the juvenile cerebellum, which we have previously shown to be significantly higher in females than males (Abel et al., 2011).

Materials and Methods

Animals and breeding:

C57BL/6J mice used in these experiments were generated at the Biological Resources Facility at NC State University. The progenitor mice were purchased from Jackson Labs (Bar Harbor, ME). All animals were maintained on a 12:12 light/dark cycle (lights off at 1200) and provided food and water *ad libitum*. The Institutional Animal Care and Use Committee at NC State University approved all procedures.

Transgenerational F3 BPA and control lineages were generated using methods published previously (Goldsby et al., 2017; Wolstenholme et al., 2013, 2012). Females were randomly assigned to consume one of two custom diets: phytoestrogen free chow (Teklad 95092, "Control"), or the same chow containing 5 mg/kg BPA (Teklad 09386, "BPA"). Plasma levels of BPA levels in pregnant dams consuming this BPA diet averaged 3.9 ng/ml (Wolstenholme et al., 2012), which is within the range (0.3–4.0 ng/ml) reported in pregnant women (Schönfelder et al., 2002). The females consumed their assigned diet for 7-10 days prior to mating with a male and throughout

pregnancy. To eliminate potential effects of BPA on maternal behavior, all pups were fostered to a dam who had been on control food throughout pregnancy.

At weaning on postnatal day 21, F1 generation offspring were transferred to standard rodent chow (Teklad 7012). Offspring were housed in same-sex same-exposure groups until reaching sexual maturity. In adulthood, F1 offspring were mating to non-siblings of the same gestational diet exposure (i.e. control females paired with control males and BPA paired with BPA males) to produce the F2 generation. Adult F2 generation mice were paired with non-siblings in a similar manner to produce the F3 generation. For all breeding pairs, the sire was removed from the breeding cage prior to the birth of a litter. All F2 and F3 mice consumed standard rodent chow.

F3 generation juveniles (27-29 days old) were euthanized via carbon dioxide. Brains were carefully removed from the skull and quickly dissected on ice. The cerebellum was gently separated from the midbrain and brainstem, then rapidly frozen on dry ice and stored at -80°C until processing. No more than 2 mice per sex per litter were used for Westerns.

F3 generation adults (15 weeks old) were deeply anesthetized with a lethal dose of sodium pentobarbital and transcardially perfused with cold 0.9% NaCl followed by 4% paraformaldehyde. Brains were removed from the skull and post-fixed in 4% paraformaldehyde for 2 hours, then cryoprotected in 0.1 M sodium phosphate buffer containing a 30% sucrose overnight at 4°C. The brains were rapidly frozen on dry ice and stored at -80°C until sectioning. Fixed brains were cut into 3 series of 40 µm sections on a cryostat and stored in antifreeze (sucrose, polyvinylpyrrolidone-40, and ethylene glycol in tris-buffered saline (TBS)) at -20°C until processing. One series of sections was processed for TH/ERα and a second was used for calbindin staining. No more than 2 mice per sex per litter were used for immunohistochemistry.

Immunohistochemistry:

We analyzed 10 Control males, 9 BPA males, 11 Control females, and 10 BPA females for TH/ERα immunofluorescence. Sections were washed 0.02 M TBS 6 times over 1 hour, followed

by pre-incubation in TBS containing 2% normal donkey serum and 0.3% Triton-X (D-TBS). The sections were then incubated in a cocktail of primary antibodies in D-TBS overnight at 4°C (monoclonal mouse anti-TH (Chemicon, 1:5000) and polyclonal rabbit anti-ERα (Upstate, C1355, 1:1000). After washing, sections were incubated with fluorescent-labeled secondary antibodies for 1 hour (Donkey anti-mouse IgG, Alexa Fluor 555, A31570; Donkey Anti-rabbit IgG, Alexa Fluor 488, A21206; Invitrogen, 1:200 in D-TBS). Sections were mounted onto gel-coated slides, cover-slipped, and stored at -20°C until visualization.

10 control males, 10 BPA males, 12 Control females, and 10 BPA females were analyzed for Calb-ir SDN-POA volume. Sections for calbindin immunohistochemistry were processed as previously published (Bodo and Rissman, 2008). Briefly, sections were washed in 0.02 M TBS, incubated in 0.3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, pre-incubated in the primary carrier solution (L-TBS: 0.25% λ-carrageenan, 0.5% bovine serum albumin (BSA), and 0.3% Triton-X in TBS) for 1 hour, then incubated in primary antibody (monoclonal mouse anti-calbindin, Sigma C9848, 1:5000 in L-TBS) overnight at 4°C. Sections were then incubated in biotinylated goat anti-mouse secondary antibody (Vector Labs, 1:500 in L-TBS) for 75 minutes. The biotin signal was amplified using an avidin-biotin complex (ABC, 1:1000, Vector Elite Kit, Vector Labs) for 1 hour and developed in nickel-3,3′ diaminobenzidine (DAB, Sigma) chromagen activated by 0.1% hydrogen peroxide. The developed sections were mounted on gel-coated slides, dehydrated, and coverslipped with Permount (Fisher).

Confocal Microscopy:

Immunofluorescent staining was visualized at 10X magnification on a Nikon TE2000-U confocal microscope. For each animal, one side of an anatomically matched section containing the AVPV, as identified using the Allen Mouse Brain Atlas (Lein et al., 2007), was analyzed. A set of serial image planes (z-step distance = 3 μm) was collected through the entire thickness of the

section. Using NIS Elements software (Nikon Instruments), an experimenter blind to the sex and treatment of the animal hand-counted the number of TH-ir cells and TH-ir/ERα-ir cells within a region of interest (dimensions: 200 x 600 μm) encompassing a rostral portion of the AVPV. The percentage of TH-ir/ERα-ir cells was calculated as the number of TH-ir/ERα-ir cells ÷ total number of TH-ir cells x 100%.

Stereology:

Unbiased stereology measurements of SDN-POA volume were performed on a Leica DM2500P microscope (Leica Microsystems) using Stereologer™ (Stereology Resource Center) (McCaffrey et al., 2013; Schmitz and Hof, 2005; Skledar et al., 2016). Calbindin immunolabeling clearly defined the boundaries of the SDN-POA and the location was confirmed using the Allen Mouse Brain Atlas (Lein et al., 2007). The entire nucleus was contained within 2-3 sections. A unilateral border of the SDN-POA was traced on each section at 5X magnification and the volumes were estimated using the Cavalieri estimator probe. The mean coefficient of error for Calb-ir SDN-POA volume was 0.07.

Western blotting:

7 control males, 8 BPA males, 8 control females, and 7 BPA females were analyzed for juvenile cerebellum calbindin levels. Cerebellum tissue was thawed and homogenized in RIPA buffer containing proteinase inhibitors. Following centrifugation, the total protein concentrations in the tissue lysates was measured by bicinchoninic acid protein assay. 25 μg of protein per sample was separated on 12% polyacrylamide-SDS gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were washed prior to each immunoblotting step with TBS containing 0.1% Tween-20 (TBST). Non-specific binding was blocked in 5% milk in TBST for 1 hour, then incubated with primary antibodies directed against calbindin (Polyclonal rabbit anti-calbindin,

Millipore, AB1778, 1:2000) and β-actin (Mouse monoclonal, Sigma, 1:5000) in 5% milk overnight at 4°C. The membranes were incubated with HRP-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, 1:20,000, Vector Labs) in 5% milk for 1 hour followed by detection with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemicals). Signal intensities for each band were quantified using LICOR Odyssey Imaging software. Values for each sample are represented as the signal for the calbindin band / β-actin band.

Statistics:

Data were analyzed by two-way ANOVA in NCSS 2007 with sex and BPA exposure lineage as the independent variables.

Results

Calbindin protein in juvenile cerebellum

Contrary to our previous study, we found no significant sex difference in the amount of calbindin protein in the cerebellum of juvenile mice as quantified by chemilumiscent western blots ($F(1,26)=0.04$, $p>0.05$; Figure 1). Likewise, there was no effect of ancestral BPA exposure on calbindin protein in the cerebellum ($F(1,26)=2.54$, $p>0.05$; Figure 1).

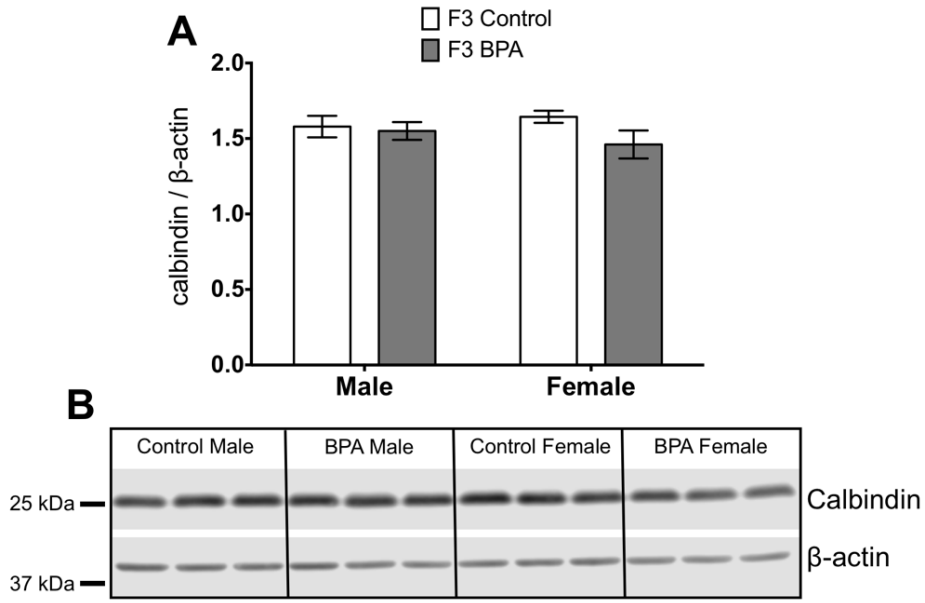

Figure 1: Calbindin protein levels in juvenile cerebellum
A) Mean ± SEM ratio of calbindin/B-actin band intensities B) representative Western blot, each of the 12 pairs of bands represents one individual cerebellum sample.
7 control males, 8 BPA males, 8 control females, and 7 BPA females

TH and ERα immunoreactivity in the AVPV

Consistent with the literature in rodents, we found that a population of TH-ir cells within AVPV was sexually dimorphic. We also found that the sub-population of TH-ir neurons co-expressing ERα also differed according to sex (Figure 2). Females had significantly more TH-ir cells in the AVPV than males ($F(1,36)=80.9$, $p<0.0001$; Figure 3A) However, the number of TH-ir cells was unaffected by ancestral BPA exposure ($F(1,36)=0.78$, $p=0.38$). The number of cells co-expressing TH and ERα within the AVPV was greater in females than males ($F(1,36)=58.6$, $p<0.0001$; Figure 3B). The percentage of TH-ir cells expressing ERα was also significantly greater in females than males ($F(1,36)=81.8$, $p<0.0001$, Figure 3C). Neither the number nor the percentage of ERα-ir TH cells was significantly affected by ancestral BPA exposure ($F(1,36)=.70$, 0.98 respectively; $p>0.05$).

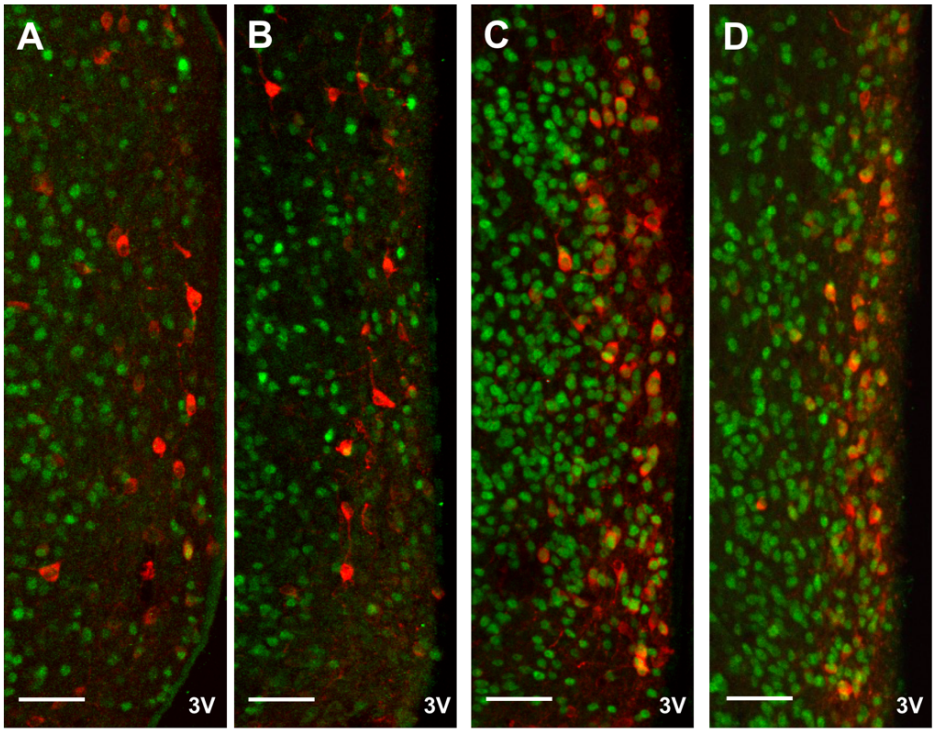

Figure 2: TH-ir and ERα-ir cells in AVPV
Representative maximum intensity projections of TH (red) and ERα (green) immunofluorescence in the AVPV generated from confocal image stacks; A) control male B) BPA male C) control female D) BPA female. Double-labeled cells appear as green/yellow nuclei within red cells. (Scale bars = 50 µm, 3V = third ventricle)

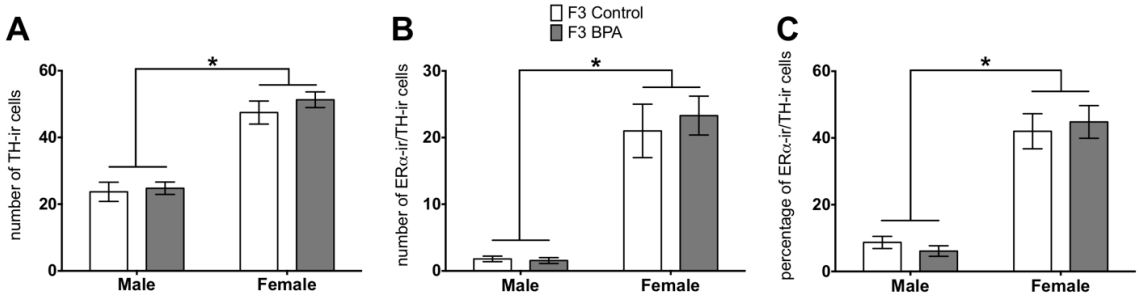

Figure 3: Quantification of TH-ir and ERα-ir cells in AVPV
Mean ± SEM A) number of TH-ir cells in the AVPV, B) number of TH/ERα double labeled cells, C) percentage of TH cells labeled with ERα
White bars represent F3 Control lineages and gray bars represent F3 BPA lineages.
* significant sex difference females > males; 10 control males, 9 BPA males, 11 control females, and 10 BPA females

Volume of calbindin-ir in SDN-POA

The boundaries of the SDN-POA were clearly defined by calbindin immunoreactivity (Figure 4). As expected, the volume of calbindin-ir in the SDN-POA was significantly larger in males compared to females ($F(1,38)=17.3$, $p<0.001$; Table 1 and Figure 4). BPA lineage did not affect calbindin-ir SDN-POA volume ($F(1,38)=0.01$, $p>0.05$).

	Male			**Female**		
	Mean volume (μm^3)	SEM	n	Mean volume (μm^3)	SEM	n
Control	8113584	1918516	10	2082115*	812768	12
BPA	7851855	1543963	10	2544583*	1084379	10

Table 1: Calbindin-ir SDN-POA Volume: Mean ± SEM volume of calbindin-ir SDN-POA.
* significant sex difference males>females, $p<0.001$

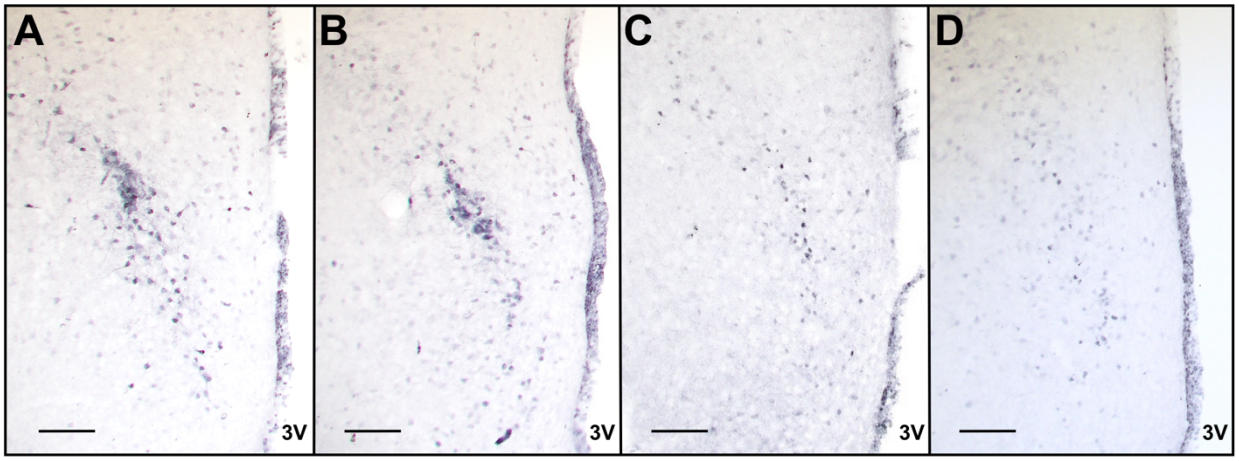

Figure 4: **Calbindin-ir SDN-POA**
Representative photomicrographs of calbindin-ir in the SDN-POA, A) control male, B) BPA male, C) control female, D) BPA female. (Scale bars = 100 µm, 3V = third ventricle)

Discussion

We hypothesized that ancestral exposure to BPA would impact the sexual differentiation of the AVPV and SDN in F3 generation mice. We found that females possess twice as many TH-

ir neurons in the AVPV as males. Additionally, the percentage of TH-ir cells in AVPV with ERα-ir nuclei also was approximately 5 times higher in females than males. Conversely, the volume of the SDN-POA as outlined by calbindin immunoreactivity was three times larger in males compared to females. We found no effect of transgenerational BPA exposure in either brain area. Additionally, we found no effect of BPA or sex on calbindin protein in juvenile cerebellum tissue.

In studies that assess the effect of direct, perinatal exposure to BPA on the volume and number of calb-ir neurons in the SDN, results vary across doses and species. Some studies show a hypermasculinizing effect of BPA on the male SDN in rats (He et al., 2012; Patisaul et al., 2007) and one found a demasculinizing effect on calb-ir cell number in F1 male rat offspring (McCaffrey et al., 2013). However, three rat studies and one study in C57BL6 mice report no significant effect of BPA exposure on SDN-POA volume or calb-SDN cells (Kwon et al., 2000; Nagao et al., 1999; Naulé et al., 2014; Takagi et al., 2004). BPA effects on SDN-POA volume or calb-ir SDN cells/volume seem to vary based on dose, timing of exposure, and species (the only effects on SDN are from rat studies). The direct effects of BPA on the AVPV appear to be more consistent across reports. Developmental BPA exposure attenuates the sex difference in TH cell numbers in the AVPV either by masculinizing female cell numbers (McCaffrey et al., 2013; Rubin et al., 2006) or demasculinizing male cell numbers (Patisaul et al., 2006). In one rat study, BPA exposure did not affect the number of TH cells in females, but did decrease the localization of ERα in the nuclei of TH-ir neurons in the AVPV (Patisaul et al., 2006). Pre- and postnatal BPA exposure did not affect the volume or number of TH-ir cells in AVPV in rats at postnatal day 21, but this could be attributed to the pre-pubertal age (Ferguson et al., 2015).

Our lab recently reported a significant increase in the number of ERα-ir cells in AVPV of F3 BPA females compared to F3 control females (Goldsby et al., 2017). Therefore, we hypothesized that transgenerational BPA exposure could also affect the number of TH cells or TH/ERα double labeled cells in this area. We were able to replicate the finding that the number

and percentage of TH/ERα double-labeled neurons in the AVPV was substantially greater in females than in males (Patisaul et al., 2006). However, we reported no significant effects of transgenerational BPA exposure on the number of TH/ERα colocalized cells or the total number of TH-ir cells in the AVPV.

In our previous study, gestational BPA exposure attenuated the sex difference in ERα-ir cells in the ventromedial hypothalamus (VMH), but did not affect ERα in the AVPV or MPOA of F1 offspring (Goldsby et al., 2017). It is therefore important to note that it remains undetermined whether direct exposure to the dose of BPA used to produce the F3 generation mice affects TH-ir/ERα-ir neurons in the AVPV or calbindin-ir SDN volume in *F1* generation offspring. In the most similar developmental BPA exposure study in C57 mice, BPA did not significantly impact calb-ir SDN cell numbers nor ERα cells in mPOA of females. However, the male offspring were not studied (Naulé et al., 2014). It is unclear whether BPA affects brain sexual differentiation in F1 offspring and these changes do not persist to the F3 generation or if BPA affects neither generation significantly.

Prenatal and peripubertal exposure to ethinylestradiol (EE2), a synthetic derivative of estradiol used in oral contraceptives, induced hypermasculinization of the male SDN and concomitant increases in male sexual behaviors. Intriguingly, this neurobehavioral phenotype persisted in male progeny through the F4 generation (Derouiche et al., 2015). These data elegantly demonstrate the plausibility of multigenerational transmission of an environmentally induced phenotype. There is some evidence for a transgenerational effect of BPA on reproductive outcomes, such as reproductive capacity in female mice (Berger et al., 2016; Ziv-Gal et al., 2015) and delayed puberty onset in rats at high doses (Tyl et al., 2002). However, none of these studies examined the transgenerational effect of BPA on sexual dimorphism in the brain, so it is impossible to state whether the effects can be traced to a neuronal origin.

Our lab has previously shown that calbindin mRNA in juvenile cerebellum is higher in

females than males, and ERα knockouts significantly decreased calbindin expression (Abel et al., 2011). However, we were unable to detect a sex difference in calbindin levels in juvenile cerebellum samples from F3 generation mice as quantified by western blot. One potential cause for this discrepancy is that the juveniles in the current study were 3-7 days older than the weanling offspring used in the previous study. Peripubertal changes in hormone signaling cause substantial reorganization of the brain, potentially leading to increased variability among groups. We hypothesized that calbindin protein levels in cerebellum might be impacted in F3 BPA juvenile because of BPA's estrogenic activity. However, we did not find any significant effect of ancestral BPA exposure on calbindin protein. It is unknown whether direct, F1 generation exposure to BPA would affect calbindin or ERα expression in the juvenile cerebellum at this dose.

Conclusion

We found the expected sex differences in TH-ir neurons in the AVPV and calbindin-ir volume of the SDN. We also confirmed the presence of sexually dimorphic ERα-ir colocalization within a distinct subset of TH-ir neurons in AVPV. However, we did not detect a significant sex difference in calbindin protein levels in juvenile cerebellum tissue. Although it has not been tested whether these outcomes are affected by gestation exposure to this dose of BPA in F1 offspring, we showed that there are no significant transgenerational effects in F3 BPA offspring. Nevertheless, it is important to note that despite finding no effect of ancestral BPA on the outcomes measured here, we have previously reported significant effects of both direct and transgenerational BPA exposure on social behaviors and the mechanisms underlying these behavioral changes have yet to be identified.

Chapter IV

Calbindin knockout alters sex-specific regulation of behavior and gene expression in amygdala and prefrontal cortex

Introduction

Calbindin-D(28K) (*Calb1*) is abundantly expressed in the brain where it functions as a high-affinity calcium buffer/sensor in neurons (Baimbridge et al., 1982; Bastianelli, 2003; Kojetin et al., 2006; Schwaller, 2009). *Calb1* is expressed in both pyramidal and non-pyramidal neurons in the brain, and in GABAergic inhibitory neurons including the Purkinje cells of the cerebellum and interneurons residing in the prefrontal cortex, amygdala, and hippocampus (Celio, 1990; DeFelipe, 1997; Druga, 2009; Hof et al., 1999). Moreover, *Calb1* mRNA and protein are sexually dimorphic (females have more than males) in the cerebellum and prefrontal cortex of juvenile mice, two brain regions associated with cognitive and affective behaviors impacted by sex-specific neurobehavioral disorders (Abel et al., 2011). Calbindin protein is also a marker for the sexually dimorphic nucleus of the preoptic area (SDN): males have significantly greater numbers of calbindin-positive cells than females (Edelmann et al., 2007; Gilmore et al., 2012). This population of *Calb1* cells is regulated by both estrogen receptor alpha and androgen receptors during development (Bodo and Rissman, 2008; Orikasa and Sakuma, 2010). However, the effect is strictly organizational in nature, as calbindin immunoreactivity in the SDN in not affected by adult exposure to gonadal hormones (Orikasa and Sakuma, 2010).

Reduced calbindin has been associated with a number of neurobehavioral diseases, many of which are sexually dimorphic in incidence. In the cortex, there are significantly fewer calbindin-immunopositive neurons in the cortex of brains of schizophrenic, but not bipolar patients, in comparison to control subjects (Sakai et al., 2008; Torrey et al., 2005). During aging, calbindin-containing neurons in the basal forebrain gradually die, and this process is accelerated in Alzheimer's patients (Riascos et al., 2011). Decreased calbindin immunoreactivity in the hippocampus is also associated with temporal lobe epilepsy (Abraham et al., 2011, 2009). Additionally, post mortem studies report fewer and smaller Purkinje cells in the cerebellum of autistic patients (Fatemi et al., 2002; Palmen et al., 2004). Transgenic mouse models of fragile X syndrome, an X chromosome-linked neurodevelopmental disorder, have lower than normal

numbers of calbindin-immunoreactive neurons (Giraldez-Perez et al., 2013; Real et al., 2011) and significantly decreased *Calb1* expression (Tessier and Broadie, 2011). In other transgenic mouse models of neurological disease, altering *Calb1* levels affects the phenotype. For example, suppressing *Calb1* expression worsens the disease phenotype in an Alzheimer's disease model (Kook et al., 2014), whereas *Calb1* over-expression has a neuroprotective effect in a Parkinson's model (H. H. Yuan et al., 2013).

Behavioral and physiological endpoints are also affected by experimental manipulations of calbindin levels. In rats, overexpression of calbindin in the dentate gyrus produces deficits in water maze learning and T-maze reversal learning (Dumas et al., 2004). Male transgenic *Calb1* knock down (KD) mice show deficits in spatial learning in the Morris water maze and the 8-arm radial maze compared to control males (Molinari et al., 1996). Additionally, complete calbindin knockout (Calb-KO) mice have disrupted circadian rhythms and impaired hippocampal long-term potentiation maintenance as compared to wildtypes (Kriegsfeld et al., 2008; Westerink et al., 2012).

In this study, we investigated novel aspects of calbindin function on social behavior, anxiety-like behavior, and fear conditioning in adult mice of both sexes by comparing wild type (WT) to littermate Calb-KO mice. We hypothesized that lack of calbindin would alter inhibitory neurotransmission by disrupting downstream genes related to synaptic plasticity. Since neocortical GABAergic interneurons temper excessive stimulation from excitatory neurons, and molecular and cellular dysfunction of these neurons leads to cognitive, affective, and behavioral impairments (Druga, 2009; Rossignol, 2011), we hypothesized that Calb-KOs would differ in the behaviors tested compared to WT littermates. Moreover, we predicted sex differences in the behavioral responses of wildtype mice would be eliminated in Calb-KO mice. Next, we examined gene expression in the amygdala and prefrontal cortex (PFC), two areas of the brain intimately connected with limbic system control of the behaviors tested, in response to sex and genotype.

Materials and Methods

Animals

All procedures were approved by, and conducted in accordance with, the University of Virginia Animal Care and Use Committee guidelines. Breeding dams and sires, both of which were heterozygous for the null *Calb1* allele, produced the mice used for all experiments (Airaksinen et al., 1997). To set up the breeding colony mice were ordered from Jackson Labs (Stock # 003079; Bar Harbor ME), the background strain was C57BL/6J. Offspring were genotyped before weaning (postnatal day 21) and all homozygotes (wild type and complete knockouts) were group housed by sex and age and maintained on a 12:12 light cycle (lights on at 1300). At all times animals had access to water and food (#7912 Harlan Teklad, Madison, WI) ad libitum.

Between 50-70 days of age, all mice were gonadectomized and each received an estradiol-filled Silastic implant (Dow Corning, Corp., Midland, MI tubing: 1.98 mm ID X 3.17 mm OD). Estradiol was prepared in sesame oil (estradiol-17β 50μg/ml) and placed subcutaneously in the back of the neck. This procedure was used to provide equivalent hormone level regardless of the genotype or the sex of the mouse. Thus, sex differences cannot be attributed to differences in the levels of gonadal hormones at the time of the test. After surgery, mice were housed individually. Ten to fourteen days after surgery mice were tested for one of the three behaviors. The observers who scored the behaviors were blind to the sex and genotype of the mice. All behavioral tests were conducted in the light.

At the end of the experiment, all mice were anesthetized using sodium pentobarbital and euthanized. Brains were rapidly removed and frozen on dry ice. Using a cryostat, brains were cut in coronal sections (120μm) onto slides. A tissue punch (1 mm) was used to dissect the complete amygdala and the prefrontal cortex using anatomical guidelines established by visually comparing slices to figures in the Mouse Brain Atlas(Lein et al., 2007). The amygdala was collected in 2 bilateral punches from 8 sections corresponding to Atlas figures 31 through 40. The prefrontal

cortex was collected in two medial punches from prelimbic and infralimbic areas in atlas figures 14-20.

Fear Conditioning

We used a modified fear conditioning protocol(Paylor et al., 1994). Testing was conducted between 0800-1200h. Before testing, mice were moved one at a time into the testing room. On the first test day, the subject was placed in one half of a mouse shuttle box (Med Associates: # ENV-010MC; each interior compartment: 20.3 cm x 15.9 cm x 21.3 cm) for 10 min to explore the novel environment and then returned to their home cage. Twenty-four hours later the animals were returned to the same test chamber. Freezing behavior was observed at 10-second intervals for two minutes (*baseline, trial 1*). Freezing was defined as no movement other than natural respiratory motions. Next, an 80 dB, white noise was presented for 30 seconds immediately followed by a 2-second 0.35 mA foot shock. This pairing was repeated after a 2-minute inter-trial interval (*training*). Each animal was returned to the home cage 30 seconds after the second shock. The next day, freezing behavior was recorded in the same environment for 5 minutes (*context, trial 2*) and then mice were returned to their cages. Between 1-2 hours later, mice were introduced to a novel side of the shuttle box chamber, which had been altered in the following way: A divider was placed on the diagonal to divide the chamber into a triangularly shaped area. Pieces of dark tape were affixed to the walls and the floor was covered with a solid piece of plastic to yield a smooth surface. Finally, a drop of orange extract was placed on a cotton swab suspended from the ceiling out of reach of the mouse. In this altered context, the animals were observed for a total of 6 min. During the first 3 min (*pre-conditioned stimulus (CS), trial 3*), no auditory stimulus was presented. During the last 3 min, the auditory stimulus was presented (*CS, trial 4*). Freezing behavior was recorded at 10-second intervals for the entire 6 min. testing period. The amount of time in context-dependent freezing is expressed as a percentage of baseline freezing: [(Trial 2 freezing time - Trial 1 freezing time) / Total time] x 100%. Percent time in cue-dependent freezing was calculated in the same way:

[(Trial 4 freezing time - Trial 3 freezing time) / Total time] x 100%. In total, data from 36 mice were used in the analysis. (KO n=10 of each sex, WT males n=7 and WT females n=9)

Elevated Plus Maze

Mice were habituated to the testing room for at least 30 minutes before the test began. At the start of the test, each subject was placed in the center of the clean elevated plus maze (EPM) facing an open arm. Mice were allowed to explore the maze for 10 minutes and videotaped from above. Between subjects, the EPM was thoroughly cleaned with ethyl alcohol. The floors and walls of the EPM (ENV-560A; Med Associates, Inc., St. Albans, VT) were black polypropylene. Each runway measured 6 cm wide x 35 cm long x 71.25 cm tall. The walls on the closed arms were 20.3 cm high. Observers, blind to the sex and genotypes of the mice, scored the time spent in each arm (open or closed) and the center of the maze, as well as the number of entries into each arm. In addition, because others have reported motor coordination differences between KO and WT mice (Barski et al., 2003), we scored the number of times each animal turned around in the open arms of the maze and the total amount of time spent turning. Time spent turning was subtracted from total time in the open arms. (WT males, females, and KO males n=7; KO females n=9)

Social Preference

Mice were tested in a large, Plexiglas cage divided into three chambers: two equal-size end areas (31.5 x 25.5 cm each) and a smaller neutral section between them (10.5 x 25.5 cm). Both end areas contained an empty "holding cell" (10.16 cm in diameter and 13.97 cm tall). During the habituation phase, the mouse was placed in the center of the box and allowed to explore the entire box for 10 minutes. The subject was then restricted in the center by closing the openings to the outer chambers. A gonad-intact adult male was placed under the holding cell on one side and an ovariectomized, estrogen-implanted adult female (both were C57BL/6 wildtype mice) on the opposite side (sides were randomly selected). These stimulus mice were habituated to the holding

cell in their home cages for at least 10 minutes before the test. During the test phase, the doors were opened again and the subjects were allowed to investigate the box for 10 minutes. Observers blind to sex and genotype scored the number of entrances into each side and the time spent investigating the stimulus mouse. Investigation was defined as the test mouse's nose touching the stimulus mouse through the bars or sniffing within 1cm of the mouse. The test arena was carefully cleaned between subjects to eliminate olfactory stimuli from previous trials. (WT males n=9, all other groups n=10).

Quantitative Real-Time PCR (qRT-PCR)

We selected genes that are either related to the behaviors we chose or associated with calbindin neurons/signaling. We chose several BDNF signaling targets because BDNF has been shown to regulate calbindin (Fiumelli et al., 2000). Since *Calb1* is primarily expressed in inhibitory neurons, we also selected two GABA-related genes. We examined both nuclear estrogen receptors because estradiol regulates *Calb1* expression. We also investigated potential effects on epigenetic regulators, like *Hdac3, Hdac4,* and *Mecp2*. Other targets, like *Nr3c1, Crhr1*, and *Avp,* are involved in the behaviors we tested. Additionally, many of these genes have been shown to have sexually dimorphic expression in the brain.

An RNeasy®Lipid Tissue Mini Kit (Qiagen, Valencia, CA) was used to isolate total RNA according to the manufacturer's protocol. The quantity and quality of the RNA were determined using a NanoVue™ Spectrophotometer. cDNA templates were prepared using an AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The ABI StepOnePlus real-time PCR system was used to perform qRT-PCR. Either TaqMan®Probe or SYBR®Green-Based Detection (Applied Biosystems, Carlsbad, CA) were used to detect PCR products of interest. The following TaqMan®Gene Expression Assays were used: estrogen receptor α (*Esr1*, Mm00433149m1), estrogen receptor β (Esr2, Mm00599821 m1), histone deacetylase 4 (*Hdac4*, Mm01299557_m1), methyl CpG binding protein 2 (*Mecp2*, Mm01193537 g1) and neurotrophic tyrosine kinase, receptor, type 2 (*Ntrk2*,

Mm00435422_m1). In these assays, all samples were normalized to mouse beta-actin (*Actb*, #4352933E). Oligonucleotide primers (Table 1) were either designed for SYBR-Green based analysis using consensus sequences and Blast from the NCBI genomic alignment database or derived from prior publications and were synthesized by Invitrogen (Carlsbad, CA). The following primers were based on previously published constructs: *Bdnf–exon IV* (Zheng et al., 2012); *Calb1* (Abel et al., 2011); *Gabrb1* and *Gad67* (Gilabert-Juan et al., 2011). All other primers were designed to span an exon-exon junction to prevent binding to genomic DNA. For all SYBR assays, samples were normalized to the endogenous control, beta-2 microglobulin (*B2m*). Validation experiments were conducted to test for equally efficient target and endogenous control gene amplification and primers were between 90 and 110% efficient for all amplifications. In each qRT-PCR reaction using SYBRGreen, primers were verified for a single PCR product of expected size with the disassociation melting curve stage. For TaqMan and SYBR Green based detection, target and endogenous control genes were measured in triplicate for each cDNA sample during each real-time run to avoid inter-sample variance. All genes of interest were analyzed with Step One™ software using the comparative cycle thresholds method (CT) method.

Gene	Sequence (5′–3′)
Avp	F, TGCTCGCCAGGATGCTCAACAC
	R, TTGCCGCCTCTGGGCAGTT
B2M	F, GGCTCACACTGAATTCACCCCC
	R, ACATGTCTCGATCCCAGTAGAC
Bdnf, all exons	F, TTAAGCGGCTTCACAGGAG
	R, CCTGCTGCCATGCATAAAAC
Bdnf, exon IV[a]	F, CTCCGCCATGCAATTTCCAC
	R, GCCTTCATGCAACCGAAGTA
Calb[b]	F, ACTCTCAAACTAGCCGCTGCA
	R, TCAGCGTCGAAATGAAGCC
Crhr1	F, CTGAACAGTGAGGTCCGCTC
	R, GGCTCTGATGGAGTGCTT
Gabrb1[c]	F, GCCATGGACTGGTTTATTGC
	R, CCACGCATACCCTCTCTTGGTG
Gad67[c]	F, GGGTTCCAGATAGCCCTGAGCGA
	R, TGGCCTTGTCCCCTTGAGGCT
Hdac3	F, ATGACAGGACTGACGAGGCCGA
	R, TGGGTGCTTCTGGCCTGCTGTA
Nr3c1	F, GGATGCCATTATGGGGTGC
	R, TCGTTTTTCGAGCTTCCAGG

Table 1: Quantitative RT-PCR primer sequences
F: forward; R: reverse; *Primer from (Zheng et al., 2012); **Primer from (Abel et al., 2011); ***Primers from (Gilabert-Juan et al., 2011).

Serum corticosterone

Blood was collected from awake, adult male mice of both genotypes (n=6 WT and n= 7 KO). These mice had been tested in the EPM, at least two weeks prior to blood collection. Each mouse was sampled twice: once for a baseline measurement and again following 15 minutes of restraint stress. Samples were collected at least one week apart and the order of the conditions was counter-balanced by genotype. Samples were obtained by submandibular punctures using a 4mm Goldenrod Lancet (Mineola, NY) during the first hour of the light period. This blood collection method is rapid (under three minutes) and requires no anesthesia(Golde et al., 2005). Samples were centrifuged at 12000 rpm for 10 minutes, and serum was collected and frozen until assay. We assessed the serum concentrations of corticosterone using an enzyme immunoassay kit (DetectX® Corticosterone Immunoassay kit, Arbor Assays, Ann Arbor, MI). Control serum pools were assayed in duplicate and interpolated from the standard curve, which was fitted with a 4PLC nonlinear regression in GraphPad Prism 6 for Mac (La Jolla, CA). The sensitivity of the kit was 18.6 pg/mL; the limit of detection was 16.9 pg/ml. The average coefficient of variance was 3.89%.

Statistics

All data were analyzed using NCSS Software (2000). Data points greater than two standard deviations from the mean were tested using Grubb's Outlier test. For data analyses, two-way or repeated measures ANOVAs were used, as appropriate, to assess the contributions of genotype and sex. Paired comparisons were conducted using Fisher's LSD multiple comparison tests.

Results

Male KO mice display reduced conditioned fear in response to an auditory cue

A repeated measures ANOVA revealed an interaction between sex and genotype

(F(1,95)=5.73, p<0.025) for freezing time across trials 1-4. This effect was produced by male KO mice, which displayed less freezing behavior than male WT or female KO mice over all trials (p<0.05). Interestingly, there was an interaction between trial and genotype (F(1,95)=3.21, p<0.03). The genotype difference was only noted on the cued conditioned stimulus trial (trial 4). In response to the conditioned cue, WT animals displayed more freezing than did KO mice (p<0.05) (Figure 1). To further investigate this interaction, we ran repeated measures analysis on the final two trials. Again, a significant interaction between sex and genotype was indicated along with interactions between sex and trial as well as genotype and trial (F(1,95)=4.13, 5.73, 8.67, respectively, p<0.05 or less). A large main effect of trial was also noted (F(1,95)=343.19, p<0.0001). The sex by genotype interaction was caused by a difference between KO and WT males, whereby freezing behavior was significantly lower in KO males (p<0.05). The interaction between sex and trial was caused by a sex difference on the conditioned stimulus (tone) trial; males froze more than females (p<0.05). On the other hand, the genotype by trial effects were noted on the final trial when KO mice were less reactive to the tone than WT mice (p<0.05).

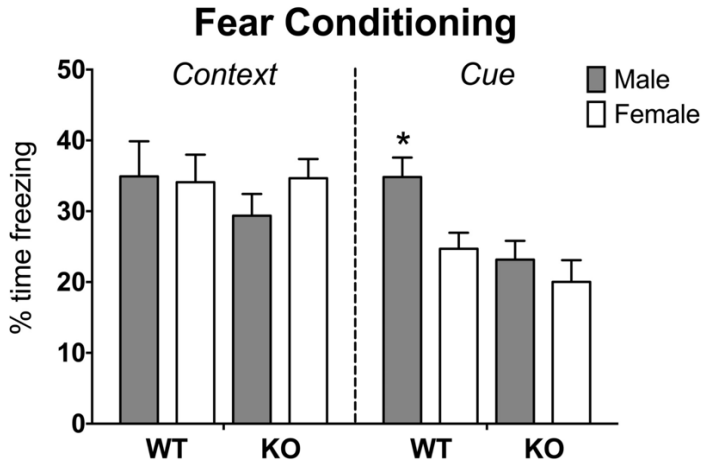

Figure 1: Impaired retention of cued fear memory in Calb-KOs
Mean ± SEM percent of time spent freezing for context-specific and cue-specific fear conditioning expressed as a percentage of baseline freezing: (Context trial freezing - Baseline trial freezing / Total time) X 100% and (Cue trial freezing – Baseline trial freezing / Total time) X 100%. * = Male WT significantly different from all other groups, p<0.05.

Calb-KO mice show less anxiety-like behavior on the elevated plus maze

Calbindin knockout mice spent more time than wild type mice exploring the open arms of the EPM ($F(1,29)=9.78$; $p<0.004$) (Figure 2a). KO mice spent more time turning around in the open arms ($F(1,29)=14.40$, $p<0.001$) (Figure 2c) and made more turns than WT mice ($F(1,29)=9.67$, $p<0.005$). The genotype effect on time spent exploring the open arms mice remained significant even after we subtracted the amount of time spent turning around from total time in the open arms $F(1,29)=10.81$; $p<0.004$). There were no effects of sex nor were there any sex by genotype interactions. Additionally, WT mice spent more time in the closed arms than KO animals ($F(1,29)=10.67$; $p<0.003$) (Figure 2b) but no other effects or interactions were noted. No significant effect of sex was found in the total number of crosses (open, closed, and center areas) made during the test. However, there was a trend towards a genotype effect ($F(1,29)=3.9$ $p=0.06$): KO mice made more crosses in the maze than WT mice (Figure 2d).

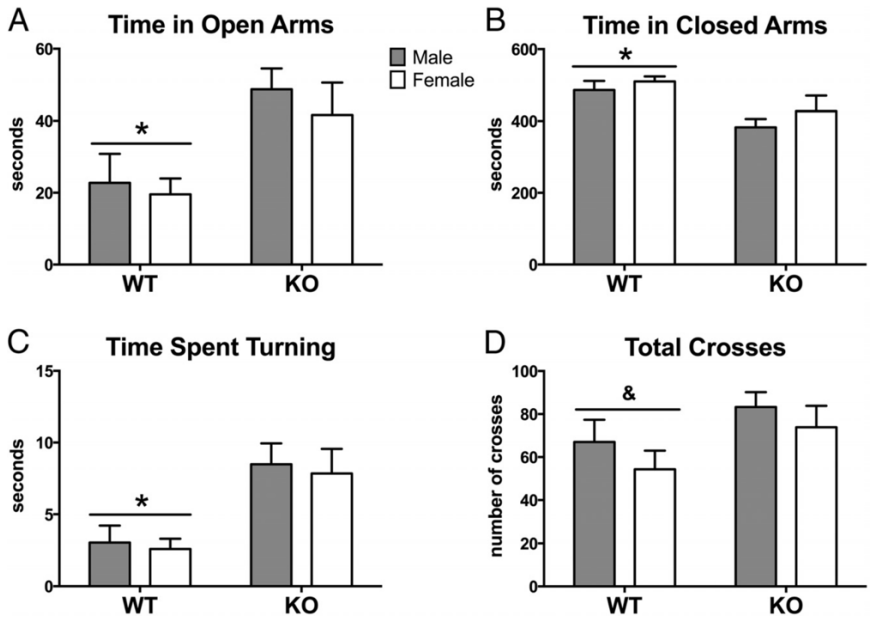

Figure 2: Decreased anxiety-like behavior in Calb-KO mice on the elevated plus maze
Mean ± SEM time (seconds) spent (a) in the open arms, (b) in the closed arms, and (c) turning around in the open arms during the 10-minute (600-second) test. Total crosses between open, closed, and center portions of the maze represented in (d). * = Significant main effect of genotype, $p<0.05$. & = Genotype trend, $p=0.06$

Male Calb-KO mice have enhanced social interactions

The duration of social interactions during the social preference test proved to differ by genotype and/or sex. Analyses revealed main effect of genotype, with KO mice spending more time than WT mice investigating the female stimulus mouse ($F(1,35)=4.58$, $p<0.04$) (Figure 3a). There was also an overall effect of sex, in which females of both genotypes investigated the male stimulus mouse less than males of both genotypes ($F(1,35)=11.23$, $p=0.002$) (Figure 3b), however no interaction was noted. No group showed a significant sexual preference, calculated by subtracting time spent with male from time spent with the female, nor were there differences between the groups for this measure. Interestingly, the time spent investigating both stimulus mice of either sex (male and female) was significantly affected by sex, genotype, and we noted an interaction between these factors ($F(1,35)=16.04$, 5.13, 4.46; $p<0.0003$, 0.03, 0.05 respectively).

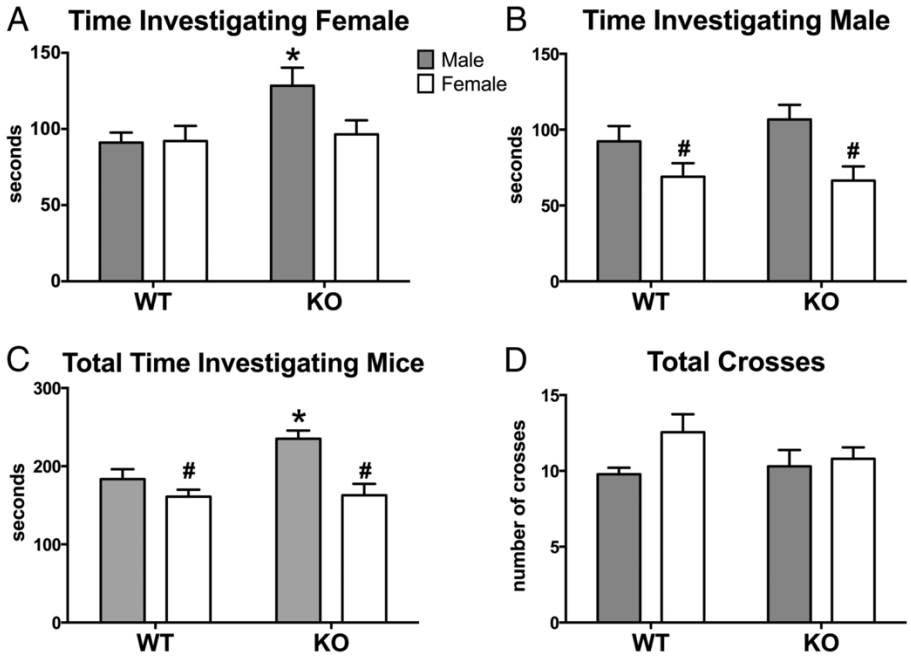

Figure 3: Increased investigation during the social preference task by male KOs
Mean ± SEM time (seconds) spent investigating (a) female stimulus mouse, (b) male stimulus mouse, (c) total time with either stimulus. Total number of transitions between different compartments of the three-chambered box represented in (d). # = Significant main effect of sex, $p<0.05$. * = Significantly different from all other groups, $p<0.05$.

The effects were caused by the male KO mice, which spent more time with the stimulus animals than females or WT males ($p<0.05$) (Figure 3c). Lastly, the total number of entries into each chamber was not significantly different by genotype nor was there a sex by genotype interaction (Figure 3d). There was a trend for a sex difference in which females were more active than males ($F(1,35)=3.74$, $p=0.06$).

Sexually dimorphic genes in the prefrontal cortex and amygdala

Consistent with our previous research (Abel et al., 2011), wild-type females expressed more *Calb1* mRNA than males in the prefrontal cortex ($T=2.85$, $p<.007$), but not in the amygdala (data not shown). We also noted region-specific, sexually dimorphic expression of estrogen receptor alpha (*Esr1*), GABA-A receptor subunit beta (*Gabrb1*), and methyl CpG binding protein 2 (*Mecp2*). In the amygdala, all three genes were more highly expressed in females than males regardless of genotype (*Esr1*: $F(1,29)=12.7$, $p<.001$; *Gabrb1*: $F(1,31)=4.92$, $p<.034$; *Mecp2*: $F(1,28)=4.93$, $p<.035$) (Figure 4a). In contrast, the opposite pattern was true in the prefrontal cortex: males had higher expression than females (*Esr1*: $F(1,27)=8.59$, $p<.007$; *Gabrb1*: $F(1,30)=17.03$, $p<.00001$; *Mecp2*: no sex difference) (Figure 4b). Additionally, there was significantly higher

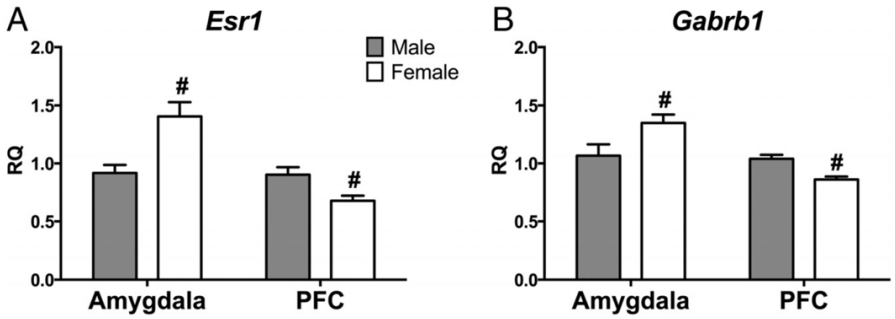

Figure 4: Sexually dimorphic expression of *Esr1* and *Gabrb1* in amygdala and PFC independent of genotype
Relative quantity (RQ) (a) estrogen receptor alpha (*Esr1)* and (b) GABA receptor subunit beta (*Gabrb1*) mRNA in two brain areas: amygdala and prefrontal cortex (PFC). Wildtype and knockout RQ values are combined by sex. Grey bars = Mean + SEM wildtype male + knockout male, white bars = Mean + SEM wildtype female + knockout female. # = Significant main effect of sex within brain region, $p<0.05$.

expression of brain-derived neurotrophic factor (*Bdnf*) in the amygdala of WT males than in WT females (F(1,29)=8.17, p<.009) (Figure 5a). There were no significant effects noted in the expression of *Bdnf exon IV* in the amygdala (Figure 5b).

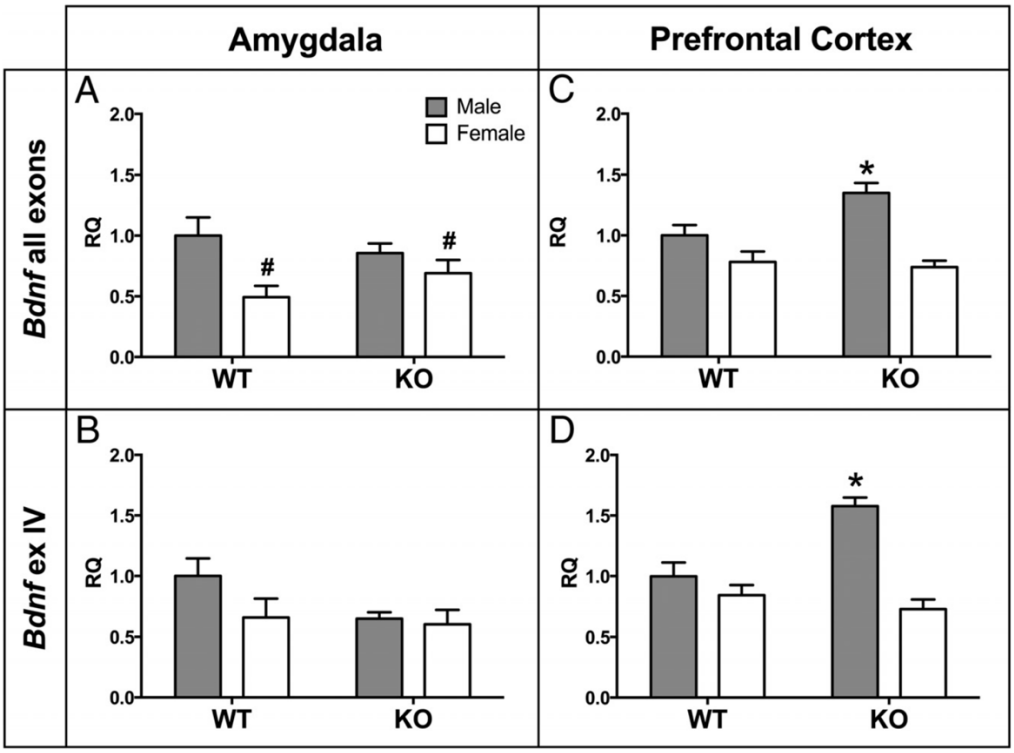

Figure 5: Elevated *Bdnf* expression in male Calb-KO PFC, but not amygdala
Mean + SEM Relative quantity (RQ) *Bdnf* (all exons) mRNA expressed in the (a) amygdala and (c) prefrontal cortex. RQ values of *Bdnf* exon IV mRNA in the (b) amygdala and (d) prefrontal cortex. # = Significant main effect of sex, p<0.05 * = Significantly different from all other groups, p<0.05.

Genes affected by Calb-KO in the amygdala and PFC

In the amygdala, there was a significant main effect of genotype (KO<WT) on corticotropin releasing hormone receptor 1 (*Crhr1*: F(1,30)=4.69, p<.04) (Figure 7a), histone deacetylase 4 (*Hdac4*: F(1,30)=4.48, p<.04) (Figure 6b), and BDNF receptor TrkB (*Ntrk2*: F(1,31)=12.08, p<.002) (Figure 6c). However, genotype effects on *Hdac4* and *Ntrk2* predominated in the males as indicated by significant sex by genotype interactions for both genes [*Hdac4:* (F(1,30)=8.76, p<.006) and *Ntrk2*: (F(1,31)=16.54, p<.00001)]. Moreover, post-hoc tests revealed

an effect on *Crhr1* limited to males in which WT males had higher levels than KO males ($p<.05$).

Similar to the amygdala, the male knockout group also drove several genotype effects in the prefrontal cortex. Male KO mice expressed significantly more *Bdnf* in the PFC compared to WT males as indicated by a significant sex by genotype interaction ($F(1,29)=6.52$, $p<.017$) (Figure 5c). More specifically, exon IV of *Bdnf* was increased in KO compared to WT mice ($F(1,29)=5.97$, $p<.02$) and a significant interaction between sex and genotype ($F(1,29)=14.29$, $p<.0008$) limited the knockout effect to males (Figure 5d). Expression of the GABA synthesis enzyme glutamate decarboxylase (*Gad67*) in the PFC was also significantly reduced in KO males as compared to WT males, but female knockouts were unaffected ($p<.05$) (Figure 6d). Unlike the amygdala, however, expression of *Hdac4* was unaffected by genotype or sex (Figure 6e). In contrast, gene expression was reduced in KO mice of both sexes for the BDNF receptor gene, *Ntrk2* ($F(1,30)=5.97$, $p<.021$) (Figure 6f).

Only two of the genes investigated showed significant main effects driven by female knockouts. In the prefrontal cortex, we noted a significant sex effect in glucocorticoid receptor (*Nr3c1*) expression where females were greater than males ($F(1,28)=6.13$, $p<.02$). However, a significant interaction between sex and genotype ($F(1,28)=6.52$, $p<.02$) showed that the sex effect was specific for KO mice: female KOs had higher expression than male KOs only ($p<.05$) (Figure 7b). Histone deacetylase 3 (*Hdac3*) expression was also significantly higher in the amygdala of KO as compared to WT mice ($F(1,31)=4.18$, $p<.05$) (Figure 6a). *Hdac3* expression also tended to be higher in females compared to males, however, the interaction between sex and genotype was not statistically significant ($F(1,31)=3.93$, $p=.06$). Finally, there were no significant genotype or sex effects on the expression of vasopressin (*Avp*) and estrogen receptor beta (*Esr2*) in either brain area ($p>.05$) (Table 2).

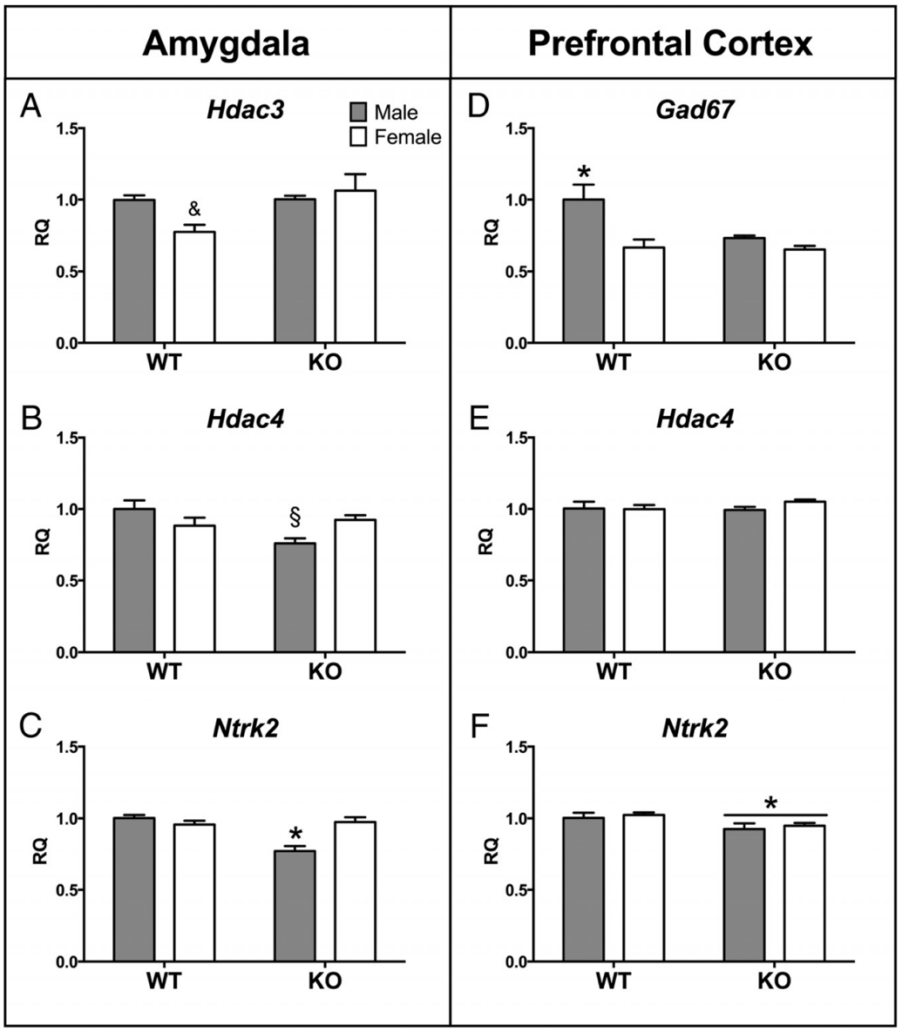

Figure 6: Genotype-dependent expression changes in amygdala and PFC
Mean + SEM RQ values for expression of (a) Histone deacetylase 3 (*Hdac3)* in the amygdala; histone deacetylase 4 (*Hdac4*) in the (b) amygdala and (e) in PFC; Neurotrophic tyrosine kinase receptor 2 (*Ntrk2*) in the (c) amygdala and (f) in PFC. & - Trend for a sex by genotype interaction: FKO>FWT, p=0.06. § = Significantly different from same-sex control group, $p<0.05$. * = Significantly different from all other groups, $p<0.05$.

Stress response is not affected by genotype

As expected, serum corticosterone concentrations significantly increased immediately following restraint stress (Repeated measures ANOVA: $F(1,25)=46.68$, $p=0.00003$). Corticosterone concentrations were unaffected by genotype, nor was there a stress by genotype interaction ($F(1,25)=0.82, 0.18$; $p>0.05$ respectively) (Figure 7c). Additionally, there was no effect

of trial (i.e. stressed on first or second trial) on serum corticosterone concentrations ($F(1,25)=0.97$, $p>0.05$).

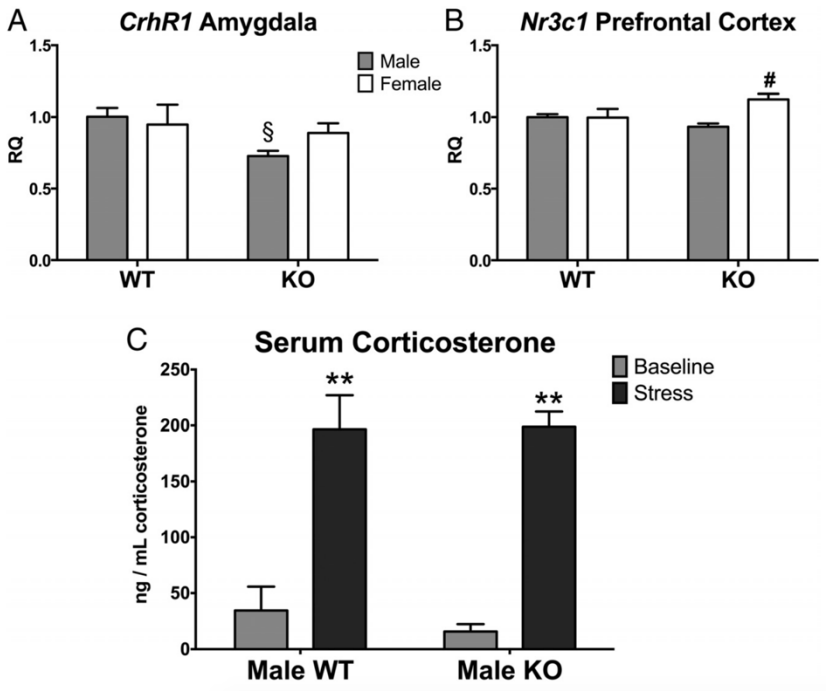

Figure 7: Calb-KO affects stress-related genes in the brain, but not serum corticosterone levels in males Mean ± SEM RQ values of (a) corticotropin releasing hormone receptor 1 (*Crhr1*) in the amygdala, (b) glucocorticoid receptor (*Nr3c1*) in the prefrontal cortex, (c) Serum corticosterone levels in wildtype and knockout males at baseline (grey bars) and 15 minutes after a 15-minute restrain stressor (dark grey bars). § = Significantly different from WT male group, $p<0.05$. # = Significantly different from KO male group, $p<0.05$. ** = Significant main effect of stress, $p<0.05$.

		Amygdala			Prefrontal Cortex		
Gene		Sex Effect	Genotype Effect	Sex-Genotype Interaction	Sex Effect	Genotype Effect	Sex-Genotype Interaction
Avp	Vasopressin	NS	NS	NS	NS	NS	NS
Bdnf (all exons)	Brain-derived neurotrophic factor	M > F, $P < .008$	NS	NS	M > F, $P < .00\,001$	NS	MKO > MWT, FWT, FKO
Bdnf exon IV	Brain-derived neurotrophic factor	NS	NS	NS	M > F, $P < .00\,001$	KO > WT, $P < .02$	MKO > MWT, FWT, FKO
Calb1 (WT only)	Calbindin	NS	NA	NA	F > M, $P < .007$	NA	NA
Crhr1	CRH receptor 1	NS	WT > KO, $P < 0.04$	NS	NS	NS	NS
Esr1	Estrogen receptor-α	F > M, $P < .001$	NS	NS	M > F, $P < .007$	NS	NS
Esr2	Estrogen receptor-β	NS	NS	NS	NS	NS	NS
Gabrb1	GABA A receptor, subunit-β	F > M, $P < .034$	NS	NS	M > F, $P < .0003$	NS	NS
Gad67	Glutamate decarboxylase 67	NS	NS	NS	M > F, $P < .005$	WT > KO, $P < .045$	MWT > FWT, FKO, MKO
Hdac3	Histone deacetylase 3	NS	KO > WT, $P < .05$	NS trend FKO > FWT	NS	NS	NS
Hdac4	Histone deacetylase 4	NS	WT > KO, $P < .043$	MWT > MKO	NS	NS	NS
Mecp2	Methyl CpG binding protein 2	F > M, $P < .035$	NS	NS	NS	NS	NS
Ntrk2	Neurotrophic tyrosine kinase receptor 2	F > M, $P < .015$	WT > KO, $P < .001$	MKO < MWT, FWT, FKO	NS	WT > KO, $P < .02$	NS
Nr3c1	Glucocorticoid receptor	NS	NS	NS	F > M, $P < .02$	NS	FKO > MKO

Abbreviations: F, female; M, male; NA, not applicable; NS, not significant. Fisher's least significant differences test was used for all interactions ($P < 0.05$).

Table 2: Statistical summary of qPCR results from amygdala and prefrontal cortex
M: male, F: female, WT: wildtype, KO: knockout, NS: not significant, NA: not applicable, Fisher LSD $p<0.05$ for all interactions

Discussion

We predicted that loss of calbindin would influence the affective and social behaviors we selected to test here. Calbindin is present in specific prefrontal cortical and limbic brain regions that underlie emotional and cognitive behavior (Baimbridge et al., 1992, 1982; Celio, 1990; Rossignol, 2011). We also predicted that, since *Calb1* is sexually dimorphic in the mouse prefrontal cortex, the elimination of *Calb1* would likewise eliminate sexual dimorphism in some of the behaviors we tested (Abel et al., 2011; Bodo and Rissman, 2008). In one of the three behaviors we tested, fear conditioning, we found that sex differences in WT mice were not present in the KO mice. In another behavior, social investigation, sex differences were revealed in the KO mice that were not present in WT animals. It is worth noting that we have previously observed a sex difference in wildtype C57 mice using a Y-maze to test partner preferences between males and females, in gonadectomized and hormone-treated adults (Bodo and Rissman, 2008; Kauffman et al., 2007). We noted a significant decrease in anxiety-like behavior on the elevated plus maze in

knockout mice. We also noted that KO mice tended to show less anxiety-like behavior and increased exploratory behavior in the other tests we performed. For example, in the social interaction test, KO males spent more time interacting with the stimulus mice than the other groups, which suggests a reduction in anxiety-like behavior. Moreover, in the cued portion of the fear conditioning task, KO mice displayed less freezing behavior than the WT males, also indicative of a reduction of anxiety-like behavior in Calb-KO mice.

Because an earlier study reported an increase in pain threshold in Calb-KO mice (Egea et al., 2012), we tested reaction times to shock and all mice reacted with the same, with very short latencies. Previously it has been documented that Calb-KO mice have no auditory impairments and we assume they can hear the tone conditioned to the shock (Airaksinen et al., 2000). An alternate interpretation of our data is that the KO male mice are impaired in their ability to form or retain memory of an audible cue. This seems unlikely given their normal performance on the context portion of the task but nevertheless should be considered. Alternatively, the genotype effect may be due to the loss of a calbindin-dependent sex difference in fear behavior. While only a trend, we did note a sex difference in WT mice. Freezing behavior in response to an auditory cue was higher in WT males than in WT females, despite the fact that all groups of mice were gonadectomized and treated with estradiol. Therefore, ruling out adult differences in circulating gonadal hormones, the normal sex difference in fear behavior may depend on the presence of calbindin. Another interpretation is that the Calb-KO mice, and WT female mice are less anxious than males and thus are less prone to freezing in response to the conditioning tone.

In fact, our data from the EPM support this notion. Calb-KO mice spent more time in the open arms than do WT mice. Conversely, WT mice spent significantly more time in the closed arms and less time exploring the open arms compared to KO mice. These data indicate that reduced anxiety-like behavior is associated with the loss of calbindin. Due to potential differences in motor coordination(Barski et al., 2003), we predicted that KO mice would take longer to turn around in the open arms of the maze than WT mice. Even after correcting for the time spent turning around

in the open arms, the genotype effect remained significant. Given that the *Calb-KO* mice appeared to have difficulty maneuvering in the EPM, it is particularly impressive that they select to spend more time in open arms and less time in the secure closed arms of the maze as compared with WT mice.

We did not observe any sex differences in any aspect of EPM behavior for either genotype, unlike sex differences observed in most studies when gonad-intact mice are tested (Rilett et al., 2015; Rodgers and Cole, 1993; Xu et al., 2015). Thus, our data also suggest that differences in circulating gonadal hormones between adult males and females produce previously noted sex differences in anxiety-like behavior on the EPM. However, research using gonadectomized, testosterone, or blank implanted four core genotype mice indicates that sex chromosome complement also affects anxiety-like behavior. In the elevated plus maze, mice with two X chromosomes spent significantly more time in the open arms than XY mice, regardless of gonadal sex (Seney et al., 2013a). However, we were unable to distinguish between gonadal and chromosomal sex differences in the current study.

In order to begin to unravel the molecular underpinnings of the behavioral results described above, we used qPCR to quantify gene expression changes in amygdala and prefrontal cortex tissue harvested from wildtype and knockout gonadectomized adult mice of both sexes. Three distinct classes of the genes examined were impacted by *Calb1* knockout. These include genes associated with BDNF signaling, GABA neurotransmission, and histone protein deacetylation. Many of these genes are strongly associated with many of the behaviors we examined. In the PFC, the BDNF receptor *Ntrk2* was significantly down regulated in both male and female KO animals. The differences between male Calb-KO mice and the other groups drove the sex-specific genotype effects we observed in the expression of *Ntrk2* in the amygdala (male KO less than all groups) and *Bdnf/Bdnf exon IV* in PFC (male KO greater than all groups). Numerous studies have shown a causal link between BDNF and calbindin. For example, BDNF induced the expression of *Calb1* mRNA and protein in cultured hippocampal and cortical neurons (Fiumelli et al., 2000; Ip et al.,

1993). Additionally, *Bdnf* KO mice have reduced *Calb1* expression in the hippocampus and cortex despite a normal density of GABAergic neurons(Jones et al., 1994). A plethora of evidence has linked BDNF to *Calb1* expression, but to our knowledge, we are the first to demonstrate reciprocity between the two genes by showing changes in *Bdnf-Ntrk2* gene expression in the amygdala and PFC as a result of calbindin knockout.

The latter finding could offer another explanation as to why male KO mice failed to freeze in the cued fear conditioning paradigm. The acquisition of conditioned fear is dependent on normal Bdnf signaling through TrkB receptors in the amygdala(Heldt et al., 2014; Musumeci et al., 2009; Rattiner et al., 2004). Changes in *Bdnf* expression in the PFC also align with our other behavioral results. Overexpression of *Bdnf* in forebrain neurons decreased anxiety-like behavior of mice on the EPM, similar to the anxiolytic phenotype of the Calb-KO mice(Weidner et al., 2014). We also noted that social investigation in male Calb-KO mice is increased as compared with WT males, an effect that parallels the genotype effects on *Bdnf* expression in the PFC. These data are supported by a study in mice which showed that increased affiliative behavior during a social interaction test is associated with significantly higher expression of *Bdnf* in the frontal cortex of mice (Branchi et al., 2013). *Bdnf IV* transcription is regulated by Ca^{2+} influx through voltage-sensitive calcium channels (Ca_v1 L-type CA^{2+}) that are activated in cortical neurons (Ghosh et al., 1994). Calbindin co-localizes with these calcium channels in mouse cortex (Fiumelli et al., 2000; Ip et al., 1993), which suggests one possible route by which calbindin could regulate the expression of *Bdnf*. However, it remains to be determined whether the effects of *Calb1* knockout on TrkB are due to the loss of calbindin itself, or secondary effects caused by feedback regulation of the receptor by BDNF (Haapasalo et al., 2002).

Expression of the GABA synthesis enzyme, glutamate decarboxylase 67 (GAD67), in the prefrontal cortex was reduced in male Calb-KO mice compared to WT controls. We also found a sex difference in the WT mice, wherein males were greater than females; this was attenuated by the loss of calbindin. This expression pattern parallels the changes we observed in the social

preference task. KO males spent significantly more time investigating the stimulus mice during the test than any other group. Similarly, maternal immune activation is associated with decreases in *Gad67* expression in the medial prefrontal cortex of the offspring and concomitant changes in behavior in a social interaction task (Basta-Kaim et al., 2015). Others have reported associations between of *Gad67* and social behavior, such as social odor preference (conspecific urine vs. water), sociability (novel mouse vs. novel object), and social preference (familiar mouse vs. novel mouse) (Sandhu et al., 2014; Zhang et al., 2014). In the prefrontal cortex of schizophrenic patients, expression of *Gad67* and the TrkB receptor are positively correlated (Hashimoto et al., 2005). The authors suggest that decreased TrkB may underlie dysfunction of GABAergic neurons in schizophrenia. TrkB signaling may also mediate the effect we observed in the PFC of Calb-KO mice, as *TrkB* and *Gad67* expression were both decreased compared to controls.

In the amygdala, two classes of histone deacetylases were affected in response to a loss of calbindin. Male Calb-KO mice showed reduced levels of *Hdac4* and increased expression of *Hdac3* in the amygdala. Class I and IIa HDACs have opposite effects on learning and memory. Class I HDACs, such as HDAC3, tend to impair memory and cognition, whereas HDAC4, a class IIa HDAC, improves learning (Ronan et al., 2013). In the rat amygdala, cued fear conditioning resulted in increased histone acetyl-transferase activity and treatment with HDAC3 reduces fear memory and conditioning (Yeh et al., 2004). Similarly, pharmacological inhibition of HDAC3 activity significantly improved long-term memory in spatial learning tasks (McQuown et al., 2011). In contrast, silencing *Hdac4* disrupts spatial learning and memory (Sando 3rd et al., 2012). Conditional forebrain only *Hdac4* knockout animals spend more time exploring the open arms of the EPM and exhibit reduced fear in a context-dependent fear conditioning paradigm (Kim et al., 2012). Similarly, *Calb1* knockouts show decreased anxiety, reduced fear, and express less *Hdac4*. In addition, because the amygdala is required for cue-dependent associative fear memory (Phillips and LeDoux, 1992), it follows that we only observed HDAC expression changes in the amygdala and not the PFC. Synaptic activity-dependent calcium signaling affects the nuclear export of

HDAC4, which may explain calbindin's role in regulating HDAC activity and expression (Backs et al., 2008). We speculate that the synergistic interaction of the two HDACs in the amygdala are part of a network of genes that underlie the impaired retention of cued fear and decreased anxiety in *Calb1* knockouts.

Changes in corticotropin releasing hormone receptor (*Crhr1*) expression may also partially explain the fear and anxiety phenotypes of calbindin knockouts. Male Calb-KO mice express significantly less *Crhr1* in the amygdala than WT males. CRH and its receptor in the amygdala act as crucial regulators of fear memory. Activation of CRHR1 receptors in the basolateral amygdala enhances fear memory consolidation (Hubbard et al., 2007), whereas antagonizing CRHR1 in the central amygdala attenuates stress-induced freezing behavior (Swiergiel et al., 1993). Genetic variations in the *Crhr1* gene have even been shown to affect fear acquisition behavior in humans (Heitland et al., 2013). Additionally, *Crhr1* in the amygdala and forebrain is known to play a critical role in anxiety-related behaviors, which serves to further explain the anxiolytic phenotype of Calb-KO mice (Gray et al., 2015; Muller et al., 2003; Timpl et al., 1998). We also noted gene expression changes in another regulator of the stress and anxiety response, the glucocorticoid receptor (*Nr3c1*). Female Calb-KO mice express significantly more *Nr3c1* in the prefrontal cortex than male KO mice, whereas there was no sex difference in WT mice. Based on the genotype-dependent effects on expression of *Crhr1* and *Nr3c1* in the amygdala and prefrontal cortex, respectively, we hypothesized that the stress axis (hypothalamic-pituitary-adrenal, HPA) would be affected by the loss of calbindin. For example, mice lacking *Crhr1* have a decreased stress-induced corticosterone release and atrophy of the adrenal gland (Timpl et al., 1998). However, in this first-pass assessment of the HPA axis we did not note any differences in serum corticosterone levels at baseline or post-stress between male Calb-KO and WT mice. Therefore, we conclude that the significant behavioral differences between genotypes are due to changes in gene expression within the limbic system, and not via a secondary effect of stress hormones.

We found several sex differences in gene expression in the amygdala and PFC. Firstly, we

replicated our previous work by showing that *Calb1* expression is sexually dimorphic and biased to females in the prefrontal cortex (Abel et al., 2011). As expected, we did not find a sex difference in *Calb1* expression in the amygdala. Estrogen receptor alpha (*Esr1*) mRNA was sexually dimorphic in the PFC; expression in males was significantly greater than in females regardless of genotype, and the opposite was true in the amygdala. This finding is in contrast to other studies that reported the opposite direction of the sex differences in *Esr1* expression in postnatal day 10 prefrontal cortex (Wilson et al., 2011), and no sex difference in the PFC of postnatal day 21-25 mice (Abel et al., 2011). Early post-natal and pre-pubertal mice have very low levels of circulating sex hormones, as they have yet to undergo hormonal reorganization of the brain during puberty. This may explain why our current results using adult estradiol-implanted gonadectomized mice are different from prior work.

GABA-A receptor subunit beta (*Gabrb1*) expression was also sexually dimorphic in opposing directions in both brain areas: males had higher expression than females in the PFC and the opposite was true for the amygdala. This sex difference is interesting considering the important role of *Gabrb1* in sexually dimorphic disorders, like autism (Fatemi et al., 2010; Ma et al., 2005). We also noted a sex difference in expression of methyl CpG binding protein 2 (*Mecp2*) in the amygdala only. Females expressed higher levels of this X-chromosome gene associated with the neurodevelopmental disorder, Rett Syndrome, than did males. Others have reported a similar sex difference in *Mecp2* expression in the post-natal day one rat amygdala (Kurian et al., 2007), which indirectly supports our result by suggesting that the sex difference is organized by early estradiol exposure. The same group also showed that sexually dimorphic *Mecp2* expression in the amygdala is required for the development of behavioral sex differences in later life (Kurian et al., 2008).

Notably, we reported no effect of *Calb1* knockout on any of the genes just described. However, it remains unknown if androgen signaling plays an important role in modulating the effects of calbindin in the brain. In this study, all mice were gonadectomized and estrogen-treated as adults to minimize activational effects of gonadal hormones. The sex differences described

above were significant despite equalized estradiol levels, which suggests an organizational effect of steroid hormones/receptors and/or sex chromosome complement (Abel et al., 2011; Seney et al., 2013b). Therefore, we interpret our findings to reflect sex differences in gene expression caused by organizational effects of hormones and sex chromosomal complement rather than the activational effects of sex hormones.

A number of previous studies have indirectly examined the relationship between calbindin and affective and social behavior via manipulations of the rodent environment. For example, early maternal deprivation imposes restraints on the density of calbindin-GABA positive neurons in the paraventricular region of the hypothalamus of peri-adolescent rats. These animals interact more with an unfamiliar conspecific and demonstrated less anxiety-like behavior compared to controls (Giachino et al., 2007). Likewise, in juvenile *Octodon degus*, a rodent species that shows biparental care, the absence of the sire in the nest causes brain region- and age-specific changes in *Calb1* expression in the hippocampus, cortex, and bed nucleus of the stria terminalis, areas that regulate stress and anxiety (K. Braun et al., 2011; Gos et al., 2013). Finally, early social isolation reduces the amount of calbindin in prefrontal cortex (PFC) neurons in male rats (Pascual et al., 2007). The authors suggested that a reduction of calbindin in GABAergic interneurons compromised their ability to interact normally with medial PFC pyramidal neurons, which connect to subcortical limbic networks underlying fear and anxiety. Therefore, these behaviors would be disrupted if calbindin is reduced.

Conclusion

To our knowledge, this is the first set of studies to directly examine how calbindin affects affective and social behaviors in rodents. In this study, we chose a more direct route by using *Calb1* knockout mice. Moreover, we chose to focus on global, as opposed to a region-specific, knockout because *Calb1* is expressed ubiquitously across multiple brain regions that are intimately involved in regulating emotional behaviors including fear, anxiety, and sociability. We reported significant

genotype- and sex-dependent gene expression effects of *Calb1* knockout in two of these brain areas: the prefrontal cortex and amygdala. We also demonstrated that loss of calbindin significantly alters fear, anxiety, and social behaviors in adult mice, thereby directly linking calbindin with affective behaviors.

Chapter V

Discussion and conclusions

Summary

The aim of this dissertation was to examine the impact of specific genetic and environmental factors on neurobehavioral outcomes in mice. Firstly, I designed an experiment to test the effect of gestational or paternal exposure to BPA on offspring behaviors. BPA primarily affected behavior via gestational exposure. I found that gestational BPA significantly increased the median frequency and duration of ultrasonic vocalizations in infant mice. Juvenile mice exposed to BPA during gestation also displayed significantly more anxiety-like behavior than controls, in agreement with the literature. However, juvenile social recognition was unaffected by both paternal and maternal BPA administration. In adults, I found significant sex differences in operant responding in a reversal learning task. Females had higher accuracy of responding and received more rewards than males. Additionally, control females earned significantly more rewards during the training phase than BPA females. Although we found no notable effects of preconception paternal exposure to BPA, the sires exposed to BPA weighed significantly more than control sires after mating.

The Rissman lab had previously demonstrated that direct and transgenerational exposure to BPA impacted social behaviors in juvenile mice (Wolstenholme et al., 2013, 2012). However, the mechanism behind these behavioral changes was not entirely clear. A study from our lab indicated that transgenerational BPA exposure altered ERα immunoreactivity in sexually dimorphic brain areas in adult F3 females (Goldsby et al., 2017). Therefore, I hypothesized that molecular markers in other brain areas regulated by sex hormone exposure would be impacted in F3 generation BPA lineage adults. In the AVPV and SDN-POA, I found the expected sex differences in immunoreactivity. Female dopaminergic cells (particularly those co-expressing ERα) greatly outnumbered male cells in the AVPV. The volume of calbindin immunoreactivity in the SDN was significantly larger in males than in females. However, unlike our previous study (Abel et al., 2011), I found no significant effect of sex on calbindin protein levels in the cerebellum.

Transgenerational exposure to BPA did not significantly affect sexually dimorphic dopaminergic cell populations in the AVPV or calbindin immunoreactivity in the SDN-POA. Likewise, calbindin protein in the cerebellum was unaffected by transgenerational exposure to BPA.

Lastly, I examined the effects of calbindin knockout on anxiety-like and social behaviors, fear conditioning, and gene expression in the amygdala and PFC. Calbindin expression has sexually dimorphic expression across several areas of the brain (Abel et al., 2011), so I hypothesized that the loss of calbindin would disrupt sex-specific behaviors and gene expression. In Calb-KO mice of both sexes, anxiety-like behavior on the EPM was decreased compared to WT. The lack of calbindin was associated with an increase in social investigation, specifically in Calb-KO males. Calbindin knockout also attenuated the sex difference in cued fear conditioning by decreasing the freezing response of Calb-KO males compared to wildtype males. These behavioral differences were accompanied by several gene expression differences in key brain areas that regulate emotional behaviors. I found an increase in *Bdnf* mRNA in KO males in the PFC. There were also male-specific decreases in amygdala expression of *Hdac4* and *Ntrk2*, the receptor for BDNF. In the PFC, calbindin KO decreased expression of *Ntrk2* in both sexes and decreased sexually dimorphic expression of the GABA synthesis enzyme, *Gad67*. Lastly, I also reported decreased *Crhr1* expression of knockouts in the amygdala and increased glucocorticoid receptor expression in the PFC of KO females. Despite differences in genes regulating anxiety and fear, calbindin KO males displayed a normal corticosterone response to an acute stressor.

Anxiety and the HPA axis

Calbindin knockout and BPA exposure had opposing effects on anxiety-like behavior as measured by activity on the EPM. Calb-KO adults spent more time in the open arms than wildtypes, whereas juvenile BPA mice spent less time in the open arms compared to controls. Despite their divergent effects, they have roots in the same neuroendocrine system, the HPA axis. In Calb-KO mice, this was demonstrated by gene expression results in the amygdala and PFC. Male Calb-KO

mice expressed less *CrhR1* in the amygdala than wildtype males. Calb-KO females tended to have more glucocorticoid receptor mRNA in the PFC than Calb-KO males, whereas there was no sex difference in wildtypes in PFC.

Although I did not directly test the effect of BPA on the neuroendocrine stress response, there is strong evidence suggesting that early-life BPA exposure can affect the HPA axis. An *in silico* study showed that BPA binds the glucocorticoid receptor as an agonist (Prasanth et al., 2010). Several studies in rats have found that BPA exposure affects basal HPA axis physiology, stress reactivity, *Crhr1* in the pituitary as well as GR levels in hippocampus and PVN in a sex-specific manner (Chen et al., 2014; Panagiotidou et al., 2014; Poimenova et al., 2010; Zhou et al., 2015)**.** These changes were accompanied by increases in anxiety-related behaviors in all of those studies. BPA also affects corticotropin-releasing hormone (CRH) neurons in the bed nucleus of the stria terminalis (BNST), a key brain region in the regulation of the stress response. Prenatal exposure to low doses of BPA resulted in a loss of sexual dimorphism in the number of CRH positive cells in the BNST, but not the preoptic area (Funabashi et al., 2004). These results taken together suggest another mechanism by which BPA can affect neurodevelopment.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), another steroidogenic enzyme, catalyzes the conversion of deoxycorticosterone to corticosterone. Recent research has shown that low doses of BPA *increase* the activity and expression of 11β-HSD1 in human-derived adipocytes (Wang et al., 2013) but *decrease* 11β-HSD1 activity in adult rat Leydig cells (Guo et al., 2012). Facilitation of 11β-HSD1 by BPA in adipocytes promoted adipogenesis and was attenuated by an 11β-HSD1 inhibitor, carbenoxelone, and also by glucocorticoid receptor antagonist, RU486 (Wang et al., 2013). 11β-HSD1 is important for local regulation of corticosterone levels, particularly in the hippocampus (Yau et al., 2015).

BPA also affects transcription of 11β-HSD type 2, which catalyzes the reverse reaction: converting active corticosterone to the inactive metabolite. BPA significantly increases 11β-HSD2

mRNA and protein in human placenta trophoblast cells (Rajakumar et al., 2015). 11β-HSD2 expression in the placenta and fetus serves to protect developing tissues from excess maternal stress (Chapman et al., 2013). Pharmacological inhibition of 11β-HSD2 results in a detrimental behavioral/physiological phenotype similar to prenatally stressed offspring (Welberg et al., 2000). Low activity of 11β-HSD2 in umbilical cord blood is also associated with adverse pregnancy outcomes such as low birth weight and pre-eclampsia (Cottrell et al., 2013). This further suggests that BPA may via the placenta to affect fetal development.

Ultrasonic vocalizations

USVs in infant rodents can be affected by genetic, pharmacological, or early-life environmental manipulations, such as chronic stress. Infant USVs during maternal separation are also correlated with anxiety-life behaviors in later life (Barua et al., 2014; Trezza et al., 2008; Veronesi et al., 2017), as was the case with gestational exposure to BPA. However, there have been no other reports on infant USVs in a BPA exposure model. The mechanism behind the increase in median frequency and call duration in BPA pups reported in chapter two is unclear. In a recent study in rats, perinatal hypothyroidism affected the acoustic characteristics and developmental timeline of pup USVs during isolation (Wada, 2017). Production of USVs decreases after auditory function develops, but the hypothyroid pups continued vocalizing after this point, indicating an auditory developmental delay. Mice are also born deaf, but their auditory function begins around postnatal day 10, which is when the number of USVs in produced by mice sharply decreases (Ehret, 2005; Wiaderkiewicz et al., 2013). The hypothyroid rat pups weighed significantly less than controls (Wada, 2017), though, so this may also reflect a difference in sound production from potentially underdeveloped vocal cords. Although we have not noted any differences in body weight due to BPA exposure at this dose, several studies have reported that BPA may disrupt thyroid hormone function (Chevrier et al., 2013; Sheng et al., 2012). The effect of gestational BPA exposure on infant USVs may be mediated by thyroid hormone disruption.

A study in a rats demonstrated that dopamine receptor D2 function in the striatum and nucleus accumbens both regulate USV production during maternal separation in rat pups (Muller et al., 2008, 2005; Muller and Shair, 2016). BPA affects dopamine D2 receptor and TH expression in the PFC of male rats (Castro et al., 2015a, 2013), which is particularly interesting given that the PFC sends dopaminergic inputs to nucleus accumbens (Brady and O'Donnell, 2004). Ultrasonic vocalizations in adult rodents of both sexes are responsive to changes in dopamine, as well as and gonadal steroid hormone levels (Heckman et al., 2016; Wang et al., 2008). Ovariectomized female rats given estradiol implants emit fewer USVs than OVX females with blank implants in response to their cage mate after social separation (Garcia et al., 2017). Micro-implants of E2 or testosterone within the mPOA of castrated male gerbils restored mounting behavior and USVs during sex behaviors (Holman et al., 1991). In castrated mice, administration of DHT and E2 both restore vocalizations in response to a female stimulus (Nunez et al., 1978; Pomerantz et al., 1983). Similarly, DHT and estradiol benzoate treatment in castrated ArKO males restores social recognition ability and female-oriented USVs (Pierman et al., 2008).

Given the results presented here, future research should investigate the effect of gestational BPA exposure on ultrasonic calling during social or sexual behaviors. Integrating USVs and behavioral interactions may give a more comprehensive picture about potential social deficits (Kabitzke et al., 2015). We have previously demonstrated direct and transgenerational effects of gestational BPA on social behaviors in mice (Wolstenholme et al., 2013, 2012). BPA exposure is associated with decreased *Foxp2* expression, a critical regulator of sex-specific USVs in mice (Wada, 2017), and decreased dopaminergic projections in the cortex neonatal rats (Komada et al., 2014). Therefore, I hypothesize that BPA would affect USVs in juveniles and adults in conjunction with social behavior, potentially across F1 and F3 generations.

Furthermore, considering the connection between anxiety and infant USVs as discussed in chapter two, Calb-KO mice may also exhibit altered USVs. Calb-KO mice had decreased anxiety-like behavior on the EPM as adults. Therefore, I would hypothesize that infant USVs during

maternal separation would be decreased in Calb-KO pups as well. Calbindin is important in the auditory learning circuit of zebra finches (Braun et al., 1991; Pinaud et al., 2007). Calbindin is expressed in the sex-specific premotor pathway in male zebra finches (Martin Wild et al., 2001). An isoform of FOXP2 colocalizes with calbindin in purkinje cells in the cerebellum beginning during embryonic development in mice, further suggesting a role for calbindin in vocalizations (Y. Tanabe et al., 2012).

Calbindin and the cerebellum

Given the functional importance of calbindin in the hypothalamus, cortex, and cerebellum, the disruption of fear, anxiety, and social behaviors in Calb-KO mice makes sense. Calbindin is highly expressed in the cerebellum as a marker of purkinje cells. The cerebellum has bidirectional connections with the limbic system (hippocampus, thalamus, amygdala, hypothalamus, and basal ganglia) as well as brainstem nuclei that innervate the cortex and limbic system (Dean and McCarthy, 2008). In the 1970s, doctors implanted patients with electrodes in the cerebellum in an attempt to control epilepsy. When stimulated, patients reported experiencing intense fear and anger, suggesting that the cerebellum has the potential to regulate mood state in humans (Konarski et al., 2005). Perhaps the loss of calbindin dampened the signals between the cerebellum and limbic system, resulting in the reduced anxiety-like behavior and decreased freezing in response during cued fear conditioning in knockout animals.

The cerebellum also has a strong connection to the regulation of social behavior as indicated by the cerebellar pathology common in individuals with autism spectrum disorder (ASD) (Becker and Stoodley, 2013). In postmortem tissue, purkinje cell size (Fatemi et al., 2002), number and density is reduced in individuals with ASD compared to control tissue (Wegiel et al., 2014). However, standard Nissl staining of the human cerebellum may not yield accurate numbers of purkinje cells and specific calbindin staining in the cerebellum has been proposed as a more reliable marker of purkinje cells (Elizabeth R. Whitney et al., 2008). In a small study of calbindin-

immunopositive purkinje cells in postmortem human cerebellum, found that 3 of the ASD patient brains had normal numbers of purkinje cell but the other 3 patients had abnormal purkinje cell numbers compared to control brains (Elizabeth R Whitney et al., 2008). Volumetric analysis of the cerebellum with magnetic resonance imaging (MRI) has revealed cerebellar enlargement is common in young children with ASD, but by adulthood, cerebellar volume is reduced in ASD brains compared to control (Becker and Stoodley, 2013).

I did not test the effect of direct exposure to BPA on the cerebellum in my experiments, although BPA has documented effects on the cerebellum as discussed in chapter three. Therefore, disturbing cerebellar functioning (as in Calb-KO mice) might have impacted the behavior of BPA offspring. BPA exposure and Calb-KO produced opposite effects on anxiety-like behavior on the EPM. In addition, altered USVs in BPA pups potentially indicate an increased distress response during isolation. Perhaps BPA exposure enhances purkinje cell function, increasing anxiety-like behavior and USVs. The cerebellum also plays a role in motor learning, such as eye blink conditioning, at which females tend to perform better than males (Dalla and Shors, 2009). Differences in cerebellar physiology might also explain the masculinizing effect of BPA on adult female offspring during operant behavior training. Unfortunately, it is impossible to conclude whether the cerebellum mediates the behavioral changes associated with BPA exposure without directly examining the cerebellum of exposed mice.

Dopamine

Another potential mediator of behavioral changes associated with BPA actions is the dopaminergic system. Several rodent studies implicate dopamine the potential mechanism underlying behavioral changes. Increased anxiety-like behavior associated with perinatal BPA exposure in male mice was accompanied by reduced activity of monoamine oxidase B (MAO-B), the enzyme that metabolizes dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC), in the medulla (Matsuda et al., 2012). Furthermore, BPA decreased the ratio of DOPAC to dopamine in

the hippocampus, medulla, and amygdala, indicating reduced activity of MAO-B in those areas. Interestingly, nuclear receptor targets of BPA have been shown to affect MAO-B expression. ERRγ stimulates the expression of MAO-B, but this effect dampened by ERα and ERβ activation *in vitro* (Zhang et al., 2006). Several other papers have shown that the dopaminergic system is sensitive to BPA (Castro et al., 2015a, 2015b; Elsworth et al., 2013; Jones and Miller, 2008; Masuo et al., 2004). For example, BPA exposure in mice decreased the density of tyrosine hydroxylase immunoreactivity in substantia nigra, a midbrain nucleus involved in dopaminergic control of motor activity and reward systems (Tando et al., 2007).

Additionally, BPA inhibited dopamine D3 receptor-mediated G-protein activation in the limbic forebrain (Mizuo et al., 2004). BPA also facilitated dopaminergic activity, most likely via D1 receptors, which enhanced morphine-induced hyperlocomotion and reward behavior (Narita et al., 2006). This could be further explored as a potential mechanism in regulating the decreased responding of F1 BPA females during operant training. Due to the lack of an effect of ancestral BPA exposure on dopaminergic neurons in the AVPV, this seems an unlikely mechanism to explain the transgenerational social behavior changes we have previously observed (Wolstenholme et al., 2013, 2012). However, this does not preclude the involvement of other dopaminergic brain regions in F3 BPA mice.

Operant responding

Disruption of learning and memory processes might impact the capability of mice to perform in an operant conditioning task. Estradiol has a well-documented effect on learning and memory processes (Duarte-Guterman et al., 2015). Additionally, mice lacking neuronal ERRγ in the hippocampus and cortex show significant deficits in spatial learning and memory and hippocampal long-term potentiation (Pei et al., 2015). However, since BPA affected the *amount* of responding and not the *accuracy* of responding in the operant task, it seems unlikely that this effect is due to a disruption of learning and memory. Estradiol also impacts perseverative and compulsive

behaviors. Perseverative behaviors, like marble burying in rodents, are influenced by estrous cycle (Kokras and Dalla, 2014). Estrous cycle influences extinction in a food-reinforced operant conditioning protocol in rats (Flaisher-Grinberg et al., 2009). Additionally, male ArKO mice develop compulsive-like behaviors as adults (obsessive grooming and wheel running), which also suggests a role of sex hormones in perseverative/compulsive behavior (Hill et al., 2007).

Future studies should investigate whether the difference in responding during FR1 and FR3 training of BPA females can be attributed to motor activity, motivation, or compulsive behaviors. Using a progressive ratio schedule, the test animal must systematically increase the number of responses for each successive reinforcer within the same trial, until the requirement becomes so large that the animal stops responding (break point). The higher the break point, the more motivated an animal is to seek a reward. Testing the break point of males and females exposed to BPA for a sucrose or a non-sucrose reward pellet may give insight as to whether BPA and/or sex affect motivation for a palatable reward versus an unpalatable reward. Is this a difference in overall motivation for food or hedonic responding for a palatable reward? At the end of a progressive ratio experiment, an extinction protocol might also give more information about a compulsive responding. In this type of experiment, responding in any of nose-poke holes is not reinforced or rewarded. Perhaps BPA females would extinguish responding before control females, indicating a decrease in compulsive responding. However, as estrous cycle influences these kinds of behaviors, ovariectomy and hormone replacement might be considered in order to reduce variability across testing days.

Conclusions

I described several significant behavioral effects of gestational BPA exposure in mice in chapter two. These included altered ultrasonic vocalizations in pups, increased anxiety-like behavior in juveniles, and decreased responding of adult BPA females during operant conditioning acquisition. Our lab has also published direct and transgenerational social behavior differences in

response to gestational BPA exposure. We have shown that gene expression is impacted by BPA in F1 and F4 in embryonic whole brain tissue (Wolstenholme et al., 2012), and ERα-immunoreactive neurons in sexually dimorphic brain areas is affected in F1 and F3 adult BPA females.

In chapter three I discussed the results of an immunohistochemistry study of the AVPV and SDN-POA in F3 adult brains, as well as analysis of calbindin protein in F3 juvenile cerebellum. I reported significant sex differences in TH/ERα cells in the AVPV and volume of calbindin immunoreactivity in the SDN-POA, as expected. However, in contrast to a previous report from our lab (Abel et al., 2011), I found no sex difference in the amount of calbindin in juvenile cerebellum. There were no significant differences in any of these brain regions due to ancestral BPA exposure, but this does not rule out potential differences in other areas of the adult or juvenile brain.

I also described the results of a study of the calbindin knockout mouse on various behaviors and gene expression in the amygdala and prefrontal cortex. In contrast to BPA exposure, Calb-KO was associated with decreased anxiety-like behavior. Calb-KO also increased social investigation behaviors and decreased cue-induced freezing in a fear conditioning paradigm specifically in males. These behavioral differences were reflected in the gene expression changes I observed in the amygdala and PFC. Genes that were specifically affected in Calb-KO males tended to be associated with the behaviors that had sex-specific patterns. Also, despite changes in some two genes affiliated with HPA axis function, I found no effect of Calb-KO on corticosterone responses to an acute stressor.

Lastly, I proposed several potential mechanistic explanations for the behavioral changes I observed in my BPA F1 experiments and their connections to the Calb-KO study. For example, gestational BPA exposure and Calb-KO had opposite effects on anxiety-like behavior. Ultrasonic vocalizations in pups and anxiety-like behavior seem to be correlated, and both phenotypes are

affected by HPA axis functioning and dopaminergic signaling, which are both systems that are affected by BPA in the literature.

In conclusion, the calbindin knockout study establishes calbindin as an important regulator of social, anxiety, and fear behavior in mice and connects the loss of calbindin to several novel gene expression changes in the amygdala and PFC. Moreover, the results of this dissertation contribute novel data to the ever-expanding literature on behavioral impacts of gestational BPA exposure. (See figure 1)

Lastly, in order to further expand on the research of this dissertation I propose three hypotheses to be addressed in the future of this research:

1. I hypothesize that significant differences in juvenile social behaviors in response to gestational BPA exposure (Wolstenholme et al., 2013, 2012, 2011b) are associated with alterations in ultrasonic vocalizations produced during social interactions, particularly considering published effects of BPA on dopaminergic signaling, HPA axis regulation, and the cerebellum.
2. Furthermore, given the transgenerational effects of BPA on juvenile social behaviors (Wolstenholme et al., 2013, 2012), I hypothesize that ancestral BPA exposure will also increase distress-related USVs of pups during maternal separation and that F3 BPA juveniles will display altered USVs during social interactions.
3. Based on the decreased anxiety-like behavior of Calb-KO mice in adulthood, I hypothesize that calbindin will likewise decrease ultrasonic vocalizations in infants during maternal separation. Also, given the important roles of the hypothalamus and cerebellum in regulating social behavior, the loss of calbindin in those areas may be connected to sex-specific alterations of USVs in juveniles during social interactions as well.

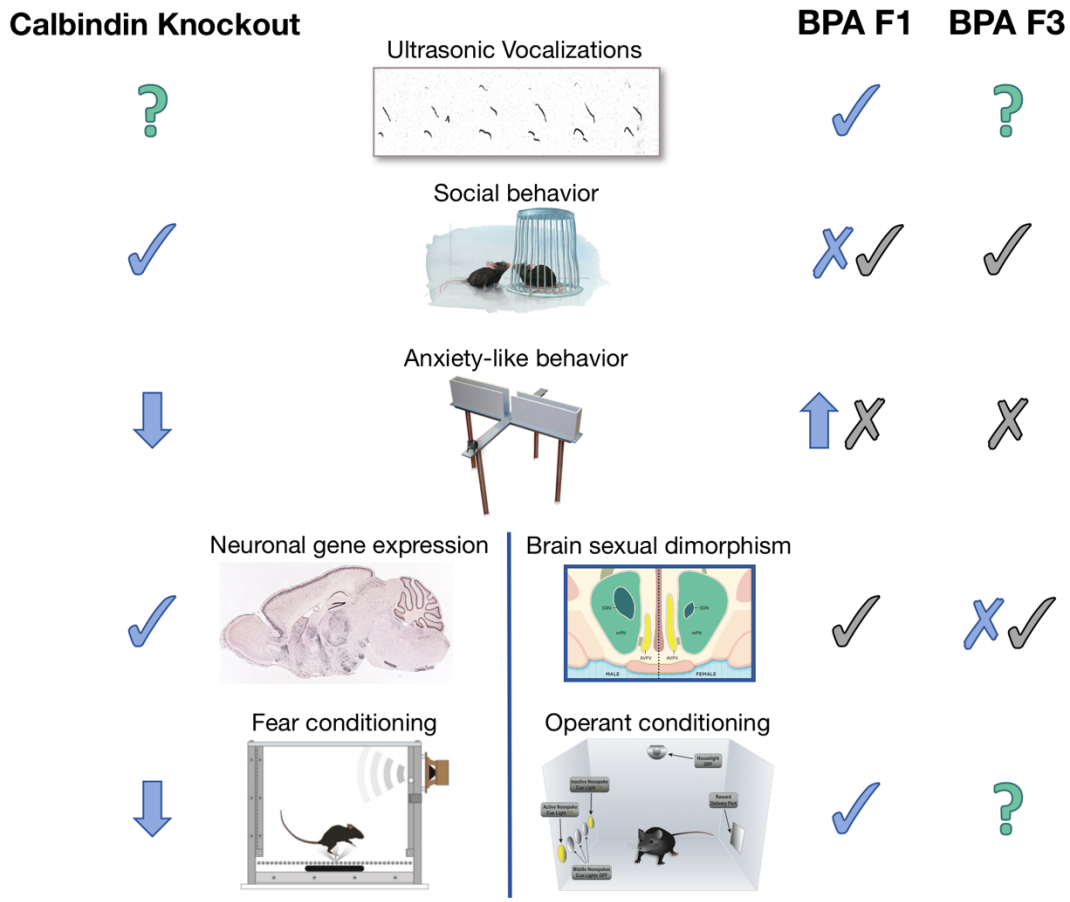

Figure 1: Summary model figure
Blue symbols represent the results presented in chapters 2-4. Gray symbols represent findings from the literature and our previous research. Green question marks represent potential future directions. An ”X” means there was no effect of BPA. A check mark indicates a significant effect of BPA or Calb-KO. Arrows up or down indicate the direction of the difference (i.e. upward arrow indicates an increase)

Appendix A

Sim1 expression in the developing hypothalamus

Introduction

Our previous research in mice indicates that gestational exposure to low doses of the endocrine disrupting chemical bisphenol A (BPA) significantly alters juvenile social behaviors as well as expression of neuropeptides and steroid hormone receptors in the brain (Wolstenholme et al., 2013, 2012, 2011b). Other researchers have shown that gestational exposure to BPA disrupts normal neocortical development by accelerating neuronal differentiation/migration (Nakamura et al., 2007a). Prenatal BPA exposure has also been shown to alter neuronal migration/positioning patterns during cortical histogenesis (Ling et al., 2016; Nakamura et al., 2007b). Oral exposure to BPA from gestational day 8.5 to 13.5 is associated with cortical hyperplasia, accelerated cell cycle exit, and decreased numbers of neural stem/progenitor cells (Komada et al., 2012). Interestingly, very low-dose (0.0068 μM) exposure to BPA leads to precocious neurogenesis in the hypothalamus of zebrafish, whereas a moderate dose (1 μM) did not affect hypothalamic neurogenesis (Kinch et al., 2015).

A complex genetic pattern controls the differentiation of hypothalamic neural progenitor cells (NPCs) that will eventually form the neuroendocrine hypothalamus. Transcription factors with temporal and regional specificities guide the proliferation, migration, and differentiation of these progenitors (Maggi et al., 2015). Single-minded homolog 1, *Sim1*, is important member of the basic helix–loop–helix (bHLH) family of transcription factors required for the development of hypothalamic neurons expressing the neuropeptides oxytocin (OT), vasopressin (AVP), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH), and somatostatin (SS) (Michaud et al., 1998). In *Sim1*$^{-/-}$ mutant mice do not survive past birth: the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus are severely hypocellular and the expression of *OT*, *AVP*, *Trh*, and *Crh* could not be detected (Michaud et al., 1998).

A recent RNA-sequencing experiment from our lab revealed that *Sim1* is differentially expressed in the brains of post-natal day 28 (PN28) males directly exposed to BPA during gestation

(F1 generation) and their progeny, two generations removed from the initial BPA exposure (F3 generation). *Sim1* is therefore an excellent candidate gene given the behavioral phenotypes we have described resulting from BPA exposure in F1 and F3 generations, and the gene's crucial importance in orchestrating the development of the neuroendocrine hypothalamus (Table 1). We conducted qPCR experiments to validate the results of the RNA-sequencing analysis. *Sim1* expression was significantly decreased in the BNST/POA of PN28 F3 mice of both sexes transgenerationally exposed to BPA compared to control. This result, however, is opposite to the 1.339-fold change **increase** in the RNA-seq results. In post-natal day 0 (PN0) hypothalamus, there was a trend for an increase in *Sim1* expression for BPA-lineage mice of both sexes, but this was not statistically significant. The difference in the effect of BPA between these two qPCR experiments may be age-related, or due to brain area-specific expression of *Sim1*.

Generation	Fold Change	P value
F1	2.285	1.39 E-13
F3	1.339	1.69 E-05

Table 1: RNA-seq Results from PN28 BNST/POA BPA Males vs. Control males

Based on the expression levels observed in these experiments, we suspected that *Sim1* expression may change across development. To test this, we compared the levels of *Sim1* across several time points: gestational day 12 (E12) whole brain, E16 hypothalamus, E18 hypothalamus, PN0 hypothalamus, PN6 hypothalamus, and adult hypothalamus. There was a signficant effect of age on Sim1 expression from these samples (Figure 1). *Sim1* expression decreases signficantly from post-natal day 6 through adulthood. No sex differences were observed.

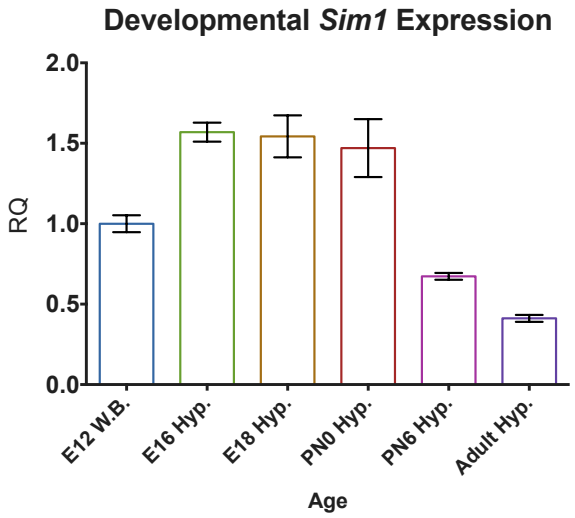

Figure 1: *Sim1* mRNA expression across neurodevelopment
Mean ± SEM relative quantity (RQ) of *Sim1* mRNA. In embryonic day 12 (E12) whole brain (W.B.) n=5 , E16 hypothalamus n=4 , E18 hypothalamus n=6, postnatal day 0 (PN0) hypothalamus n=4, PN 6 hypothalamus n=5, and adult hypothalamus n=6. The endogenous control gene was *Rpl19*.

Given these results, I decided to look at *Sim1* at embryonic/neonatal time points when its expression is higher and its function in neurodevelopment is more relevant. *Sim1* expression in from F1 generation E18 whole brain did not differ significantly between BPA and control, nor was there a sex difference (Two-way ANOVA main effect of BPA: $F(1,28)=0.16$, $p>0.05$; Figure 2A). Similarly, neither BPA nor sex significantly affected expression of *Sim1* in F1 generation PN0 whole brains (Two-way ANOVA main effect of BPA: $F(1,17)=0.03$, $p>0.05$; Figure 2B). However,

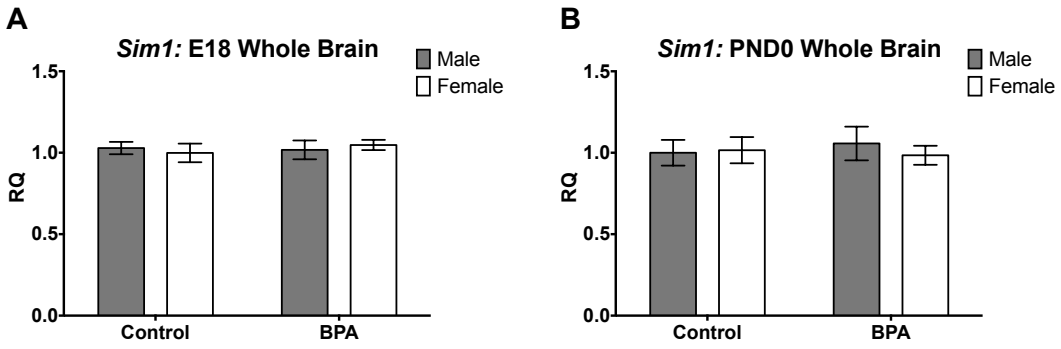

Figure 2: *Sim1* expression in E18 and PN0 Whole Brain
Mean ± SEM relative quantity (RQ) of *Sim1* mRNA in A) embryonic day 18 whole brain n=8 per group, and B) postnatal day 0 whole brain n=5-6 per group. Gray bars represent males and white bars are females. The endogenous control gene was *Rpl19*

because *Sim1* is most highly expressed within the hypothalamus, the lack of a BPA effect could potentially be due to the dilution of transcript in a whole brain sample.

Specific Aim: Determine the effect of gestation BPA exposure on Sim1 expression during hypothalamic development

Rationale: Neurons of the PVN and SON are born between E10.5 and E12.5 and their terminal differentiation takes place between E13.5 and E15.5. *Sim1* expression is first detectible at E10.5 and is restricted to the mantle layer, indicating that PVN/SON neuronal precursors only begin expressing *Sim1* once they leave the ventricular zone and stop proliferating (Chen-Ming Fan et al., 1996; Michaud et al., 1998). Estradiol and BPA are both known to affect proliferation and differentiation of neural stem/progenitors cells (NS/PCs) (Agarwal et al., 2016; Huang et al., 2016; M. Okada et al., 2008; Okada et al., 2010; Tiwari et al., 2015; Yin et al., 2015). Preliminary data discussed above indicate a potential effect of BPA on expression of *Sim1* in F3 juvenile and neonatal brain. The direct effects of gestational exposure on hypothalamic *Sim1* expression have yet to be fully described.

Hypothesis: BPA increases the expression of *Sim1* in the developing hypothalamus.

Approach: To determine the effect of BPA on expression of *Sim1* in the developing hypothalamus, I exposed C57 females to one of two diets: phytoestrogen-free diet (control diet) or a phytoestrogen-free diet with 5 mg/kg BPA added (BPA diet) for 10 days before mating. paired with males checked for copulation plugs the next morning. The day of copulation plug was considered embryonic day 0.5 (E0.5). After a plug is found, the male was removed and the female remains singly housed with the proper diet for two weeks. Tissue was collected on embryonic day 16 during the dark cycle. In order to ensure similar levels of BPA between animals, food was removed from the hopper for 2 hours. After food restriction, each dam received a pre-weighed amount of food in the hopper and for 1 hour. The remaining food was weighed to determine how

much food was consumed during this interval. Females were then weighed, anesthetized with isoflurane, euthanized by cervical dislocation. Embryos and placentas were carefully removed from the uterus, sex recorded, and intra-uterine position noted. A piece of tissue was taken for genotyping to confirm sex. The whole brain was extracted from the head, the hypothalamic area was dissected on ice, and rapidly frozen on dry ice and stored at -80 until further processing. Following RNA extraction, quantification, and reverse transcription of cDNA, the expression of *Sim1* was analyzed by real-time quantitative PCR (qPCR).

Potential Outcomes:

I expect that BPA will increase the expression of *Sim1* in E16 hypothalamus. BPA is known to affect proliferation and differentiation of neuronal progenitors. *Sim1* is expressed in differentiating neurons, therefore, if BPA accelerates the differentiation of hypothalamic neurons, then there would be increased expression of *Sim1* in BPA-exposed embryo brains. Accordingly, I would expect the neuropeptides expressed in *Sim1* neurons would also increase.

Brn2, a POU domain-encoding gene, is a downstream target of Sim1. *Brn2* expression in the hypothalamus is lost in knockouts of *Sim1* (Michaud et al., 1998). *Brn2* knockouts have a selective defect in CRH-, AVP-, and OT-expressing neurons (Hosoya et al., 2001; C. Liu et al., 2003).

We have previously reported decreased levels of *Avp* in E18 whole brains from BPA exposed embryos compared to control (Wolstenholme et al., 2012). This may indicate that BPA could be decreasing *Sim1* levels in embryonic brain, contrary to our RNA-sequencing result from juvenile brains, but similar to the significant decrease described in F3 juvenile BPA mice.

Results and conclusion:

In E16 hypothalamus, neither BPA exposure nor sex significantly impacted the expression of *Sim1* (Two-way ANOVA main effect of BPA: $F(1,38)=0.27$, $p=0.62$; Main effect of sex:

F(1,38)=0.35, p=.56; Figure 3). Expression of *Sim1* in the whole brains of E18 and PN0 mice was not significantly affected by direct, gestational exposure to BPA. Likewise, I found no effect of BPA on a more specific area of the embryonic brain, the hypothalamus, where *Sim1* is most highly expressed. Thus, at this dose of BPA, I could not detect any significant BPA effect on *Sim1* expression in the developing brain using quantitative PCR.

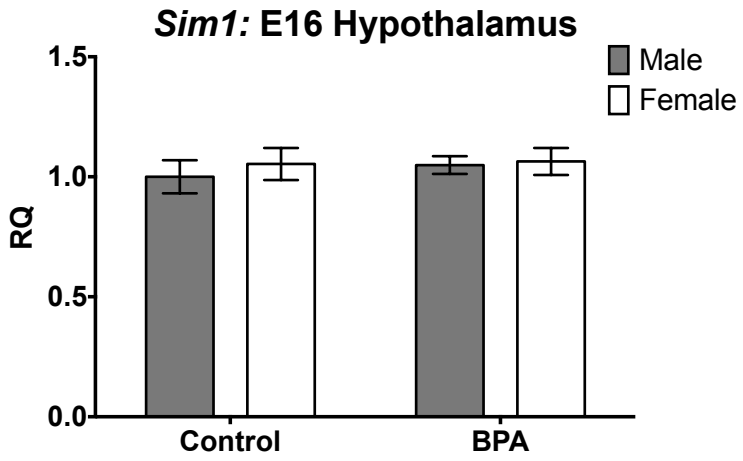

Figure 3: *Sim1* expression in E16 Hypothalamus
Mean ± SEM relative quantity (RQ) of *Sim1* mRNA in E16 hypothalamus, n=9-11 per group. Gray bars represent males and white bars are females. The endogenous control gene was *Rpl19*

References:

Abel, J.M., Witt, D.M., Rissman, E.F., 2011. Sex Differences in the Cerebellum and Frontal Cortex: Roles of Estrogen Receptor Alpha and Sex Chromosome Genes. Neuroendocrinology 93, 230–240. doi:10.1159/000324402

Abraham, H., Richter, Z., Gyimesi, C., Horvath, Z., Janszky, J., Doczi, T., Seress, L., 2011. Degree and pattern of calbindin immunoreactivity in granule cells of the dentate gyrus differ in mesial temporal sclerosis, cortical malformation- and tumor-related epilepsies. Brain Res 1399, 66–78. doi:10.1016/j.brainres.2011.05.010

Abraham, H., Veszpremi, B., Kravjak, A., Kovacs, K., Gomori, E., Seress, L., 2009. Ontogeny of calbindin immunoreactivity in the human hippocampal formation with a special emphasis on granule cells of the dentate gyrus. Int J Dev Neurosci 27, 115–127. doi:10.1016/j.ijdevneu.2008.12.004

ADDM, 2014. Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2010. MMWR. Surveill. Summ. 63 Suppl 2, 1–21.

Agarwal, S., Yadav, A., Tiwari, S.K., Seth, B., Chauhan, L.K.S., Khare, P., Ray, R.S., Chaturvedi, R.K., 2016. Dynamin-related protein 1 inhibition mitigates Bisphenol-A mediated alterations in mitochondrial dynamics and neural stem cells proliferation and differentiation. J. Biol. Chem. jbc.M115.709493. doi:10.1074/jbc.M115.709493

Airaksinen, L., Virkkala, J., Aarnisalo, A., Meyer, M., Ylikoski, J., Airaksinen, M.S., 2000. Lack of calbindin-D28k does not affect hearing level or survival of hair cells in acoustic trauma. ORL. J. Otorhinolaryngol. Relat. Spec. 62, 9–12.

Airaksinen, M.S., Eilers, J., Garaschuk, O., Thoenen, H., Konnerth, A., Meyer, M., 1997. Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. Proc Natl Acad Sci U S A 94, 1488–1493.

Akingbemi, B.T., Sottas, C.M., Koulova, A.I., Klinefelter, G.R., Hardy, M.P., 2004. Inhibition of Testicular Steroidogenesis by the Xenoestrogen Bisphenol A Is Associated with Reduced Pituitary Luteinizing Hormone Secretion and Decreased Steroidogenic Enzyme Gene Expression in Rat Leydig Cells. Endocrinology 145, 592–603. doi:10.1210/en.2003-1174

Alyea, R.A., Watson, C.S., 2009. Differential regulation of dopamine transporter function and location by low concentrations of environmental estrogens and 17beta-estradiol. Environ. Health Perspect. 117, 778–83. doi:10.1289/ehp.0800026

Anderson, O.S., Peterson, K.E., Sanchez, B.N., Zhang, Z., Mancuso, P., Dolinoy, D.C., 2013. Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course. FASEB J. 27, 1784–92. doi:10.1096/fj.12-223545

Andreescu, C.E., Milojkovic, B.A., Haasdijk, E.D., Kramer, P., De Jong, F.H., Krust, A., De Zeeuw, C.I., De Jeu, M.T.G., 2007. Estradiol Improves Cerebellar Memory Formation by Activating Estrogen Receptor. J. Neurosci. 27, 10832–10839. doi:10.1523/JNEUROSCI.2588-07.2007

Arambula, S.E., Belcher, S.M., Planchart, A., Turner, S.D., Patisaul, H.B., 2016. Impact of Low Dose Oral Exposure to Bisphenol A (BPA) on the Neonatal Rat Hypothalamic and Hippocampal Transcriptome: A CLARITY-BPA Consortium Study. Endocrinology 157, en20161339. doi:10.1210/en.2016-1339

Atif, M., Ahmed, A.S., Eid, R.A., 2015. Effect of Bisphenol-A on the Post-Natal Development and Structure of Rat Cerebellum. Int. J. Curr. Microbiol. Appl. Sci. 4, 14–35.

Azzouni, F., Godoy, A., Li, Y., Mohler, J., 2012. The 5 alpha-reductase isozyme family: a review of basic biology and their role in human diseases. Adv. Urol. 2012, 530121. doi:10.1155/2012/530121

Backs, J., Backs, T., Bezprozvannaya, S., McKinsey, T.A., Olson, E.N., 2008. Histone

deacetylase 5 acquires calcium/calmodulin-dependent kinase II responsiveness by oligomerization with histone deacetylase 4. Mol Cell Biol 28, 3437–3445. doi:10.1128/MCB.01611-07

Baimbridge, K.G., Celio, M.R., Rogers, J.H., 1992. Calcium-binding proteins in the nervous system. Trends Neurosci 15, 303–308.

Baimbridge, K.G., Miller, J.J., Parkes, C.O., 1982. Calcium-binding protein distribution in the rat brain. Brain Res 239, 519–525. doi:0006-8993(82)90526-1 [pii]

Balakrishnan, B., Henare, K., Thorstensen, E.B., Ponnampalam, A.P., Mitchell, M.D., 2010. Transfer of bisphenol A across the human placenta. Am. J. Obstet. Gynecol. 202, 393.e1-7. doi:10.1016/j.ajog.2010.01.025

Bale, T.L., Baram, T.Z., Brown, A.S., Goldstein, J.M., Insel, T.R., McCarthy, M.M., Nemeroff, C.B., Reyes, T.M., Simerly, R.B., Susser, E.S., Nestler, E.J., 2010. Early life programming and neurodevelopmental disorders. Biol. Psychiatry 68, 314–9. doi:10.1016/j.biopsych.2010.05.028

Barker, D.J., 1999. Fetal origins of cardiovascular disease. Ann. Med. 31 Suppl 1, 3–6.

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., Nilsson, S., 1998. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol. Pharmacol. 54, 105–12.

Barski, J.J., Hartmann, J., Rose, C.R., Hoebeek, F., Morl, K., Noll-Hussong, M., De Zeeuw, C.I., Konnerth, A., Meyer, M., 2003. Calbindin in cerebellar Purkinje cells is a critical determinant of the precision of motor coordination. J Neurosci 23, 3469–3477.

Barua, S., Chadman, K.K., Kuizon, S., Buenaventura, D., Stapley, N.W., Ruocco, F., Begum, U., Guariglia, S.R., Brown, W.T., Junaid, M.A., 2014. Increasing Maternal or Post-Weaning Folic Acid Alters Gene Expression and Moderately Changes Behavior in the Offspring. PLoS One 9, e101674. doi:10.1371/journal.pone.0101674

Basta-Kaim, A., Fijal, K., Slusarczyk, J., Trojan, E., Glombik, K., Budziszewska, B., Leskiewicz, M., Regulska, M., Kubera, M., Lason, W., Wedzony, K., 2015. Prenatal administration of lipopolysaccharide induces sex-dependent changes in glutamic acid decarboxylase and parvalbumin in the adult rat brain. Neuroscience 287, 78–92. doi:10.1016/j.neuroscience.2014.12.013

Bastianelli, E., 2003. Distribution of calcium-binding proteins in the cerebellum. Cerebellum 2, 242–262.

Becker, E.B.E., Stoodley, C.J., 2013. Autism spectrum disorder and the cerebellum, 1st ed, International Review of Neurobiology. Elsevier Inc. doi:10.1016/B978-0-12-418700-9.00001-0

Belcher, S.M., 1999. Regulated expression of estrogen receptor α and β mRNA in granule cells during development of the rat cerebellum. Dev. Brain Res. 115, 57–69. doi:10.1016/S0165-3806(99)00050-4

Bell, M.R., Thompson, L.M., Rodriguez, K., Gore, A.C., 2016. Two-hit exposure to polychlorinated biphenyls at gestational and juvenile life stages: 1. Sexually dimorphic effects on social and anxiety-like behaviors. Horm. Behav. 78, 168–177. doi:10.1016/j.yhbeh.2015.11.007

Benachour, N., Aris, A., 2009. Toxic effects of low doses of Bisphenol-A on human placental cells. Toxicol. Appl. Pharmacol. 241, 322–8. doi:10.1016/j.taap.2009.09.005

Berger, A., Ziv-Gal, A., Cudiamat, J., Wang, W., Zhou, C., Flaws, J.A., 2016. The effects of in utero bisphenol A exposure on the ovaries in multiple generations of mice. Reprod. Toxicol. 60, 39–52. doi:10.1016/j.reprotox.2015.12.004

Bhandari, R.K., vom Saal, F.S., Tillitt, D.E., 2015. Transgenerational effects from early developmental exposures to bisphenol A or 17?-ethinylestradiol in medaka, Oryzias latipes. Sci. Rep. 5, 9303. doi:10.1038/srep09303

Bielsky, I.F., Hu, S.-B., Ren, X., Terwilliger, E.F., Young, L.J., 2005. The V1a vasopressin

receptor is necessary and sufficient for normal social recognition: a gene replacement study. Neuron 47, 503–13. doi:10.1016/j.neuron.2005.06.031

Bodo, C., Kudwa, A.E., Rissman, E.F., 2006. Both estrogen receptor-α and -β are required for sexual differentiation of the anteroventral periventricular area in mice. Endocrinology 147, 415–420. doi:10.1210/en.2005-0834

Bodo, C., Rissman, E.F., 2008. The androgen receptor is selectively involved in organization of sexually dimorphic social behaviors in mice. Endocrinology 149, 4142–50. doi:10.1210/en.2008-0183

Bodo, C., Rissman, E.F., 2007. Androgen receptor is essential for sexual differentiation of responses to olfactory cues in mice. Eur. J. Neurosci. 25, 2182–90. doi:10.1111/j.1460-9568.2007.05484.x

Bonefeld-Jørgensen, E.C., Long, M., Hofmeister, M. V, Vinggaard, A.M., 2007. Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. Environ. Health Perspect. 115 Suppl, 69–76. doi:10.1289/ehp.9368

Brady, A.M., O'Donnell, P., 2004. Dopaminergic Modulation of Prefrontal Cortical Input to Nucleus Accumbens Neurons In Vivo. J. Neurosci. 24, 1040–1049. doi:10.1523/JNEUROSCI.4178-03.2004

Branchi, I., Curley, J.P., D'Andrea, I., Cirulli, F., Champagne, F.A., Alleva, E., 2013. Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology 38, 522–532. doi:10.1016/j.psyneuen.2012.07.010

Brand, T., Kroonen, J., Mos, J., Slob, A.K., 1991. Adult partner preference and sexual behavior of male rats affected by perinatal endocrine manipulations. Horm. Behav. 25, 323–341. doi:10.1016/0018-506X(91)90005-3

Braun, J.M., Kalkbrenner, A.E., Calafat, A.M., Yolton, K., Ye, X., Dietrich, K.N., Lanphear, B.P., 2011. Impact of early-life bisphenol A exposure on behavior and executive function in children. Pediatrics 128, 873–82. doi:10.1542/peds.2011-1335

Braun, J.M., Kalkbrenner, A.E., Just, A.C., Yolton, K., Calafat, A.M., Sj?din, A., Hauser, R., Webster, G.M., Chen, A., Lanphear, B.P., 2014. Gestational Exposure to Endocrine-Disrupting Chemicals and Reciprocal Social, Repetitive, and Stereotypic Behaviors in 4- and 5-Year-Old Children: The HOME Study. Environ. Health Perspect. doi:10.1289/ehp.1307261

Braun, J.M., Yolton, K., Dietrich, K.N., Hornung, R., Ye, X., Calafat, A.M., Lanphear, B.P., 2009. Prenatal bisphenol A exposure and early childhood behavior. Environ. Health Perspect. 117, 1945–52. doi:10.1289/ehp.0900979

Braun, K., Scheich, H., Heizmann, C.W., Hunziker, W., 1991. Parvalbumin and calbindin-D28K immunoreactivity as developmental markers of auditory and vocal motor nuclei of the zebra finch. Neuroscience 40, 853–869. doi:10.1016/0306-4522(91)90017-I

Braun, K., Seidel, K., Weigel, S., Roski, C., Poeggel, G., 2011. Paternal deprivation alters region- and age-specific interneuron expression patterns in the biparental rodent, Octodon degus. Cereb Cortex 21, 1532–1546. doi:10.1093/cercor/bhq208

Brunelli, S.A., Hofer, M.A., 2007. Selective breeding for infant rat separation-induced ultrasonic vocalizations: Developmental precursors of passive and active coping styles. Behav. Brain Res. 182, 193–207. doi:10.1016/j.bbr.2007.04.014

Calafat, A.M., Weuve, J., Ye, X., Jia, L.T., Hu, H., Ringer, S., Huttner, K., Hauser, R., 2009. Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants. Environ. Health Perspect. 117, 639–44. doi:10.1289/ehp.0800265

Cao, X.-L., Popovic, S., Arbuckle, T.E., Fraser, W.D., 2015. Determination of free and total bisphenol A in human milk samples from Canadian women using a sensitive and selective GC-MS method. Food Addit. Contam. Part A 32, 120–125.

doi:10.1080/19440049.2014.980855
Carwile, J.L., Luu, H.T., Bassett, L.S., Driscoll, D.A., Yuan, C., Chang, J.Y., Ye, X., Calafat, A.M., Michels, K.B., 2009. Polycarbonate bottle use and urinary bisphenol A concentrations. Environ. Health Perspect. 117, 1368–72. doi:10.1289/ehp.0900604
Casas, M., Forns, J., Martínez, D., Avella-garcía, C., 2015. Exposure to bisphenol A during pregnancy and child neuropsychological development in the INMA-Sabadell cohort. Environ. Res. 142, 671–679. doi:10.1016/j.envres.2015.07.024
Castro, B., Sánchez, P., Miranda, M.T., Torres, J.M., Ortega, E., 2015a. Identification of dopamine- and serotonin-related genes modulated by bisphenol A in the prefrontal cortex of male rats. Chemosphere 139, 235–239. doi:10.1016/j.chemosphere.2015.06.061
Castro, B., Sánchez, P., Torres, J.M., Ortega, E., 2015b. Bisphenol A, bisphenol F and bisphenol S affect differently 5α-reductase expression and dopamine-serotonin systems in the prefrontal cortex of juvenile female rats. Environ. Res. 142, 281–287. doi:10.1016/j.envres.2015.07.001
Castro, B., Sánchez, P., Torres, J.M., Ortega, E., 2013. Effects of Adult Exposure to Bisphenol A on Genes Involved in the Physiopathology of Rat Prefrontal Cortex. PLoS One 8, e73584. doi:10.1371/journal.pone.0073584
CDC (Centers for Disease Control and Prevention), 2009. Fourth National Report on Human Exposure to Environmental Chemicals, Department of Health and Human Services.
Celio, M.R., 1990. Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35, 375–475.
Champagne, F.A., Curley, J.P., 2009. Epigenetic mechanisms mediating the long-term effects of maternal care on development. Neurosci. Biobehav. Rev. 33, 593–600. doi:10.1016/j.neubiorev.2007.10.009
Champagne, F.A., Francis, D.D., Mar, A., Meaney, M.J., 2003. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol. Behav. 79, 359–371. doi:10.1016/S0031-9384(03)00149-5
Chapman, K., Holmes, M., Seckl, J., 2013. 11B-Hydroxysteroid Dehydrogenases: Intracellular Gate-Keepers of Tissue Glucocorticoid Action. Physiol. Rev. 93, 1139–206. doi:10.1152/physrev.00020.2012
Chen-Ming Fan, E.K., Bulfone, A., Colin F. Fletcher, Neal G. Copeland, N.A.J., Stephen Crews, Salvador Martinez, L.P., John L. R. Rubenstein, and M.T.-L., 1996. Expression Patterns of Two Murine Homologs of. Mol. Cell. Neurosci. 16, 1–16.
Chen, F., Zhou, L., Bai, Y., Zhou, R., Chen, L., 2015. Hypothalamic-pituitary-adrenal axis hyperactivity accounts for anxiety- and depression-like behaviors in rats perinatally exposed to bisphenol A. J. Biomed. Res. 29, 250–8. doi:10.7555/JBR.29.20140058
Chen, F., Zhou, L., Bai, Y., Zhou, R., Chen, L., 2014. Sex differences in the adult HPA axis and affective behaviors are altered by perinatal exposure to a low dose of bisphenol A. Brain Res. 1571, 12–24. doi:10.1016/j.brainres.2014.05.010
Chevrier, J., Gunier, R.B., Bradman, A., Holland, N.T., Calafat, A.M., Eskenazi, B., Harley, K.G., 2013. Maternal urinary bisphenol a during pregnancy and maternal and neonatal thyroid function in the CHAMACOS study. Environ. Health Perspect. 121, 138–44. doi:10.1289/ehp.1205092
Chizhikov, V., Millen, K.J., 2003. Development and malformations of the cerebellum in mice. Mol. Genet. Metab. 80, 54–65. doi:10.1016/j.ymgme.2003.08.019
Chung, E., Genco, M.C., Megrelis, L., Ruderman, J. V., 2011. Effects of bisphenol A and triclocarban on brain-specific expression of aromatase in early zebrafish embryos. Proc. Natl. Acad. Sci. 108, 17732–17737. doi:10.1073/pnas.1115187108
Corbel, T., Gayrard, V., Viguié, C., Puel, S., Lacroix, M.Z., Toutain, P.-L., Picard-Hagen, N., 2013. Bisphenol A disposition in the sheep maternal-placental-fetal unit: mechanisms determining fetal internal exposure. Biol. Reprod. 89, 11.

doi:10.1095/biolreprod.112.106369
Cottrell, E.C., Seckl, J.R., Holmes, M.C., Wyrwoll, C.S., 2013. Foetal and placental 11β-HSD2: a hub for developmental programming. Acta Physiol. (Oxf). 288–295. doi:10.1111/apha.12187
Coughtrie, M.W., Burchell, B., Leakey, J.E., Hume, R., 1988. The inadequacy of perinatal glucuronidation: immunoblot analysis of the developmental expression of individual UDP-glucuronosyltransferase isoenzymes in rat and human liver microsomes. Mol. Pharmacol. 34, 729–35.
Cowley, S.M., Hoare, S., Mosselman, S., Parker, M.G., 1997. Estrogen Receptors {alpha} and {beta} Form Heterodimers on DNA. J. Biol. Chem. 272, 19858–19862.
Cox, K.H., Gatewood, J.D., Howeth, C., Rissman, E.F., 2010. Gestational exposure to bisphenol A and cross-fostering affect behaviors in juvenile mice. Horm. Behav. 58, 754–761. doi:10.1016/j.yhbeh.2010.07.008
Crawley, J.N., 2007. Mouse behavioral assays relevant to the symptoms of autism. Brain Pathol. 17, 448–59. doi:10.1111/j.1750-3639.2007.00096.x
Crews, D., 2011. Epigenetic modifications of brain and behavior: theory and practice. Horm. Behav. 59, 393–8. doi:10.1016/j.yhbeh.2010.07.001
Cummings, J. a, Clemens, L.G., Nunez, a a, 2010. Mother counts: how effects of environmental contaminants on maternal care could affect the offspring and future generations. Front. Neuroendocrinol. 31, 440–51. doi:10.1016/j.yfrne.2010.05.004
Dalla, C., Shors, T.J., 2009. Sex differences in learning processes of classical and operant conditioning. Physiol. Behav. 97, 229–238. doi:10.1016/j.physbeh.2009.02.035
Davis, E.C., Shryne, J.E., Gorski, R.A., 1996. Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. Neuroendocrinology 63, 142–8.
De Jonge, F.H., Louwerse, A.L., Ooms, M.P., Evers, P., Endert, E., Van De Poll, N.E., 1989. Lesions of the SDN-POA inhibit sexual behavior of male wistar rats. Brain Res. Bull. 23, 483–492. doi:10.1016/0361-9230(89)90194-9
Dean, S.L., McCarthy, M.M., 2008. Steroids, sex and the cerebellar cortex: Implications for human disease. Cerebellum 7, 38–47. doi:10.1007/s12311-008-0003-6
Dean, S.L., Wright, C.L., Hoffman, J.F., Wang, M., Alger, B.E., McCarthy, M.M., 2012. Prostaglandin E2 stimulates estradiol synthesis in the cerebellum postnatally with associated effects on purkinje neuron dendritic arbor and electrophysiological properties. Endocrinology 153, 5415–5427. doi:10.1210/en.2012-1350
DeFelipe, J., 1997. Types of neurons, synaptic connections and chemical characteristics of cells immunoreactive for calbindin-D28K, parvalbumin and calretinin in the neocortex. J Chem Neuroanat 14, 1–19. doi:S0891061897100138 [pii]
Della Seta, D., Minder, I., Dessì-Fulgheri, F., Farabollini, F., 2005. Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res. Bull. 65, 255–60. doi:10.1016/j.brainresbull.2004.11.017
Derouiche, L., Keller, M., Duittoz, A.H., Pillon, D., 2015. Developmental exposure to Ethinylestradiol affects transgenerationally sexual behavior and neuroendocrine networks in male mice. Sci. Rep. 5, 17457. doi:10.1038/srep17457
Dessì-Fulgheri, F., Porrini, S., Farabollini, F., 2002. Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environ. Health Perspect. 110 Suppl, 403–7.
Dichter, G.S., Brunelli, S.A., Hofer, M.A., 1996. Elevated plus-maze behavior in adult offspring of selectively bred rats. Physiol. Behav. 60, 299–304. doi:10.1016/0031-9384(95)02222-8
Dirks, A., Fish, E.W., Kikusui, T., van der Gugten, J., Groenink, L., Olivier, B., Miczek, K.A., 2002. Effects of corticotropin-releasing hormone on distress vocalizations and locomotion in maternally separated mouse pups. Pharmacol. Biochem. Behav. 72, 993–999.

doi:10.1016/S0091-3057(02)00809-2
Dobrzyńska, M.M., Gajowik, A., Radzikowska, J., Tyrkiel, E.J., Jankowska-Steifer, E.A., 2015. Male-mediated F1 effects in mice exposed to bisphenol A, either alone or in combination with X-irradiation. Mutat. Res. Toxicol. Environ. Mutagen. 789–790, 36–45. doi:10.1016/j.mrgentox.2015.06.015
Dohler, K.D., Coquelin, A., Davis, F., Hines, M., Shryne, J.E., Gorski, R.A., 1984. Pre- and postnatal influence of testosterone propionate and diethylstilbestrol on differentiation of the sexually dimorphic nucleus of the preoptic area in male and female rats. Brain Res. 302, 291–295. doi:10.1016/0006-8993(84)90242-7
Dohler, K.D., Coquelin, A., Davis, F., Hines, M., Shryne, J.E., Gorski, R.A., 1982. Differentiation of the sexually dimorphic nucleus in the preoptic area of the rat brain is determined by the perinatal hormone environment. Neurosci. Lett. 33, 295–298. doi:10.1016/0304-3940(82)90388-3
Dolinoy, D.C., Das, R., Weidman, J.R., Jirtle, R.L., 2007a. Metastable epialleles, imprinting, and the fetal origins of adult diseases. Pediatr. Res. 61, 30R–37R. doi:10.1203/pdr.0b013e31804575f7
Dolinoy, D.C., Weidman, J.R., Jirtle, R.L., 2007b. Epigenetic gene regulation: linking early developmental environment to adult disease. Reprod. Toxicol. 23, 297–307. doi:10.1016/j.reprotox.2006.08.012
Druga, R., 2009. Neocortical inhibitory system. Folia Biol. (Praha). 55, 201–217.
Duarte-Guterman, P., Yagi, S., Chow, C., Galea, L.A.M., 2015. Hippocampal learning, memory, and neurogenesis: Effects of sex and estrogens across the lifespan in adults. Horm. Behav. 74, 37–52. doi:10.1016/j.yhbeh.2015.05.024
Dumas, T.C., Powers, E.C., Tarapore, P.E., Sapolsky, R.M., 2004. Overexpression of calbindin D(28k) in dentate gyrus granule cells alters mossy fiber presynaptic function and impairs hippocampal-dependent memory. Hippocampus 14, 701–709. doi:10.1002/hipo.10210
Edelmann, M., Wolfe, C., Scordalakes, E.M., Rissman, E.F., Tobet, S., 2007. Neuronal nitric oxide synthase and calbindin delineate sex differences in the developing hypothalamus and preoptic area. Dev. Neurobiol. 67, 1371–1381. doi:10.1002/dneu.20507
Edlow, A.G., Chen, M., Smith, N.A., Lu, C., McElrath, T.F., 2012. Fetal bisphenol A exposure: concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters. Reprod. Toxicol. 34, 1–7. doi:10.1016/j.reprotox.2012.03.009
Egea, J., Malmierca, E., Rosa, A.O., del Barrio, L., Negredo, P., Nunez, A., Lopez, M.G., 2012. Participation of calbindin-D28K in nociception: results from calbindin-D28K knockout mice. Pflugers Arch. 463, 449–458. doi:10.1007/s00424-011-1063-x
Ehret, G., 2005. Infant rodent ultrasounds -- a gate to the understanding of sound communication. Behav. Genet. 35, 19–29. doi:10.1007/s10519-004-0853-8
Elsworth, J.D., Jentsch, J.D., Groman, S.M., Roth, R.H., Redmond, E.D., Leranth, C., 2015. Low circulating levels of bisphenol-A induce cognitive deficits and loss of asymmetric spine synapses in dorsolateral prefrontal cortex and hippocampus of adult male monkeys. J. Comp. Neurol. 523, 1248–1257. doi:10.1002/cne.23735
Elsworth, J.D., Jentsch, J.D., VandeVoort, C.A., Roth, R.H., Jr, D.E.R., Leranth, C., 2013. Prenatal exposure to bisphenol A impacts midbrain dopamine neurons and hippocampal spine synapses in non-human primates. Neurotoxicology 35, 113–120. doi:10.1016/j.neuro.2013.01.001
Espejo, E.F., 1997. Structure of the mouse behaviour on the elevated plus-maze test of anxiety. Behav. Brain Res. 86, 105–112. doi:10.1016/S0166-4328(96)02245-0
Evans, S.F., Kobrosly, R.W., Barrett, E.S., Thurston, S.W., Calafat, A.M., Weiss, B., Stahlhut, R., Yolton, K., Swan, S.H., 2014. Prenatal Bisphenol A Exposure and maternally reported behavior in boys and girls. Neurotoxicology. doi:10.1016/j.neuro.2014.10.003
Fan, Y., Ding, S., Ye, X., Manyande, A., He, D., Zhao, N., Yang, H., Jin, X., Liu, J., Tian, C.,

Xu, S., Ying, C., 2013. Does preconception paternal exposure to a physiologically relevant level of bisphenol A alter spatial memory in an adult rat? Horm. Behav. 64, 598–604. doi:10.1016/j.yhbeh.2013.08.014

Fatemi, S.H., Halt, A.R., Realmuto, G., Earle, J., Kist, D.A., Thuras, P., Merz, A., 2002. Purkinje cell size is reduced in cerebellum of patients with autism. Cell Mol Neurobiol 22, 171–175.

Fatemi, S.H., Reutiman, T.J., Folsom, T.D., Rooney, R.J., Patel, D.H., Thuras, P.D., 2010. mRNA and protein levels for GABAAalpha4, alpha5, beta1 and GABABR1 receptors are altered in brains from subjects with autism. J. Autism Dev. Disord. 40, 743–50. doi:10.1007/s10803-009-0924-z

Ferguson, J.N., Young, L.J., Hearn, E.F., Matzuk, M.M., Insel, T.R., Winslow, J.T., 2000. Social amnesia in mice lacking the oxytocin gene. Nat. Genet. 25, 284–8. doi:10.1038/77040

Ferguson, S.A., Law, C.D., Abshire, J.S., 2011. Developmental treatment with bisphenol A or ethinyl estradiol causes few alterations on early preweaning measures. Toxicol. Sci. 124, 149–60. doi:10.1093/toxsci/kfr201

Ferguson, S.A., Paule, M.G., He, Z., 2015. Pre- and postnatal bisphenol A treatment does not alter the number of tyrosine hydroxylase-positive cells in the anteroventral periventricular nucleus (AVPV) of weanling male and female rats. Brain Res. doi:10.1016/j.brainres.2015.07.013

Findlay, L.C., Kohen, D.E., 2015. Bisphenol A and child and youth behaviour: Canadian Health Measures Survey 2007 to 2011. Heal. reports 26, 3–9.

Fiumelli, H., Kiraly, M., Ambrus, A., Magistretti, P.J., Martin, J.L., 2000. Opposite regulation of calbindin and calretinin expression by brain-derived neurotrophic factor in cortical neurons. J. Neurochem. 74, 1870–1877.

Flaisher-Grinberg, S., Albelda, N., Gitter, L., Weltman, K., Arad, M., Joel, D., 2009. Ovarian hormones modulate “compulsive” lever-pressing in female rats. Horm. Behav. 55, 356–365. doi:10.1016/j.yhbeh.2008.10.002

Flint, S., Markle, T., Thompson, S., Wallace, E., 2012. Bisphenol A exposure, effects, and policy: a wildlife perspective. J. Environ. Manage. 104, 19–34. doi:10.1016/j.jenvman.2012.03.021

Funabashi, T., Kawaguchi, M., Furuta, M., Fukushima, A., Kimura, F., 2004. Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotropin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of rats. Psychoneuroendocrinology 29, 475–485. doi:10.1016/S0306-4530(03)00055-6

Garcia, A.N., Bezner, K., Depena, C., Yin, W., Gore, A.C., 2017. The effects of long-term estradiol treatment on social behavior and gene expression in adult female rats. Horm. Behav. 87, 145–154. doi:10.1016/j.yhbeh.2016.11.011

Genuis, S.J., Beesoon, S., Birkholz, D., Lobo, R.A., 2012. Human excretion of bisphenol A: Blood, urine, and sweat (BUS) study. J. Environ. Public Health 2012. doi:10.1155/2012/185731

Ghosh, A., Ginty, D.D., Bading, H., Greenberg, M.E., 1994. Calcium regulation of gene expression in neuronal cells. J. Neurobiol. 25, 294–303. doi:10.1002/neu.480250309

Giachino, C., Canalia, N., Capone, F., Fasolo, A., Alleva, E., Riva, M.A., Cirulli, F., Peretto, P., 2007. Maternal deprivation and early handling affect density of calcium binding protein-containing neurons in selected brain regions and emotional behavior in periadolescent rats. Neuroscience 145, 568–578. doi:10.1016/j.neuroscience.2006.12.042

Gilabert-Juan, J., Castillo-Gomez, E., Pérez-Rando, M., Moltó, M.D., Nacher, J., 2011. Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice. Exp. Neurol. 232, 33–40. doi:10.1016/j.expneurol.2011.07.009

Gilmore, R.F., Varnum, M.M., Forger, N.G., 2012. Effects of Blocking Developmental Cell Death on Sexually Dimorphic Calbindin Cell Groups in the Preoptic Area and Bed Nucleus of the Stria Terminalis. Biol. Sex Differ. 3, 5. doi:10.1186/2042-6410-3-5

Gioiosa, L., Fissore, E., Ghirardelli, G., Parmigiani, S., Palanza, P., 2007. Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Horm. Behav. 52, 307–16. doi:10.1016/j.yhbeh.2007.05.006

Gioiosa, L., Parmigiani, S., Vom Saal, F.S., Palanza, P., 2013. The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice. Horm. Behav. 63, 598–605. doi:10.1016/j.yhbeh.2013.02.016

Giraldez-Perez, R.M., Avila, M.N., Feijoo-Cuaresma, M., Heredia, R., De Diego-Otero, Y., Real, M.A., Guirado, S., 2013. Males but not females show differences in calbindin immunoreactivity in the dorsal thalamus of the mouse model of fragile X syndrome. J Comp Neurol 521, 894–911. doi:10.1002/cne.23209

Golde, W.T., Gollobin, P., Rodriguez, L.L., 2005. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. Lab Anim. (NY). 34, 39–43. doi:10.1038/laban1005-39

Goldsby, J.A., Wolstenholme, J.T., Rissman, E.F., 2017. Multi- and transgenerational consequences of bisphenol a on sexually dimorphic cell populations in mouse brain. Endocrinology 158, 21–30. doi:10.1210/en.2016-1188

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr. Rev. 36, E1–E150. doi:10.1210/er.2015-1010

Gorski, R.A., 1985. The 13th J. A. F. Stevenson memorial lecture. Sexual differentiation of the brain: possible mechanisms and implications. Can. J. Physiol. Pharmacol. 63, 577–94.

Gorski, R.A., Gordon, J.H., Shryne, J.E., Southam, A.M., 1978. Evidence for a morphological sex difference within the medial preoptic area of the rat brain. Brain Res. 148, 333–346. doi:10.1016/0006-8993(78)90723-0

Gos, T., Schulkin, J., Gos, A., Bock, J., Poeggel, G., Braun, K., 2013. Paternal deprivation affects the functional maturation of corticotropin-releasing hormone (CRH)- and calbindin-D28k-expressing neurons in the bed nucleus of the stria terminalis (BNST) of the biparental Octodon degus. Brain Struct Funct. doi:10.1007/s00429-013-0617-4

Gould, J.C., Leonard, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D.P., Gaido, K.W., 1998. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol. Cell. Endocrinol. 142, 203–14.

Gray, J.M., Vecchiarelli, H.A., Morena, M., Lee, T.T., Hermanson, D.J., Kim, A.B., McLaughlin, R.J., Hassan, K.I., Kuhne, C., Wotjak, C.T., Deussing, J.M., Patel, S., Hill, M.N., 2015. Corticotropin-releasing hormone drives anandamide hydrolysis in the amygdala to promote anxiety. J Neurosci 35, 3879–3892. doi:10.1523/JNEUROSCI.2737-14.2015

Greathouse, K.L., Bredfeldt, T., Everitt, J.I., Lin, K., Berry, T., Kannan, K., Mittelstadt, M.L., Ho, S., Walker, C.L., 2012. Environmental estrogens differentially engage the histone methyltransferase EZH2 to increase risk of uterine tumorigenesis. Mol. Cancer Res. 10, 546–57. doi:10.1158/1541-7786.MCR-11-0605

Groves, N.J., Burne, T.H.J., 2016. Sex-specific attentional deficits in adult vitamin D deficient BALB/c mice. Physiol. Behav. 157, 94–101. doi:10.1016/j.physbeh.2016.01.033

Gu, G.B., Simerly, R.B., 1997. Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. J. Comp. Neurol. 384, 142–164. doi:10.1002/(SICI)1096-9861(19970721)384:1<142::AID-CNE9>3.0.CO;2-1

Guo, J., Yuan, X., Qiu, L., Zhu, W., Wang, C., Hu, G., Chu, Y., Ye, L., Xu, Y., Ge, R.-S., 2012. Inhibition of human and rat 11β-hydroxysteroid dehydrogenases activities by bisphenol A. Toxicol. Lett. 215, 126–30. doi:10.1016/j.toxlet.2012.10.002

Haapasalo, A., Sipola, I., Larsson, K., Akerman, K.E.O., Stoilov, P., Stamm, S., Wong, G., Castren, E., 2002. Regulation of TRKB surface expression by brain-derived neurotrophic factor and truncated TRKB isoforms. J. Biol. Chem. 277, 43160–7.

doi:10.1074/jbc.M205202200
Haraguchi, S., Sasahara, K., Shikimi, H., Honda, S., Harada, N., Tsutsui, K., 2012. Estradiol Promotes Purkinje Dendritic Growth, Spinogenesis, and Synaptogenesis During Neonatal Life by Inducing the Expression of BDNF. The Cerebellum 11, 416–417. doi:10.1007/s12311-011-0342-6
Harley, K.G., Gunier, R.B., Kogut, K., Johnson, C., Bradman, A., Calafat, A.M., Eskenazi, B., 2013. Prenatal and early childhood bisphenol A concentrations and behavior in school-aged children. Environ. Res. 126, 43–50. doi:10.1016/j.envres.2013.06.004
Hashimoto, T., Bergen, S.E., Nguyen, Q.L., Xu, B., Monteggia, L.M., Pierri, J.N., Sun, Z., Sampson, A.R., Lewis, D.A., 2005. Relationship of Brain-Derived Neurotrophic Factor and Its Receptor TrkB to Altered Inhibitory Prefrontal Circuitry in Schizophrenia. J. Neurosci. 25, 372–383. doi:10.1523/jneurosci.4035-04.2005
He, Z., Paule, M.G., Ferguson, S. a., 2012. Low oral doses of bisphenol A increase volume of the sexually dimorphic nucleus of the preoptic area in male, but not female, rats at postnatal day 21. Neurotoxicol. Teratol. 34, 331–337. doi:10.1016/j.ntt.2012.03.004
Heckman, J., McGuinness, B., Celikel, T., Englitz, B., 2016. Determinants of the mouse ultrasonic vocal structure and repertoire. Neurosci. Biobehav. Rev. 65, 313–325. doi:10.1016/j.neubiorev.2016.03.029
Heitland, I., Groenink, L., Bijlsma, E.Y., Oosting, R.S., Baas, J.M., 2013. Human fear acquisition deficits in relation to genetic variants of the corticotropin releasing hormone receptor 1 and the serotonin transporter. PLoS One 8, e63772. doi:10.1371/journal.pone.0063772
Heldt, S. a, Zimmermann, K., Parker, K., Gaval, M., Ressler, K.J., 2014. Bdnf Deletion or TrkB Impairment in Amygdala Inhibits Both Appetitive and Aversive Learning. J. Neurosci. 34, 2444–2450. doi:10.1523/JNEUROSCI.4085-12.2014
Heyser, C.J., Fienberg, A.A., Greengard, P., Gold, L.H., 2000. DARPP-32 knockout mice exhibit impaired reversal learning in a discriminated operant task. Brain Res. 867, 122–130. doi:10.1016/S0006-8993(00)02272-1
Hill, R.A., McInnes, K.J., Gong, E.C.H., Jones, M.E.E., Simpson, E.R., Boon, W.C., 2007. Estrogen Deficient Male Mice Develop Compulsive Behavior. Biol. Psychiatry 61, 359–366. doi:10.1016/j.biopsych.2006.01.012
Hof, P.R., Glezer II, Conde, F., Flagg, R.A., Rubin, M.B., Nimchinsky, E.A., Vogt Weisenhorn, D.M., 1999. Cellular distribution of the calcium-binding proteins parvalbumin, calbindin, and calretinin in the neocortex of mammals: phylogenetic and developmental patterns. J. Chem. Neuroanat. 16, 77–116.
Hoffman, J.F., Wright, C.L., McCarthy, M.M., 2016. A Critical Period in Purkinje Cell Development Is Mediated by Local Estradiol Synthesis, Disrupted by Inflammation, and Has Enduring Consequences Only for Males. J. Neurosci. 36, 10039–10049. doi:10.1523/JNEUROSCI.1262-16.2016
Holman, S.D., Hutchison, R.E., Hutchison, J.B., 1991. Microimplants of estradiol in the sexually dimorphic area of the hypothalamus activate ultrasonic vocal behavior in male Mongolian gerbils. Horm. Behav. 25, 531–548. doi:10.1016/0018-506X(91)90019-E
Hong, H., Yang, L., Stallcup, M.R., 1999. Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J. Biol. Chem. 274, 22618–26. doi:10.1074/jbc.274.32.22618
Hong, S.B., Hong, Y.C., Kim, J.W., Park, E.J., Shin, M.S., Kim, B.N., Yoo, H.J., Cho, I.H., Bhang, S.Y., Cho, S.C., 2013. Bisphenol A in relation to behavior and learning of school-age children. J. Child Psychol. Psychiatry Allied Discip. 54, 890–899. doi:10.1111/jcpp.12050
Hosoya, T., Oda, Y., Takahashi, S., Morita, M., Kawauchi, S., Ema, M., Yamamoto, M., Fujii-Kuriyama, Y., 2001. Defective development of secretory neurones in the hypothalamus of Arnt2-knockout mice. Genes to Cells 6, 361–374. doi:10.1046/j.1365-2443.2001.00421.x

Houtsmuller, E.J., Brand, T., de Jonge, F.H., Joosten, R.N.J.M.A., van de Poll, N.E., Slob, A.K., 1994. SDN-POA volume, sexual behavior, and partner preference of male rats affected by perinatal treatment with ATD. Physiol. Behav. 56, 535–541. doi:10.1016/0031-9384(94)90298-4

Howdeshell, K.L., Furr, J., Lambright, C.R., Rider, C. V, Wilson, V.S., Gray, L.E., 2007. Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. Toxicol. Sci. 99, 190–202. doi:10.1093/toxsci/kfm069

Huang, B., Ning, S., Zhang, Q., Chen, A., Jiang, C., Cui, Y., Hu, J., Li, H., Fan, G., Qin, L., Liu, J., 2016. Bisphenol A Represses Dopaminergic Neuron Differentiation from Human Embryonic Stem Cells through Downregulating the Expression of Insulin-like Growth Factor 1. Mol. Neurobiol. doi:10.1007/s12035-016-9898-y

Huang, H., Leung, L.K., 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol. Lett. 189, 248–52. doi:10.1016/j.toxlet.2009.06.853

Hubbard, D.T., Nakashima, B.R., Lee, I., Takahashi, L.K., 2007. Activation of basolateral amygdala corticotropin-releasing factor 1 receptors modulates the consolidation of contextual fear. Neuroscience 150, 818–828. doi:10.1016/j.neuroscience.2007.10.001

Huppunen, J., Aarnisalo, P., 2004. Dimerization modulates the activity of the orphan nuclear receptor ERRγ. Biochem. Biophys. Res. Commun. 314, 964–970. doi:10.1016/j.bbrc.2003.12.194

Ikeda, Y., Nagai, A., 2006. Differential expression of the estrogen receptors alpha and beta during postnatal development of the rat cerebellum. Brain Res. 1083, 39–49. doi:10.1016/j.brainres.2006.02.025

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y., Taketani, Y., 2002. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum. Reprod. 17, 2839–2841. doi:10.1093/humrep/17.11.2839

Imamura, L., Kurashina, K., Kawahira, T., Omoteno, M., Tsuda, M., 2005. Additional repression of activity-dependent c-fos and BDNF mRNA expression by lipophilic compounds accompanying a decrease in Ca 2+ influx into neurons. Neurotoxicology 26, 17–25. doi:10.1016/j.neuro.2004.07.008

Ip, N.Y., Li, Y., Yancopoulos, G.D., Lindsay, R.M., 1993. Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J. Neurosci. 13, 3394–3405.

Ise, S., Ohta, H., 2009. Power spectrum analysis of ultrasonic vocalization elicited by maternal separation in rat pups. Brain Res. 1283, 58–64. doi:10.1016/j.brainres.2009.06.003

Jacobson, C.D., Csernus, V.J., Shryne, J.E., Gorski, R.A., 1981. The influence of gonadectomy, androgen exposure, or a gonadal graft in the neonatal rat on the volume of the sexually dimorphic nucleus of the preoptic area. J. Neurosci. 1, 1142–7.

Jakab, R.L., Wong, J.K., Belcher, S.M., 2001. Estrogen receptor beta immunoreactivity in differentiating cells of the developing rat cerebellum. J. Comp. Neurol. 430, 396–409. doi:10.1002/1096-9861(20010212)430:3<396::AID-CNE1039>3.0.CO;2-0

Jang, Y.J., Park, H.R., Kim, T.H., Yang, W.-J., Lee, J.-J., Choi, S.Y., Oh, S.B., Lee, E., Park, J.-H., Kim, H.-P., Kim, H.S., Lee, J., 2012. High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. Toxicology 296, 73–82. doi:10.1016/j.tox.2012.03.007

Jin, P., Wang, X., Chang, F., Bai, Y., Li, Y., Zhou, R., Chen, L., 2013. Low dose bisphenol A impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adult rats. J. Biomed. Res. 27, 135–44. doi:10.7555/JBR.27.20120076

Jones, D.C., Miller, G.W., 2008. The effects of environmental neurotoxicants on the dopaminergic system: A possible role in drug addiction. Biochem. Pharmacol. 76, 569–81. doi:10.1016/j.bcp.2008.05.010

Jones, K.R., Farinas, I., Backus, C., Reichardt, L.F., 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76, 989–999.

Kabitzke, P.A., Simpson, E.H., Kandel, E.R., Balsam, P.D., 2015. Social behavior in a genetic model of dopamine dysfunction at different neurodevelopmental time points. Genes, Brain Behav. 14, 503–515. doi:10.1111/gbb.12233

Kanaya, M., Tsuda, M.C., Sagoshi, S., Nagata, K., Morimoto, C., Tha Thu, C.K., Toda, K., Kato, S., Ogawa, S., Tsukahara, S., 2014. Regional difference in sex steroid action on formation of morphological sex differences in the anteroventral periventricular nucleus and principal nucleus of the bed nucleus of the stria terminalis. PLoS One 9, e112616. doi:10.1371/journal.pone.0112616

Kardas, F., Bayram, A.K., Demirci, E., Akin, L., Ozmen, S., Kendirci, M., Canpolat, M., Oztop, D.B., Narin, F., Gumus, H., Kumandas, S., Per, H., 2015. Increased Serum Phthalates (MEHP, DEHP) and Bisphenol A Concentrations in Children With Autism Spectrum Disorder: The Role of Endocrine Disruptors in Autism Etiopathogenesis. J. Child Neurol. 31, 629–635. doi:10.1177/0883073815609150

Kass, L., Durando, M., Altamirano, G.A., Manfroni-Ghibaudo, G.E., Luque, E.H., Muñoz-de-Toro, M., 2014. Prenatal Bisphenol A exposure delays the development of the male rat mammary gland. Reprod. Toxicol. doi:10.1016/j.reprotox.2014.02.001

Kauffman, a. S., Park, J.H., McPhie-Lalmansingh, a. a., Gottsch, M.L., Bodo, C., Hohmann, J.G., Pavlova, M.N., Rohde, a. D., Clifton, D.K., Steiner, R. a., Rissman, E.F., 2007. The Kisspeptin Receptor GPR54 Is Required for Sexual Differentiation of the Brain and Behavior. J. Neurosci. 27, 8826–8835. doi:10.1523/JNEUROSCI.2099-07.2007

Kim, M.S., Akhtar, M.W., Adachi, M., Mahgoub, M., Bassel-Duby, R., Kavalali, E.T., Olson, E.N., Monteggia, L.M., 2012. An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. J. Neurosci. 32, 10879–10886. doi:10.1523/JNEUROSCI.2089-12.2012

Kinch, C.D., Ibhazehiebo, K., Jeong, J.-H., Habibi, H.R., Kurrasch, D.M., 2015. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. Proc. Natl. Acad. Sci. 112, 1475–1480. doi:10.1073/pnas.1417731112

Kitraki, E., Nalvarte, I., AlavianGhavanini, A., Rüegg, J., 2015. Developmental Exposure to Bisphenol A Alters Expression and DNA Methylation of Fkbp5, an Important Regulator of the Stress Response. Mol. Cell. Endocrinol. doi:10.1016/j.mce.2015.09.028

Klinge, C.M., Jernigan, S.C., Mattingly, K.A., Risinger, K.E., Zhang, J., 2004. Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors alpha and beta by coactivators and corepressors. J. Mol. Endocrinol. 33, 387–410. doi:10.1677/jme.1.01541

Kobayashi, K., Miyagawa, M., Wang, R.-S., Suda, M., Sekiguchi, S., Honma, T., 2005. Effects of in utero and lactational exposure to bisphenol A on thyroid status in F1 rat offspring. Ind. Health 43, 685–90.

Kojetin, D.J., Venters, R.A., Kordys, D.R., Thompson, R.J., Kumar, R., Cavanagh, J., 2006. Structure, binding interface and hydrophobic transitions of Ca2+-loaded calbindin-D(28K). Nat Struct Mol Biol 13, 641–647.

Kokras, N., Dalla, C., 2014. Sex differences in animal models of psychiatric disorders., British journal of pharmacology. doi:10.1111/bph.12710

Komada, M., Asai, Y., Morii, M., Matsuki, M., Sato, M., Nagao, T., 2012. Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses. Toxicology 295, 31–38. doi:10.1016/j.tox.2012.02.013

Komada, M., Itoh, S., Kawachi, K., Kagawa, N., Ikeda, Y., Nagao, T., 2014. Newborn mice exposed prenatally to bisphenol A show hyperactivity and defective neocortical

development. Toxicology 323, 51–60. doi:10.1016/j.tox.2014.06.009
Konarski, J.Z., McIntyre, R.S., Grupp, L.A., Kennedy, S.H., 2005. Is the cerebellum relevant in the circuitry of neuropsychiatric disorders? J. Psychiatry Neurosci. 30, 178–186.
Kook, S.Y., Jeong, H., Kang, M.J., Park, R., Shin, H.J., Han, S.H., Son, S.M., Song, H., Baik, S.H., Moon, M., Yi, E.C., Hwang, D., Mook-Jung, I., 2014. Crucial role of calbindin-D28k in the pathogenesis of Alzheimer's disease mouse model. Cell Death Differ 21, 1575–1587. doi:10.1038/cdd.2014.67
Kriegsfeld, L.J., Mei, D.F., Yan, L., Witkovsky, P., Lesauter, J., Hamada, T., Silver, R., 2008. Targeted mutation of the calbindin D28K gene disrupts circadian rhythmicity and entrainment. Eur J Neurosci 27, 2907–2921. doi:EJN6239 [pii]10.1111/j.1460-9568.2008.06239.x
Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R., Aou, S., 2003. Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res. 45, 345–356. doi:10.1016/S0168-0102(02)00251-1
Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252–63. doi:10.1210/endo.139.10.6216
Kumar, D., Thakur, M.K., 2014. Perinatal Exposure to Bisphenol-A Impairs Spatial Memory through Upregulation of Neurexin1 and Neuroligin3 Expression in Male Mouse Brain. PLoS One 9, e110482. doi:10.1371/journal.pone.0110482
Kumar, P., Mendelson, C.R., 2011. Estrogen-related receptor gamma (ERRgamma) mediates oxygen-dependent induction of aromatase (CYP19) gene expression during human trophoblast differentiation. Mol. Endocrinol. 25, 1513–26. doi:10.1210/me.2011-1012
Kundakovic, M., Champagne, F.A., 2015. Early-Life Experience, Epigenetics, and the Developing Brain. Neuropsychopharmacology 40, 141–153. doi:10.1038/npp.2014.140
Kundakovic, M., Gudsnuk, K., Franks, B., Madrid, J., Miller, R.L., Perera, F.P., Champagne, F.A., 2013. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proc. Natl. Acad. Sci. U. S. A. 110, 9956–61. doi:10.1073/pnas.1214056110
Kurian, J.R., Bychowski, M.E., Forbes-Lorman, R.M., Auger, C.J., Auger, A.P., 2008. Mecp2 organizes juvenile social behavior in a sex-specific manner. J Neurosci 28, 7137–7142. doi:10.1523/JNEUROSCI.1345-08.2008
Kurian, J.R., Forbes-Lorman, R.M., Auger, A.P., 2007. Sex difference in Mecp2 expression during a critical period of rat brain development. Epigenetics 2, 173–178. doi:10.4161/epi.2.3.4841
Kurosawa, T., Hiroi, H., Tsutsumi, O., Ishikawa, T., Osuga, Y., Fujiwara, T., Inoue, S., Muramatsu, M., Momoeda, M., Taketani, Y., 2002. The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. Endocr. J. 49, 465–71.
Kwintkiewicz, J., Nishi, Y., Yanase, T., Giudice, L.C., 2010. Peroxisome proliferator-activated receptor-gamma mediates bisphenol A inhibition of FSH-stimulated IGF-1, aromatase, and estradiol in human granulosa cells. Environ. Health Perspect. 118, 400–6. doi:10.1289/ehp.0901161
Kwon, S., Stedman, D.B., Elswick, B.A., Cattley, R.C., Welsch, F., 2000. Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. Toxicol Sci 55, 399–406.
Lai, J.K.Y., Sobala-Drozdowski, M., Zhou, L., Doering, L.C., Faure, P.A., Foster, J.A., 2014. Temporal and spectral differences in the ultrasonic vocalizations of fragile X knock out mice during postnatal development. Behav. Brain Res. 259, 119–30. doi:10.1016/j.bbr.2013.10.049
Le, H.H., Carlson, E.M., Chua, J.P., Belcher, S.M., 2008. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing

cerebellar neurons. Toxicol. Lett. 176, 149–156. doi:10.1016/j.toxlet.2007.11.001

Le, W.W., Wise, P.M., Murphy, A.Z., Coolen, L.M., Hoffman, G.E., 2001. Parallel declines in Fos activation of the medial anteroventral periventricular nucleus and LHRH neurons in middle-aged rats. Endocrinology 142, 4976–4982. doi:10.1210/en.142.11.4976

Lebovic, D.I., Kavoussi, S.K., Lee, J., Banu, S.K., Arosh, J.A., 2013. PPARγ activation inhibits growth and survival of human endometriotic cells by suppressing estrogen biosynthesis and PGE2 signaling. Endocrinology 154, 4803–13. doi:10.1210/en.2013-1168

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J., Chen, L., Chen, L., Chen, T.-M., Chi Chin, M., Chong, J., Crook, B.E., Czaplinska, A., Dang, C.N., Datta, S., Dee, N.R., Desaki, A.L., Desta, T., Diep, E., Dolbeare, T.A., Donelan, M.J., Dong, H.-W., Dougherty, J.G., Duncan, B.J., Ebbert, A.J., Eichele, G., Estin, L.K., Faber, C., Facer, B.A., Fields, R., Fischer, S.R., Fliss, T.P., Frensley, C., Gates, S.N., Glattfelder, K.J., Halverson, K.R., Hart, M.R., Hohmann, J.G., Howell, M.P., Jeung, D.P., Johnson, R.A., Karr, P.T., Kawal, R., Kidney, J.M., Knapik, R.H., Kuan, C.L., Lake, J.H., Laramee, A.R., Larsen, K.D., Lau, C., Lemon, T.A., Liang, A.J., Liu, Y., Luong, L.T., Michaels, J., Morgan, J.J., Morgan, R.J., Mortrud, M.T., Mosqueda, N.F., Ng, L.L., Ng, R., Orta, G.J., Overly, C.C., Pak, T.H., Parry, S.E., Pathak, S.D., Pearson, O.C., Puchalski, R.B., Riley, Z.L., Rockett, H.R., Rowland, S.A., Royall, J.J., Ruiz, M.J., Sarno, N.R., Schaffnit, K., Shapovalova, N. V., Sivisay, T., Slaughterbeck, C.R., Smith, S.C., Smith, K.A., Smith, B.I., Sodt, A.J., Stewart, N.N., Stumpf, K.-R., Sunkin, S.M., Sutram, M., Tam, A., Teemer, C.D., Thaller, C., Thompson, C.L., Varnam, L.R., Visel, A., Whitlock, R.M., Wohnoutka, P.E., Wolkey, C.K., Wong, V.Y., Wood, M., Yaylaoglu, M.B., Young, R.C., Youngstrom, B.L., Feng Yuan, X., Zhang, B., Zwingman, T.A., Jones, A.R., 2007. Genome-wide atlas of gene expression in the adult mouse brain. Nature 445, 168–176. doi:10.1038/nature05453

Liao, C., Kannan, K., 2011. Widespread occurrence of bisphenol A in paper and paper products: Implications for human exposure. Environ. Sci. Technol. 45, 9372–9379. doi:10.1021/es202507f

Lim, M.M., Young, L.J., 2006. Neuropeptidergic regulation of affiliative behavior and social bonding in animals. Horm. Behav. 50, 506–17. doi:10.1016/j.yhbeh.2006.06.028

Ling, W., Endo, T., Kubo, K., Nakajima, K., Kakeyama, M., Tohyama, C., 2016. In Utero Bisphenol A Exposure Induces Abnormal Neuronal Migration in the Cerebral Cortex of Mice. Front. Endocrinol. (Lausanne). 7. doi:10.3389/fendo.2016.00007

Lister, R.G., 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl). 92, 180–5.

Liu, C., Duan, W., Li, R., Xu, S., Zhang, L., Chen, C., He, M., Lu, Y., Wu, H., Pi, H., Luo, X., Zhang, Y., Zhong, M., Yu, Z., Zhou, Z., 2013. Exposure to bisphenol A disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity. Cell Death Dis. 4, e676. doi:10.1038/cddis.2013.203

Liu, C., Goshu, E., Wells, A., Fan, C.M., 2003. Identification of the Downstream Targets of SIM1 and ARNT2, a Pair of Transcription Factors Essential for Neuroendocrine Cell Differentiation. J. Biol. Chem. 278, 44857–44867. doi:10.1074/jbc.M304489200

Liu, D., Zhang, Z., Teng, C.T., 2005. Estrogen-related receptor-gamma and peroxisome proliferator-activated receptor-gamma coactivator-1alpha regulate estrogen-related receptor-alpha gene expression via a conserved multi-hormone response element. J. Mol. Endocrinol. 34, 473–87. doi:10.1677/jme.1.01586

Liu, R.C., Miller, K.D., Merzenich, M.M., Schreiner, C.E., 2003. Acoustic variability and distinguishability among mouse ultrasound vocalizations. J. Acoust. Soc. Am. 114, 3412–3422. doi:10.1121/1.1623787

Liu, X., Herbison, A.E., 2013. Dopamine Regulation of Gonadotropin-Releasing Hormone Neuron Excitability in Male and Female Mice. Endocrinology 154, 340–350.

doi:10.1210/en.2012-1602
Liu, X., Matsushima, A., Okada, H., Shimohigashi, Y., 2010. Distinction of the binding modes for human nuclear receptor ERRgamma between bisphenol A and 4-hydroxytamoxifen. J. Biochem. 148, 247–54. doi:10.1093/jb/mvq056
Lombó, M., Fernández-Díez, C., González-Rojo, S., Navarro, C., Robles, V., Herráez, M.P., 2015. Transgenerational inheritance of heart disorders caused by paternal bisphenol A exposure. Environ. Pollut. 206, 667–678. doi:10.1016/j.envpol.2015.08.016
Lorke, D.E., Süsens, U., Borgmeyer, U., Hermans-Borgmeyer, I., 2000. Differential expression of the estrogen receptor-related receptor γ in the mouse brain. Mol. Brain Res. 77, 277–280. doi:10.1016/S0169-328X(00)00063-2
Ma, D.Q., Whitehead, P.L., Menold, M.M., Martin, E.R., Ashley-Koch, a E., Mei, H., Ritchie, M.D., Delong, G.R., Abramson, R.K., Wright, H.H., Cuccaro, M.L., Hussman, J.P., Gilbert, J.R., Pericak-Vance, M. a, 2005. Identification of significant association and gene-gene interaction of GABA receptor subunit genes in autism. Am. J. Hum. Genet. 77, 377–388. doi:10.1086/433195
Maggi, R., Zasso, J., Conti, L., 2015. Neurodevelopmental origin and adult neurogenesis of the neuroendocrine hypothalamus. Front. Cell. Neurosci. 8, 1–7. doi:10.3389/fncel.2014.00440
Mahalingaiah, S., Meeker, J.D., Pearson, K.R., Calafat, A.M., Ye, X., Petrozza, J., Hauser, R., 2008. Temporal variability and predictors of urinary bisphenol A concentrations in men and women. Environ. Health Perspect. 116, 173–8. doi:10.1289/ehp.10605
Markey, C.M., Wadia, P.R., Rubin, B.S., Sonnenschein, C., Soto, A.M., 2005. Long-term effects of fetal exposure to low doses of the xenoestrogen bisphenol-A in the female mouse genital tract. Biol. Reprod. 72, 1344–51. doi:10.1095/biolreprod.104.036301
Martin Wild, J., Williams, M.N., Suthers, R.A., 2001. Parvalbumin-positive projection neurons characterise the vocal premotor pathway in male, but not female, zebra finches. Brain Res. 917, 235–252. doi:10.1016/S0006-8993(01)02938-9
Masuo, Y., Morita, M., Oka, S., Ishido, M., 2004. Motor hyperactivity caused by a deficit in dopaminergic neurons and the effects of endocrine disruptors: a study inspired by the physiological roles of PACAP in the brain. Regul. Pept. 123, 225–34. doi:10.1016/j.regpep.2004.05.010
Matsuda, S., Matsuzawa, D., Ishii, D., Tomizawa, H., Sutoh, C., Nakazawa, K., Amano, K., Sajiki, J., Shimizu, E., 2012. Effects of perinatal exposure to low dose of bisphenol A on anxiety like behavior and dopamine metabolites in brain. Prog. Neuropsychopharmacol. Biol. Psychiatry 39, 273–9. doi:10.1016/j.pnpbp.2012.06.016
Matsumoto, J., Yokota, H., Yuasa, A., 2002. Developmental increases in rat hepatic microsomal UDP-glucuronosyltransferase activities toward xenoestrogens and decreases during pregnancy. Environ. Health Perspect. 110, 193–6.
Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S.-I., Kimura, M., Shimohigashi, Y., 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. J. Biochem. 142, 517–24. doi:10.1093/jb/mvm158
Matthews, J.B., Twomey, K., Zacharewski, T.R., 2001. In Vitro and in Vivo Interactions of Bisphenol A and Its Metabolite, Bisphenol A Glucuronide, with Estrogen Receptors α and β. Chem. Res. Toxicol. 14, 149–157. doi:10.1021/tx0001833
McCaffrey, K.A., Jones, B., Mabrey, N., Weiss, B., Swan, S.H., Patisaul, H.B., 2013. Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation. Neurotoxicology 36, 55–62. doi:10.1016/j.neuro.2013.03.001
McCarthy, M., 2015. Sex on the Brain [WWW Document]. Sci. Mag. URL http://www.the-scientist.com/?articles.view/articleNo/44048/title/Sex-on-the-Brain/ (accessed 6.28.17).
McCarthy, M.M., Schlenker, E.H., Pfaff, D.W., 1993. Enduring consequences of neonatal

treatment with antisense oligodeoxynucleotides to estrogen receptor messenger ribonucleic acid on sexual differentiation of rat brain. Endocrinology 133, 433–439. doi:10.1210/endo.133.2.8344188

McQuown, S.C., Barrett, R.M., Matheos, D.P., Post, R.J., Rogge, G.A., Alenghat, T., Mullican, S.E., Jones, S., Rusche, J.R., Lazar, M.A., Wood, M.A., 2011. HDAC3 is a critical negative regulator of long-term memory formation. J. Neurosci. 31, 764–774. doi:10.1523/JNEUROSCI.5052-10.2011

Meaney, M.J., 2001. Maternal Care, Gene Expression, and the Transmission of Individual Differences in Stress Reactivity Across Generations. Annu. Rev. Neurosci. 24, 1161–1192. doi:10.1146/annurev.neuro.24.1.1161

Metz, C.M., 2016. Bisphenol A: Understanding the controversy. Workplace Health Saf. 64, 28–36. doi:10.1177/2165079915623790

Miao, M., Yuan, W., He, Y., Zhou, Z., Wang, J., Gao, E., Li, G., Li, D.-K., 2011. In utero exposure to bisphenol-A and anogenital distance of male offspring. Birth Defects Res. A. Clin. Mol. Teratol. 91, 867–72. doi:10.1002/bdra.22845

Michałowicz, J., 2014. Bisphenol A - Sources, toxicity and biotransformation. Environ. Toxicol. Pharmacol. 37, 738–758. doi:10.1016/j.etap.2014.02.003

Michaud, J.L., Rosenquist, T., May, N.R., Fan, C.-M., 1998. Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. Genes Dev. 12, 3264–3275. doi:10.1101/gad.12.20.3264

Millen, K.J., Wurst, W., Herrup, K., Joyner, A.L., 1994. Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants. Development 120, 695–706.

Milligan, S.R., Khan, O., Nash, M., 1998. Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (Oncorhynchus mykiss) plasma. Gen. Comp. Endocrinol. 112, 89–95. doi:10.1006/gcen.1998.7146

Miodovnik, A., Edwards, A., Bellinger, D.C., Hauser, R., 2014. Developmental neurotoxicity of ortho-phthalate diesters: Review of human and experimental evidence. Neurotoxicology 41, 112–122. doi:10.1016/j.neuro.2014.01.007

Mizuo, K., Narita, M., Yoshida, T., Narita, M., Suzuki, T., 2004. Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice. Addict. Biol. 9, 19–25. doi:10.1080/13556210410001674059

Molinari, S., Battini, R., Ferrari, S., Pozzi, L., Killcross, A.S., Robbins, T.W., Jouvenceau, A., Billard, J.M., Dutar, P., Lamour, Y., Baker, W.A., Cox, H., Emson, P.C., 1996. Deficits in memory and hippocampal long-term potentiation in mice with reduced calbindin D28K expression. Proc Natl Acad Sci U S A 93, 8028–8033.

Monje, L., Varayoud, J., Luque, E.H., Ramos, J.G., 2007. Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor alpha transcripts with alternative 5'-untranslated regions in the female rat preoptic area. J. Endocrinol. 194, 201–12. doi:10.1677/JOE-07-0014

Moon, M.K., Jeong, I.-K., Jung Oh, T., Ahn, H.Y., Kim, H.H., Park, Y.J., Jang, H.C., Park, K.S., 2015. Long-term oral exposure to bisphenol A induces glucose intolerance and insulin resistance. J. Endocrinol. 226, 35–42. doi:10.1530/JOE-14-0714

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., Nakao, K., 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. J. Clin. Endocrinol. Metab. 87, 5185–90. doi:10.1210/jc.2002-020209

Muller, J.M., Brunelli, S. a, Moore, H., Myers, M.M., Shair, H.N., 2005. Maternally modulated infant separation responses are regulated by D2-family dopamine receptors. Behav. Neurosci. 119, 1384–1388. doi:10.1037/0735-7044.119.5.1384

Muller, J.M., Moore, H., Myers, M.M., Shair, H.N., 2008. Ventral striatum dopamine D2 receptor activity inhibits rat pups' vocalization response to loss of maternal contact. Behav. Neurosci. 122, 119–128. doi:10.1037/0735-7044.122.1.119

Muller, J.M., Shair, H.N., 2016. Isolation-induced vocalization in the infant rat depends on the nucleus accumbens. Dev. Psychobiol. 58, 1116–1123. doi:10.1002/dev.21447

Muller, M.B., Zimmermann, S., Sillaber, I., Hagemeyer, T.P., Deussing, J.M., Timpl, P., Kormann, M.S., Droste, S.K., Kuhn, R., Reul, J.M., Holsboer, F., Wurst, W., 2003. Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat Neurosci 6, 1100–1107. doi:10.1038/nn1123

Murakami, G., Hunter, R.G., Fontaine, C., Ribeiro, A., Pfaff, D., 2011. Relationships among estrogen receptor, oxytocin and vasopressin gene expression and social interaction in male mice. Eur. J. Neurosci. 34, 469–77. doi:10.1111/j.1460-9568.2011.07761.x

Mustieles, V., Pérez-Lobato, R., Olea, N., Fernández, M.F., 2015. Bisphenol A: Human exposure and neurobehavior. Neurotoxicology 49, 174–84. doi:10.1016/j.neuro.2015.06.002

Musumeci, G., Sciarretta, C., Rodriguez-Moreno, A., Al Banchaabouchi, M., Negrete-Diaz, V., Costanzi, M., Berno, V., Egorov, A. V, von Bohlen Und Halbach, O., Cestari, V., Delgado-Garcia, J.M., Minichiello, L., 2009. TrkB modulates fear learning and amygdalar synaptic plasticity by specific docking sites. J Neurosci 29, 10131–10143. doi:10.1523/JNEUROSCI.1707-09.2009

Mychasiuk, R., Gibb, R., Kolb, B., 2011. Prenatal bystander stress induces neuroanatomical changes in the prefrontal cortex and hippocampus of developing rat offspring. Brain Res. 1412, 55–62. doi:10.1016/j.brainres.2011.07.023

Nagao, T., Saito, Y., Usumi, K., Kuwagata, M., Imai, K., 1999. Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. Reprod. Toxicol. 13, 303–311. doi:10.1016/S0890-6238(99)00017-9

Nakagami, A., Negishi, T., Kawasaki, K., Imai, N., Nishida, Y., Ihara, T., Kuroda, Y., Yoshikawa, Y., Koyama, T., 2009. Alterations in male infant behaviors towards its mother by prenatal exposure to bisphenol A in cynomolgus monkeys (Macaca fascicularis) during early suckling period. Psychoneuroendocrinology 34, 1189–97. doi:10.1016/j.psyneuen.2009.03.005

Nakamura, K., Itoh, K., Sugimoto, T., Fushiki, S., 2007a. Prenatal exposure to bisphenol A affects adult murine neocortical structure. Neurosci. Lett. 420, 100–105. doi:10.1016/j.neulet.2007.02.093

Nakamura, K., Itoh, K., Sugimoto, T., Fushiki, S., 2007b. Prenatal exposure to bisphenol A affects adult murine neocortical structure. Neurosci. Lett. 420, 100–5. doi:10.1016/j.neulet.2007.02.093

Nakamura, K., Itoh, K., Yaoi, T., Fujiwara, Y., Sugimoto, T., Fushiki, S., 2006. Murine neocortical histogenesis is perturbed by prenatal exposure to low doses of Bisphenol A. J. Neurosci. Res. 84, 1197–205. doi:10.1002/jnr.21020

Narita, M., Miyagawa, K., Mizuo, K., Yoshida, T., Suzuki, T., 2006. Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect. Neurosci. Lett. 402, 249–52. doi:10.1016/j.neulet.2006.04.014

Naulé, L., Picot, M., Martini, M., Parmentier, C., Hardin-Pouzet, H., Keller, M., Franceschini, I., Mhaouty-Kodja, S., 2014. Neuroendocrine and behavioral effects of maternal exposure to oral bisphenol A in female mice. J. Endocrinol. 220, 375–88. doi:10.1530/JOE-13-0607

Negishi, T., Nakagami, A., Kawasaki, K., Nishida, Y., Ihara, T., Kuroda, Y., Tashiro, T., Koyama, T., Yoshikawa, Y., 2014. Altered social interactions in male juvenile cynomolgus monkeys prenatally exposed to bisphenol A. Neurotoxicol. Teratol. 44, 46–52. doi:10.1016/j.ntt.2014.05.004

Nishikawa, M., Iwano, H., Yanagisawa, R., Koike, N., Inoue, H., Yokota, H., 2010. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. Environ.

Health Perspect. 118, 1196–203. doi:10.1289/ehp.0901575
Nunez, A.A., Nyby, J., Whitney, G., 1978. The effects of testosterone, estradiol, and dihydrotestosterone on male mouse (Mus musculus) ultrasonic vocalizations. Horm. Behav. 11, 264–72.
Ogi, H., Itoh, K., Fushiki, S., 2013. Social behavior is perturbed in mice after exposure to bisphenol A: a novel assessment employing an IntelliCage. Brain Behav. 3, 223–8. doi:10.1002/brb3.130
Okada, H., Tokunaga, T., Liu, X., Takayanagi, S., Matsushima, A., Shimohigashi, Y., 2008. Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-gamma. Environ. Health Perspect. 116, 32–8. doi:10.1289/ehp.10587
Okada, M., Makino, A., Nakajima, M., Okuyama, S., Furukawa, S., Furukawa, Y., 2010. Estrogen stimulates proliferation and differentiation of neural stem/progenitor cells through different signal transduction pathways. Int. J. Mol. Sci. 11, 4114–4123. doi:10.3390/ijms11104114
Okada, M., Murase, K., Makino, A., Nakajima, M., Kaku, T., Furukawa, S., Furukawa, Y., 2008. Effects of estrogens on proliferation and differentiation of neural stem/progenitor cells. Biomed. Res. 29, 163–170. doi:10.2220/biomedres.29.163
Orikasa, C., Kondo, Y., Hayashi, S., McEwen, B.S., Sakuma, Y., 2002. Sexually dimorphic expression of estrogen receptor beta in the anteroventral periventricular nucleus of the rat preoptic area: implication in luteinizing hormone surge. Proc. Natl. Acad. Sci. U. S. A. 99, 3306–3311. doi:10.1073/pnas.052707299
Orikasa, C., Sakuma, Y., 2010. Estrogen configures sexual dimorphism in the preoptic area of C57BL/6J and ddN strains of mice. J. Comp. Neurol. 518, 3618–3629. doi:10.1002/cne.22419
Palanza, P., Gioiosa, L., vom Saal, F.S., Parmigiani, S., 2008. Effects of developmental exposure to bisphenol A on brain and behavior in mice. Environ. Res. 108, 150–157. doi:10.1016/j.envres.2008.07.023
Palanza, P., Nagel, S.C., Parmigiani, S., vom Saal, F.S., 2016. Perinatal exposure to endocrine disruptors: sex, timing and behavioral endpoints. Curr. Opin. Behav. Sci. 7, 69–75. doi:10.1016/j.cobeha.2015.11.017
Palanza, P.L., Howdeshell, K.L., Parmigiani, S., vom Saal, F.S., 2002. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspect. 110 Suppl, 415–22.
Palmen, S.J., van Engeland, H., Hof, P.R., Schmitz, C., 2004. Neuropathological findings in autism. Brain 127, 2572–2583. doi:10.1093/brain/awh287awh287 [pii]
Panagiotidou, E., Zerva, S., Mitsiou, D.J., Alexis, M.N., Kitraki, E., 2014. Perinatal exposure to low-dose bisphenol A affects the neuroendocrine stress response in rats. J. Endocrinol. 220, 207–218. doi:10.1530/JOE-13-0416
Pascual, R., Zamora-Leon, P., Catalan-Ahumada, M., Valero-Cabre, A., 2007. Early social isolation decreases the expression of calbindin D-28k and dendritic branching in the medial prefrontal cortex of the rat. Int J Neurosci 117, 465–476. doi:10.1080/00207450600773459
Patisaul, H.B., Bateman, H.L., 2008. Neonatal exposure to endocrine active compounds or an ERbeta agonist increases adult anxiety and aggression in gonadally intact male rats. Horm. Behav. 53, 580–8. doi:10.1016/j.yhbeh.2008.01.008
Patisaul, H.B., Fortino, A.E., Polston, E.K., 2007. Differential disruption of nuclear volume and neuronal phenotype in the preoptic area by neonatal exposure to genistein and bisphenol-A. Neurotoxicology 28, 1–12. doi:10.1016/j.neuro.2006.10.001
Patisaul, H.B., Fortino, A.E., Polston, E.K., 2006. Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. Neurotoxicol. Teratol. 28, 111–8. doi:10.1016/j.ntt.2005.11.004

Patisaul, H.B., Sullivan, A.W., Radford, M.E., Walker, D.M., Adewale, H.B., Winnik, B., Coughlin, J.L., Buckley, B., Gore, A.C., 2012. Anxiogenic effects of developmental bisphenol A exposure are associated with gene expression changes in the juvenile rat amygdala and mitigated by soy. PLoS One 7, e43890. doi:10.1371/journal.pone.0043890

Paylor, R., Tracy, R., Wehner, J., Rudy, J.W., 1994. DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. Behav. Neurosci. 108, 810–817. doi:10.1037/0735-7044.108.4.810

Pei, L., Mu, Y., Leblanc, M., Alaynick, W., Barish, G.D., Pankratz, M., Tseng, T.W., Kaufman, S., Liddle, C., Yu, R.T., Downes, M., Pfaff, S.L., Auwerx, J., Gage, F.H., Evans, R.M., 2015. Dependence of Hippocampal Function on ERRγ-Regulated Mitochondrial Metabolism. Cell Metab. 21, 628–636. doi:10.1016/j.cmet.2015.03.004

Pellegrini, M., Bulzomi, P., Lecis, M., Leone, S., Campesi, I., Franconi, F., Marino, M., 2014. Endocrine Disruptors Differently Influence Estrogen Receptor β and Androgen Receptor in Male and Female Rat VSMC. J. Cell. Physiol. 229, 1061–8. doi:10.1002/jcp.24530

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods 14, 149–67.

Perera, F., Nolte, E.L.R., Wang, Y., Margolis, A.E., Calafat, A.M., Wang, S., Garcia, W., Hoepner, L.A., Peterson, B.S., Rauh, V., Herbstman, J., 2016. Bisphenol A exposure and symptoms of anxiety and depression among inner city children at 10–12 years of age. Environ. Res. 151, 195–202. doi:10.1016/j.envres.2016.07.028

Perera, F., Vishnevetsky, J., Herbstman, J.B., Calafat, A.M., Xiong, W., Rauh, V., Wang, S., 2012. Prenatal bisphenol a exposure and child behavior in an inner-city cohort. Environ. Health Perspect. 120, 1190–4. doi:10.1289/ehp.1104492

Perez-Pouchoulen, M., Toledo, R., Garcia, L.I., Perez-Estudillo, C.A., Coria-Avila, G.A., Hernandez, M.E., Carrillo, P., Manzo, J., 2016. Androgen receptors in Purkinje neurons are modulated by systemic testosterone and sexual training in a region-specific manner in the male rat. Physiol. Behav. 156, 191–198. doi:10.1016/j.physbeh.2016.01.027

Phillips, R.G., LeDoux, J.E., 1992. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav. Neurosci. 106, 274–285. doi:10.1037/0735-7044.106.2.274

Pierman, S., Sica, M., Allieri, F., Viglietti-Panzica, C., Panzica, G.C., Bakker, J., 2008. Activational effects of estradiol and dihydrotestosterone on social recognition and the arginine-vasopressin immunoreactive system in male mice lacking a functional aromatase gene. Horm. Behav. 54, 98–106. doi:10.1016/j.yhbeh.2008.02.001

Pinaud, R., Saldanha, C.J., Wynne, R.D., Lovell, P. V., Mello, C. V., 2007. The excitatory thalamo-"cortical" projection within the song control system of zebra finches is formed by calbindin-expressing neurons. J. Comp. Neurol. 504, 601–618. doi:10.1002/cne.21457

Poimenova, A., Markaki, E., Rahiotis, C., Kitraki, E., 2010. Corticosterone-regulated actions in the rat brain are affected by perinatal exposure to low dose of bisphenol A. Neuroscience 167, 741–9. doi:10.1016/j.neuroscience.2010.02.051

Pomerantz, S.M., Fox, E., Clemens, L.G., 1983. Gonadal hormone activation of male courtship ultrasonic vocalizations and male copulatory behavior in castrated male deer mice (Peromyscus maniculatus bairdi). Behav. Neurosci. 97, 462–9.

Porrini, S., Belloni, V., Della Seta, D., Farabollini, F., Giannelli, G., Dessì-Fulgheri, F., 2005. Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res. Bull. 65, 261–6. doi:10.1016/j.brainresbull.2004.11.014

Prasanth, G.K., Divya, L.M., Sadasivan, C., 2010. Bisphenol-A can bind to human glucocorticoid receptor as an agonist: an in silico study. J. Appl. Toxicol. 30, 769–74. doi:10.1002/jat.1570

Prins, G.S., Hu, W.-Y., Shi, G.-B., Hu, D.-P., Majumdar, S., Li, G., Huang, K., Nelles, J.L., Ho, S.-M., Walker, C.L., Kajdacsy-Balla, A., van Breemen, R.B., 2014. Bisphenol A promotes human prostate stem-progenitor cell self-renewal and increases in vivo carcinogenesis in

human prostate epithelium. Endocrinology 155, 805–17. doi:10.1210/en.2013-1955
Qiu, L.-L., Wang, X., Zhang, X., Zhang, Z., Gu, J., Liu, L., Wang, Y., Wang, X., Wang, S.-L., 2013. Decreased androgen receptor expression may contribute to spermatogenesis failure in rats exposed to low concentration of bisphenol A. Toxicol. Lett. 219, 116–24. doi:10.1016/j.toxlet.2013.03.011
Rajakumar, C., Guan, H., Langlois, D., Cernea, M., Yang, K., 2015. Bisphenol A disrupts gene expression in human placental trophoblast cells. Reprod. Toxicol. 53, 39–44. doi:10.1016/j.reprotox.2015.03.001
Rattiner, L.M., Davis, M., French, C.T., Ressler, K.J., 2004. Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J. Neurosci. 24, 4796–806. doi:10.1523/JNEUROSCI.5654-03.2004
Razzaque, M.A., Masuda, N., Maeda, Y., Endo, Y., Tsukamoto, T., Osumi, T., 2004. Estrogen receptor-related receptor gamma has an exceptionally broad specificity of DNA sequence recognition. Gene 340, 275–82. doi:10.1016/j.gene.2004.07.010
Real, M.A., Simon, M.P., Heredia, R., de Diego, Y., Guirado, S., 2011. Phenotypic changes in calbindin D28K immunoreactivity in the hippocampus of Fmr1 knockout mice. J Comp Neurol 519, 2622–2636. doi:10.1002/cne.22643
Rebuli, M.E., Cao, J., Sluzas, E., Delclos, K.B., Camacho, L., Lewis, S.M., Vanlandingham, M.M., Patisaul, H.B., 2014. Investigation of the effects of subchronic low dose oral exposure to bisphenol A (BPA) and ethinyl estradiol (EE) on estrogen receptor expression in the juvenile and adult female rat hypothalamus. Toxicol. Sci. 140, 190–203. doi:10.1093/toxsci/kfu074
Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., Prossnitz, E.R., 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307, 1625–30. doi:10.1126/science.1106943
Rhees, R.W., Al-Saleh, H.N., Kinghorn, E.W., Fleming, D.E., Lephart, E.D., 1999. Relationship between sexual behavior and sexually dimorphic structures in the anterior hypothalamus in control and prenatally stressed male rats. Brain Res. Bull. 50, 193–199. doi:10.1016/S0361-9230(99)00191-4
Riascos, D., de Leon, D., Baker-Nigh, A., Nicholas, A., Yukhananov, R., Bu, J., Wu, C.K., Geula, C., 2011. Age-related loss of calcium buffering and selective neuronal vulnerability in Alzheimer's disease. Acta Neuropathol 122, 565–576. doi:10.1007/s00401-011-0865-4
Rilett, K.C., Friedel, M., Ellegood, J., MacKenzie, R.N., Lerch, J.P., Foster, J.A., 2015. Loss of T cells influences sex differences in behavior and brain structure. Brain. Behav. Immun. 46, 249–60. doi:10.1016/j.bbi.2015.02.016
Rissman, E.F., Wersinger, S.R., Fugger, H.N., Foster, T.C., 1999. Sex with knockout models: Behavioral studies of estrogen receptor. Brain Res. 835, 80–90. doi:10.1016/S0006-8993(99)01452-3
Rocio, P.L., Vicente, M., Irene, C., Inmaculada, J.D., Rosa, R., Noelia, C.C., José, L.J.F., Soledad, R., Nicolás, O., Fernandez, M.F., 2016. Exposure to bisphenol A and behavior in school-age children. Neurotoxicology 53, 12–19. doi:10.1016/j.neuro.2015.12.001
Rodgers, A.B., Morgan, C.P., Bronson, S.L., Revello, S., Bale, T.L., 2013. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. J. Neurosci. 33, 9003–12. doi:10.1523/JNEUROSCI.0914-13.2013
Rodgers, A.B., Morgan, C.P., Leu, N.A., Bale, T.L., 2015. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. Proc. Natl. Acad. Sci. 112, 13699–13704. doi:10.1073/pnas.1508347112
Rodgers, R.J., Cole, J.C., 1993. Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. Physiol. Behav. 54, 729–36.
Roen, E.L., Wang, Y., Calafat, A.M., Wang, S., Margolis, A., Herbstman, J., Hoepner, L.A., Rauh, V., Perera, F.P., 2015. Bisphenol A exposure and behavioral problems among inner

city children at 7-9 years of age. Environ. Res. doi:10.1016/j.envres.2015.01.014
Ronan, J.L., Wu, W., Crabtree, G.R., 2013. From neural development to cognition: unexpected roles for chromatin. Nat. Rev. Genet. 14, 347–359. doi:10.1038/nrg3413
Rosenfeld, C.S., 2015. Bisphenol A and phthalate endocrine disruption of parental and social behaviors. Front. Neurosci. 9, 57. doi:10.3389/fnins.2015.00057
Rossignol, E., 2011. Genetics and function of neocortical GABAergic interneurons in neurodevelopmental disorders. Neural Plast. 2011, 649325. doi:10.1155/2011/649325
Rubin, B.S., Lenkowski, J.R., Schaeberle, C.M., Vandenberg, L.N., Ronsheim, P.M., Soto, A.M., 2006. Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. Endocrinology 147, 3681–91. doi:10.1210/en.2006-0189
Rubin, B.S., Soto, A.M., 2009. Bisphenol A: Perinatal exposure and body weight. Mol. Cell. Endocrinol. 304, 55–62. doi:10.1016/j.mce.2009.02.023
Ryan, B.C., Vandenbergh, J.G., 2006. Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Horm. Behav. 50, 85–93.
Sadowski, R.N., Park, P., Neese, S.L., Ferguson, D.C., Schantz, S.L., Juraska, J.M., 2014. Effects of perinatal bisphenol A exposure during early development on radial arm maze behavior in adult male and female rats. Neurotoxicol. Teratol. 42, 17–24. doi:10.1016/j.ntt.2014.01.002
Saili, K.S., Corvi, M.M., Weber, D.N., Patel, A.U., Das, S.R., Przybyla, J., Anderson, K.A., Tanguay, R.L., 2012. Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. Toxicology 291, 83–92. doi:10.1016/j.tox.2011.11.001
Sakai, T., Oshima, A., Nozaki, Y., Ida, I., Haga, C., Akiyama, H., Nakazato, Y., Mikuni, M., 2008. Changes in density of calcium-binding-protein-immunoreactive GABAergic neurons in prefrontal cortex in schizophrenia and bipolar disorder. Neuropathology 28, 143–150. doi:NEU867 [pii]10.1111/j.1440-1789.2007.00867.x
Sakamoto, H., Mezaki, Y., Shikimi, H., Ukena, K., Tsutsui, K., 2003. Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. Endocrinology 144, 4466–4477. doi:10.1210/en.2003-0307
Salian, S., Doshi, T., Vanage, G., 2009. Impairment in protein expression profile of testicular steroid receptor coregulators in male rat offspring perinatally exposed to Bisphenol A. Life Sci. 85, 11–8. doi:10.1016/j.lfs.2009.04.005
Sandhu, K. V, Lang, D., Muller, B., Nullmeier, S., Yanagawa, Y., Schwegler, H., Stork, O., 2014. Glutamic acid decarboxylase 67 haplodeficiency impairs social behavior in mice. Genes Brain Behav 13, 439–450. doi:10.1111/gbb.12131
Sando 3rd, R., Gounko, N., Pieraut, S., Liao, L., Yates 3rd, J., Maximov, A., 2012. HDAC4 governs a transcriptional program essential for synaptic plasticity and memory. Cell 151, 821–834. doi:10.1016/j.cell.2012.09.037
Sasahara, K., Shikimi, H., Haraguchi, S., Sakamoto, H., Honda, S. -i., Harada, N., Tsutsui, K., 2007. Mode of Action and Functional Significance of Estrogen-Inducing Dendritic Growth, Spinogenesis, and Synaptogenesis in the Developing Purkinje Cell. J. Neurosci. 27, 7408–7417. doi:10.1523/JNEUROSCI.0710-07.2007
Scattoni, M.L., Crawley, J., Ricceri, L., 2009. Ultrasonic vocalizations: A tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci. Biobehav. Rev. 33, 508–515. doi:10.1016/j.neubiorev.2008.08.003
Scattoni, M.L., Gandhy, S.U., Ricceri, L., Crawley, J.N., 2008. Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. PLoS One 3, e3067. doi:10.1371/journal.pone.0003067
Schmitz, C., Hof, P.R., 2005. Design-based stereology in neuroscience. Neuroscience 130, 813–31. doi:10.1016/j.neuroscience.2004.08.050
Schönfelder, G., Wittfoht, W., Hopp, H., Talsness, C.E., Paul, M., Chahoud, I., 2002. Parent

bisphenol A accumulation in the human maternal-fetal-placental unit. Environ. Health Perspect. 110, A703-7.
Schroeder, A.C., Privalsky, M.L., 2014. Thyroid Hormones, T3 and T4, in the Brain. Front. Endocrinol. (Lausanne). 5, 40. doi:10.3389/fendo.2014.00040
Schug, T.T., Blawas, A.M., Gray, K., Heindel, J.J., Lawler, C.P., 2015. Elucidating the links between endocrine disruptors and neurodevelopment. Endocrinology 156, 1941–1951. doi:10.1210/en.2014-1734
Schug, T.T., Blawas, A.M., Gray, K., Heindel, J.J., Lawler, C.P., 2015. Elucidating the links between endocrine disruptors and neurodevelopment. Endocrinology 156, 1941–51. doi:10.1210/en.2014-1734
Schwaller, B., 2009. The continuing disappearance of "pure" Ca2+ buffers. Cell. Mol. Life Sci. 66, 275–300. doi:10.1007/s00018-008-8564-6
Seney, M.L., Chang, L.C., Oh, H., Wang, X., Tseng, G.C., Lewis, D.A., Sibille, E., 2013a. The Role of Genetic Sex in Affect Regulation and Expression of GABA-Related Genes Across Species. Front Psychiatry 4, 104. doi:10.3389/fpsyt.2013.00104
Seney, M.L., Ekong, K.I., Ding, Y., Tseng, G.C., Sibille, E., 2013b. Sex chromosome complement regulates expression of mood-related genes. Biol Sex Differ 4, 20. doi:10.1186/2042-6410-4-20
Sheng, Z.-G., Tang, Y., Liu, Y.-X., Yuan, Y., Zhao, B.-Q., Chao, X.-J., Zhu, B.-Z., 2012. Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. Toxicol. Appl. Pharmacol. 259, 133–42. doi:10.1016/j.taap.2011.12.018
Shikimi, H., Sakamoto, H., Mezaki, Y., Ukena, K., Tsutsui, K., 2004. Dendritic growth in response to environmental estrogens in the developing Purkinje cell in rats. Neurosci. Lett. 364, 114–118. doi:10.1016/j.neulet.2004.04.023
Sickel, M.J., McCarthy, M.M., 2000. Calbindin-d28k immunoreactivity is a marker for a subdivision of the sexually dimorphic nucleus of the preoptic area of the rat: Developmental profile and gonadal steroid modulation. J. Neuroendocrinol. 12, 397–402. doi:10.1046/j.1365-2826.2000.00474.x
Simerly, R.B., 2002. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu. Rev. Neurosci. 25, 507–36. doi:10.1146/annurev.neuro.25.112701.142745
Simerly, R.B., Swanson, L.W., Gorski, R.A., 1985. The distribution of monoaminergic cells and fibers in a periventricular preoptic nucleus involved in the control of gonadotropin release: Immunohistochemical evidence for a dopaminergic sexual dimorphism. Brain Res. 330, 55–64. doi:10.1016/0006-8993(85)90007-1
Simerly, R.B., Swanson, L.W., Handa, R.J., Gorski, R.A., 1985. Influence of perinatal androgen on the sexually dimorphic distribution of tyrosine hydroxylase-immunoreactive cells and fibers in the anteroventral periventricular nucleus of the rat. Neuroendocrinology 40, 501–510. doi:10.1159/000124122
Simerly, R.B., Zee, M.C., Pendleton, J.W., Lubahn, D.B., Korach, K.S., 1997. Estrogen receptor-dependent sexual differentiation of dopaminergic neurons in the preoptic region of the mouse. Neurobiology 94, 14077–14082. doi:10.1073/pnas.94.25.14077
Skinner, M.K., 2015. Endocrine disruptors in 2015: Epigenetic transgenerational inheritance. Nat. Rev. Endocrinol. 2–3. doi:10.1038/nrendo.2015.206
Skinner, M.K., Manikkam, M., Guerrero-Bosagna, C., 2011. Epigenetic transgenerational actions of endocrine disruptors. Reprod. Toxicol. 31, 337–43. doi:10.1016/j.reprotox.2010.10.012
Skledar, D.G., Schmidt, J., Fic, A., Klopčič, I., Trontelj, J., Dolenc, M.S., Finel, M., Mašič, L.P., 2016. Influence of metabolism on endocrine activities of bisphenol S. Chemosphere 157, 152–159. doi:10.1016/j.chemosphere.2016.05.027
Somogyi, V., Horváth, T.L., Tóth, I., Bartha, T., Frenyó, L.V., Kiss, D.S., Jócsák, G., Kerti, A.,

Naftolin, F., Zsarnovszky, A., 2016. Bisphenol A influences oestrogen- and thyroid hormone-regulated thyroid hormone receptor expression in rat cerebellar cell culture. Acta Vet. Hung. 64, 497–513. doi:10.1556/004.2016.046

Sosa-Ferrera, Z., Mahugo-Santana, C., Santana-Rodr?guez, J.J., 2013. Analytical Methodologies for the Determination of Endocrine Disrupting Compounds in Biological and Environmental Samples. Biomed Res. Int. 2013, 1–23. doi:10.1155/2013/674838

Stein, T.P., Schluter, M.D., Steer, R.A., Guo, L., Ming, X., 2015. Bisphenol a exposure in children with autism spectrum disorders. Autism Res. 272–283. doi:10.1002/aur.1444

Sullivan, A.W., Beach, E.C., Stetzik, L.A., Perry, A., D'Addezio, A.S., Cushing, B.S., Patisaul, H.B., 2014. A Novel Model for Neuroendocrine Toxicology: Neurobehavioral Effects of BPA Exposure in a Prosocial Species, the Prairie Vole (*Microtus ochrogaster*). Endocrinology 155, 3867–3881. doi:10.1210/en.2014-1379

Sun, S., Li, F., Gao, X., Zhu, Y., Chen, J., Zhu, X., Yuan, H., Gao, D., 2011. Calbindin-D28K inhibits apoptosis in dopaminergic neurons by activation of the PI3-kinase-Akt signaling pathway. Neuroscience 199, 359–367. doi:10.1016/j.neuroscience.2011.09.054

Swiergiel, A.H., Takahashi, L.K., Kalin, N.H., 1993. Attenuation of stress-induced behavior by antagonism of corticotropin-releasing factor receptors in the central amygdala in the rat. Brain Res 623, 229–234.

Tachibana, T., Wakimoto, Y., Nakamuta, N., Phichitraslip, T., Wakitani, S., Kusakabe, K., Hondo, E., Kiso, Y., 2007. Effects of bisphenol A (BPA) on placentation and survival of the neonates in mice. J. Reprod. Dev. 53, 509–14.

Takagi, H., Shibutani, M., Masutomi, N., Uneyama, C., Takahashi, N., Mitsumori, K., Hirose, M., 2004. Lack of maternal dietary exposure effects of bisphenol A and nonylphenol during the critical period for brain sexual differentiation on the reproductive/endocrine systems in later life. Arch. Toxicol. 78, 97–105. doi:10.1007/s00204-003-0517-0

Takao, T., Nanamiya, W., Pournajafi Nazarloo, H., Matsumoto, R., Asaba, K., Hashimoto, K., 2003. Exposure to the environmental estrogen bisphenol A differentially modulated estrogen receptor-α and -β immunoreactivity and mRNA in male mouse testis. Life Sci. 72, 1159–1169. doi:10.1016/S0024-3205(02)02364-0

Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., Shimohigashi, Y., 2006. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. Toxicol. Lett. 167, 95–105. doi:10.1016/j.toxlet.2006.08.012

Takeda, Y., Liu, X., Sumiyoshi, M., Matsushima, A., Shimohigashi, M., Shimohigashi, Y., 2009. Placenta Expressing the Greatest Quantity of Bisphenol A Receptor ERR among the Human Reproductive Tissues: Predominant Expression of Type-1 ERR Isoform. J. Biochem. 146, 113–122. doi:10.1093/jb/mvp049

Tanabe, N., Yoshino, H., Kimoto, T., Hojo, Y., Ogiue-Ikeda, M., Shimohigashi, Y., Kawato, S., 2012. Nanomolar dose of bisphenol A rapidly modulates spinogenesis in adult hippocampal neurons. Mol. Cell. Endocrinol. 351, 317–325. doi:10.1016/j.mce.2012.01.008

Tanabe, Y., Fujiwara, Y., Matsuzaki, A., Fujita, E., Kasahara, T., Yuasa, S., Momoi, T., 2012. Temporal expression and mitochondrial localization of a Foxp2 isoform lacking the forkhead domain in developing Purkinje cells. J. Neurochem. 122, 72–80. doi:10.1111/j.1471-4159.2011.07524.x

Tanaka, M., Nakaya, S., Katayama, M., Leffers, H., Nozawa, S., Nakazawa, R., Iwamoto, T., Kobayashi, S., 2006. Effect of prenatal exposure to bisphenol A on the serum testosterone concentration of rats at birth. Hum. Exp. Toxicol. 25, 369–373. doi:10.1191/0960327106ht638oa

Tando, S., Itoh, K., Yaoi, T., Ikeda, J., Fujiwara, Y., Fushiki, S., 2007. Effects of pre- and neonatal exposure to bisphenol A on murine brain development. Brain Dev. 29, 352–356. doi:10.1016/j.braindev.2006.10.003

Tanida, T., Matsuda, K.I., Yamada, S., Kawata, M., Tanaka, M., 2017. Immunohistochemical profiling of estrogen-related receptor gamma in rat brain and colocalization with estrogen receptor alpha in the preoptic area. Brain Res. 1659, 71–80. doi:10.1016/j.brainres.2017.01.024

Tejada, L.D., Rissman, E.F., 2012. Sex Differences in Social Investigation: Effects of Androgen Receptors, Hormones and Test Partner. J. Neuroendocrinol. 24, 1144–1153. doi:10.1111/j.1365-2826.2012.02322.x

Teppala, S., Madhavan, S., Shankar, A., 2012. Bisphenol A and metabolic syndrome: Results from NHANES. Int. J. Endocrinol. 2012. doi:10.1155/2012/598180

Tessier, C.R., Broadie, K., 2011. The fragile X mental retardation protein developmentally regulates the strength and fidelity of calcium signaling in Drosophila mushroom body neurons. Neurobiol. Dis. 41, 147–159. doi:10.1016/j.nbd.2010.09.002

Thomas, P., Dong, J., 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J. Steroid Biochem. Mol. Biol. 102, 175–9. doi:10.1016/j.jsbmb.2006.09.017

Thompson, C.C., 2000. Thyroid Hormone Action in Neural Development. Cereb. Cortex 10, 939–945. doi:10.1093/cercor/10.10.939

Tian, Y.-H., Baek, J.-H., Lee, S.-Y., Jang, C.-G., 2010. Prenatal and postnatal exposure to bisphenol a induces anxiolytic behaviors and cognitive deficits in mice. Synapse 64, 432–9. doi:10.1002/syn.20746

Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F., Wurst, W., 1998. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet 19, 162–166. doi:10.1038/520

Tiwari, S.K., Agarwal, S., Seth, B., Yadav, A., Ray, R.S., Mishra, V.N., Chaturvedi, R.K., 2015. Inhibitory Effects of Bisphenol-A on Neural Stem Cells Proliferation and Differentiation in the Rat Brain Are Dependent on Wnt/β-Catenin Pathway. Mol. Neurobiol. 52, 1735–1757. doi:10.1007/s12035-014-8940-1

Torrey, E.F., Barci, B.M., Webster, M.J., Bartko, J.J., Meador-Woodruff, J.H., Knable, M.B., 2005. Neurochemical markers for schizophrenia, bipolar disorder, and major depression in postmortem brains. Biol Psychiatry 57, 252–260. doi:10.1016/j.biopsych.2004.10.019

Trezza, V., Campolongo, P., Cassano, T., Macheda, T., Dipasquale, P., Carratù, M.R., Gaetani, S., Cuomo, V., 2008. Effects of perinatal exposure to delta-9-tetrahydrocannabinol on the emotional reactivity of the offspring: a longitudinal behavioral study in Wistar rats. Psychopharmacology (Berl). 198, 529–37. doi:10.1007/s00213-008-1162-3

Tsukahara, S., 2009. Sex differences and the roles of sex steroids in apoptosis of sexually dimorphic nuclei of the preoptic area in postnatal rats. J. Neuroendocrinol. 21, 370–376. doi:10.1111/j.1365-2826.2009.01855.x

Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J.C., Joiner, R.L., Butala, J.H., Dimond, S.S., Cagen, S.Z., Shiotsuka, R.N., Stropp, G.D., Waechter, J.M., 2002. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol. Sci. 68, 121–46.

USEPA, 2010. Bisphenol A Action Plan (CASRN 80-05-7) [WWW Document]. www.epa.gov/sites/production/files/2015-09/documents/bpa_action_plan.pdf. URL www.epa.gov/sites/production/files/2015-09/documents/bpa_action_plan.pdf

van Haaren, F., van Hest, A., Heinsbroek, R.P.W., 1990. Behavioral differences between male and female rats: Effects of gonadal hormones on learning and memory. Neurosci. Biobehav. Rev. 14, 23–33. doi:10.1016/S0149-7634(05)80157-5

van Hest, A., van Haaren, F., van de Poll, N.E., 1988. The behavior of male and female Wistar rats pressing a lever for food is not affected by sex differences in food motivation. Behav. Brain Res. 27, 215–221. doi:10.1016/0166-4328(88)90118-0

Vandenberg, L.N., 2014. Low-dose effects of hormones and endocrine disruptors. Vitam. Horm. 94, 129–65. doi:10.1016/B978-0-12-800095-3.00005-5

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.-H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W. V, Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455. doi:10.1210/er.2011-1050

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W. V, 2007a. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24, 139–77. doi:10.1016/j.reprotox.2007.07.010

Vandenberg, L.N., Maffini, M. V, Wadia, P.R., Sonnenschein, C., Rubin, B.S., Soto, A.M., 2007b. Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. Endocrinology 148, 116–27. doi:10.1210/en.2006-0561

Venerosi, A., Ricceri, L., Scattoni, M.L., Calamandrei, G., 2009. Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in CD-1 mouse pups. Environ. Health 8, 12. doi:10.1186/1476-069X-8-12

Veronesi, V.B., Batista, T.H., Ribeiro, A.C.A.F., Giusti-Paiva, A., Vilela, F.C., 2017. Maternal dipyrone treatment during lactation in mice reduces maternal behavior and increases anxiety-like behavior in offspring. Int. J. Dev. Neurosci. 58, 74–81. doi:10.1016/j.ijdevneu.2017.02.005

Völkel, W., Colnot, T., Csanády, G.A., Filser, J.G., Dekant, W., 2002. Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. Chem. Res. Toxicol. 15, 1281–7.

Wacker, D.W., Ludwig, M., 2012. Vasopressin, oxytocin, and social odor recognition. Horm. Behav. 61, 259–65. doi:10.1016/j.yhbeh.2011.08.014

Wada, H., 2017. Acoustic alterations of ultrasonic vocalization in rat pups induced by perinatal hypothyroidism. Neurotoxicology 59, 175–182. doi:10.1016/j.neuro.2016.05.017

Wang, H., Liang, S., Burgdorf, J., Wess, J., Yeomans, J., 2008. Ultrasonic Vocalizations Induced by Sex and Amphetamine in M2, M4, M5 Muscarinic and D2 Dopamine Receptor Knockout Mice. PLoS One 3, e1893. doi:10.1371/journal.pone.0001893

Wang, J., Sun, B., Hou, M., Pan, X., Li, X., 2013. The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. Int. J. Obes. (Lond). 37, 999–1005. doi:10.1038/ijo.2012.173

Wang, Y., 2003. Epididymal Sperm Count, in: Current Protocols in Toxicology. John Wiley & Sons, Inc., Hoboken, NJ, USA, p. 16.6.1-16.6.5. doi:10.1002/0471140856.tx1606s14

Waters, E.M., Simerly, R.B., 2009. Estrogen Induces Caspase-Dependent Cell Death during Hypothalamic Development. J. Neurosci. 29, 9714–9718. doi:10.1523/JNEUROSCI.0135-09.2009

Weber, D.N., Hoffmann, R.G., Hoke, E.S., Tanguay, R.L., 2015. Bisphenol A exposure during early development induces sex-specific changes in adult zebrafish social interactions. J. Toxicol. Environ. Health. A 78, 50–66. doi:10.1080/15287394.2015.958419

Wegiel, J., Flory, M., Kuchna, I., Nowicki, K., Ma, S., Imaki, H., Wegiel, J., Cohen, I.L., London, E., Wisniewski, T., Brown, W., 2014. Stereological study of the neuronal number and volume of 38 brain subdivisions of subjects diagnosed with autism reveals significant alterations restricted to the striatum, amygdala and cerebellum. Acta Neuropathol. Commun. 2, 141. doi:10.1186/s40478-014-0141-7

Weidner, K.L., Buenaventura, D.F., Chadman, K.K., 2014. Mice over-expressing BDNF in forebrain neurons develop an altered behavioral phenotype with age. Behav Brain Res 268, 222–228. doi:10.1016/j.bbr.2014.04.025

Welberg, L.A.M., Seckl, J.R., Holmes, M.C., 2000. Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently

programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. Eur. J. Neurosci. 12, 1047–1054. doi:10.1046/j.1460-9568.2000.00958.x

Westerink, R.H., Beekwilder, J.P., Wadman, W.J., 2012. Differential alterations of synaptic plasticity in dentate gyrus and CA1 hippocampal area of Calbindin-D28K knockout mice. Brain Res 1450, 1–10. doi:S0006-8993(12)00313-7 [pii]10.1016/j.brainres.2012.02.036

Whitney, E.R., Kemper, T.L., Bauman, M.L., Rosene, D.L., Blatt, G.J., 2008. Cerebellar Purkinje cells are reduced in a subpopulation of autistic brains: a stereological experiment using calbindin-D28k. Cerebellum 7, 406–16. doi:10.1007/s12311-008-0043-y

Whitney, E.R., Kemper, T.L., Rosene, D.L., Bauman, M.L., Blatt, G.J., 2008. Calbindin-D28k is a more reliable marker of human Purkinje cells than standard Nissl stains: A stereological experiment. J. Neurosci. Methods 168, 42–47. doi:10.1016/j.jneumeth.2007.09.009

Wiaderkiewicz, J., Głowacka, M., Grabowska, M., Barski, J.J., 2013. Ultrasonic vocalizations (USV) in the three standard laboratory mouse strains: Developmental analysis. Acta Neurobiol. Exp. (Wars). 73, 557–563.

Wiegand, S.J., Terasawa, E., Bridson, W.E., 1978. Persistent estrus and blockade of progesterone-induced LH release follows lesions which do not damage the suprachiasmatic nucleus. Endocrinology 102, 1645–8. doi:10.1210/endo-102-5-1645

Williams, M.T., Hennessy, M.B., Davis, H.N., 1998. Stress During Pregnancy Alters Rat Offspring Morphology and Ultrasonic Vocalizations. Physiol. Behav. 63, 337–343. doi:10.1016/S0031-9384(97)00428-9

Williams, M.T., Hennessy, M.B., Davis, H.N., 1995. CRF administered to pregnant rats alters offspring behavior and morphology. Pharmacol. Biochem. Behav. 52, 161–167. doi:10.1016/0091-3057(95)00082-8

Wilson, M.E., Westberry, J.M., Trout, A.L., 2011. Estrogen receptor-alpha gene expression in the cortex: Sex differences during development and in adulthood. Horm. Behav. 59, 353–357. doi:10.1016/j.yhbeh.2010.08.004

Wolstenholme, J.T., Edwards, M., Shetty, S.R.J., Gatewood, J.D., Taylor, J. a, Rissman, E.F., Connelly, J.J., 2012. Gestational exposure to bisphenol a produces transgenerational changes in behaviors and gene expression. Endocrinology 153, 3828–38. doi:10.1210/en.2012-1195

Wolstenholme, J.T., Goldsby, J.A., Rissman, E.F., 2013. Transgenerational effects of prenatal bisphenol A on social recognition. Horm. Behav. 64, 833–9. doi:10.1016/j.yhbeh.2013.09.007

Wolstenholme, J.T., Rissman, E.F., Connelly, J.J., 2011a. The role of Bisphenol A in shaping the brain, epigenome and behavior. Horm. Behav. 59, 296–305. doi:10.1016/j.yhbeh.2010.10.001

Wolstenholme, J.T., Taylor, J. a, Shetty, S.R.J., Edwards, M., Connelly, J.J., Rissman, E.F., 2011b. Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice. PLoS One 6, e25448. doi:10.1371/journal.pone.0025448

Xu, L.-C., Sun, H., Chen, J.-F., Bian, Q., Qian, J., Song, L., Wang, X.-R., 2005. Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. Toxicology 216, 197–203. doi:10.1016/j.tox.2005.08.006

Xu, X., Dong, F., Yang, Y., Wang, Y., Wang, R., Shen, X., 2015. Sex-specific effects of long-term exposure to bisphenol-A on anxiety- and depression-like behaviors in adult mice. Chemosphere 120, 258–66. doi:10.1016/j.chemosphere.2014.07.021

Xu, X., Hong, X., Xie, L., Li, T., Yang, Y., Zhang, Q., Zhang, G., Liu, X., 2012. Gestational and lactational exposure to bisphenol-A affects anxiety- and depression-like behaviors in mice. Horm. Behav. 62, 480–490. doi:10.1016/j.yhbeh.2012.08.005

Xu, X., Liu, X., Zhang, Q., Zhang, G., Lu, Y., Ruan, Q., Dong, F., Yang, Y., 2013a. Sex-specific effects of bisphenol-A on memory and synaptic structural modification in hippocampus of adult mice. Horm. Behav. 63, 766–775. doi:10.1016/j.yhbeh.2013.03.004

Xu, X., Xie, L., Hong, X., Ruan, Q., Lu, H., Zhang, Q., Zhang, G., Liu, X., 2013b. Perinatal exposure to bisphenol-A inhibits synaptogenesis and affects the synaptic morphological development in offspring male mice. Chemosphere 91, 1073–1081. doi:10.1016/j.chemosphere.2012.12.065

Yang, M., Chen, M., Wang, J., Xu, M., Sun, J., Ding, L., Lv, X., Ma, Q., Bi, Y., Liu, R., Hong, J., Ning, G., 2016. Bisphenol a promotes adiposity and inflammation in a nonmonotonic dose-response way in 5-week-old male and female C57BL/6J mice fed a low-calorie diet. Endocrinology 157, 2333–2345. doi:10.1210/en.2015-1926

Yaoi, T., Itoh, K., Nakamura, K., Ogi, H., Fujiwara, Y., Fushiki, S., 2008. Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A. Biochem. Biophys. Res. Commun. 376, 563–7. doi:10.1016/j.bbrc.2008.09.028

Yau, J.L.W., Noble, J., Kenyon, C.J., Ludwig, M., Seckl, J.R., 2015. Diurnal and stress-induced intra-hippocampal corticosterone rise attenuated in 11β-HSD1-deficient mice: a microdialysis study in young and aged mice. Eur. J. Neurosci. 41, 787–92. doi:10.1111/ejn.12836

Yeh, S.-H., Lin, C.-H., Gean, P.-W., 2004. Acetylation of nuclear factor-kappaB in rat amygdala improves long-term but not short-term retention of fear memory. Mol. Pharmacol. 65, 1286–1292. doi:10.1124/mol.65.5.1286

Yeo, M., Berglund, K., Hanna, M., Guo, J.U., Kittur, J., Torres, M.D., Abramowitz, J., Busciglio, J., Gao, Y., Birnbaumer, L., Liedtke, W.B., 2013. Bisphenol A delays the perinatal chloride shift in cortical neurons by epigenetic effects on the Kcc2 promoter. Proc. Natl. Acad. Sci. U. S. A. 110, 4315–20. doi:10.1073/pnas.1300959110

Yin, N., Yao, X., Qin, Z., Wang, Y.L., Faiola, F., 2015. Assessment of Bisphenol A (BPA) neurotoxicity in vitro with mouse embryonic stem cells. J. Environ. Sci. (China) 36, 181–187. doi:10.1016/j.jes.2015.06.004

Yolton, K., Xu, Y., Strauss, D., Altaye, M., Calafat, A.M., Khoury, J., 2011. Prenatal exposure to bisphenol A and phthalates and infant neurobehavior. Neurotoxicol. Teratol. 33, 558–566.

Young, D.M., Schenk, A.K., Yang, S.-B., Jan, Y.N., Jan, L.Y., 2010. Altered ultrasonic vocalizations in a tuberous sclerosis mouse model of autism. Proc. Natl. Acad. Sci. U. S. A. 107, 11074–9. doi:10.1073/pnas.1005620107

Yuan, H.-H., Chen, R.-J., Zhu, Y.-H., Peng, C.-L., Zhu, X.-R., 2013. The Neuroprotective Effect of Overexpression of Calbindin-D28k in an Animal Model of Parkinson's Disease. Mol. Neurobiol. 47, 117–122. doi:10.1007/s12035-012-8332-3

Yuan, H.H., Chen, R.J., Zhu, Y.H., Peng, C.L., Zhu, X.R., 2013. The neuroprotective effect of overexpression of calbindin-D(28k) in an animal model of Parkinson's disease. Mol Neurobiol 47, 117–122. doi:10.1007/s12035-012-8332-3

Zhang, K., Hill, K., Labak, S., Blatt, G.J., Soghomonian, J.J., 2014. Loss of glutamic acid decarboxylase (Gad67) in Gpr88-expressing neurons induces learning and social behavior deficits in mice. Neuroscience 275, 238–247. doi:10.1016/j.neuroscience.2014.06.020

Zhang, Y., Wang, L., 2011. Nuclear receptor SHP inhibition of Dnmt1 expression via ERRγ. FEBS Lett. 585, 1269–75. doi:10.1016/j.febslet.2011.03.059

Zhang, Z., Chen, K., Shih, J.C., Teng, C.T., 2006. Estrogen-Related Receptors-Stimulated Monoamine Oxidase B Promoter Activity Is Down-Regulated by Estrogen Receptors. Mol. Endocrinol. 20, 1547–1561. doi:10.1210/me.2005-0252

Zhang, Z., Teng, C.T., 2007. Interplay between estrogen-related receptor alpha (ERRα) and gamma (ERRγ) on the regulation of ERRα gene expression. Mol. Cell. Endocrinol. 264, 128–141. doi:10.1016/j.mce.2006.11.002

Zheng, F., Zhou, X., Moon, C., Wang, H., 2012. Regulation of brain-derived neurotrophic factor expression in neurons. Int. J. Physiol. Pathophysiol. Pharmacol. 4, 188–200.

Zhou, R., Bai, Y., Yang, R., Zhu, Y., Chi, X., Li, L., Chen, L., Sokabe, M., Chen, L., 2011. Abnormal synaptic plasticity in basolateral amygdala may account for hyperactivity and

attention-deficit in male rat exposed perinatally to low-dose bisphenol-A. Neuropharmacology 60, 789–98. doi:10.1016/j.neuropharm.2011.01.031

Zhou, R., Chen, F., Feng, X., Zhou, L., Li, Y., Chen, L., 2015. Perinatal exposure to low-dose of bisphenol A causes anxiety-like alteration in adrenal axis regulation and behaviors of rat offspring: A potential role for metabotropic glutamate 2/3 receptors. J. Psychiatr. Res. 64, 121–129. doi:10.1016/j.jpsychires.2015.02.018

Ziv-Gal, A., Wang, W., Zhou, C., Flaws, J.A., 2015. The effects of in utero bisphenol A exposure on reproductive capacity in several generations of mice. Toxicol. Appl. Pharmacol. 284, 354–62. doi:10.1016/j.taap.2015.03.003

Zsarnovszky, A., Le, H.H., Wang, H.S., Belcher, S.M., 2005. Ontogeny of rapid estrogen-mediated extracellular signal-regulated kinase signaling in the rat cerebellar cortex: Potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinology 146, 5388–5396. doi:10.1210/en.2005-0565