Perinatal Bisphenol A administration alters reproductive and affective behaviours and physiology in adulthood.

by

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Abstract

Bisphenol A (BPA) is a ubiquitous endocrine disrupting compound that is detectable in over 90% of Canadians and Americans. It was originally found to be an estrogenic compound, but can also act through a variety of other hormone systems, including glucocorticoids and, according to *in vitro* studies, also exerts anti-androgenic activity though the *in vivo* evidence for this claim is inconclusive. Some work has suggested that reproductive behaviours are impaired in males and there is conflicting evidence as to how emotionality may be affected. However, this field of research has been somewhat controversial, as governments worldwide have accepted that BPA is safe at low doses, despite the experimental and epidemiological evidence that low doses are in fact more likely to exert negative effects. As such, this research program is examining a wide range of doses of BPA administered to rats both pre- and postnatally. Reproduction, anxiety, learning and learned helplessness were then tested in adulthood. This data indicates that low doses of BPA administered perinatally can inhibit the reproductive behaviour of males even after multiple experiences, can demasculinize males in anxiety and depressive-like behaviours, and alters the expression of estrogen receptors in the medial amygdala. However, perinatal BPA administration did not alter the survival or soma size of motor neurons controlling penile musculature. Chronic administration during adulthood did reduce soma size in both sexually dimorphic and monomorphic motor neurons, suggesting a non-androgenic mechanism of action. The implication of these data on policy surrounding human exposure to BPA is discussed.

Keywords: Bisphenol A; Endocrine Disruptors; Estrogen; Androgen; Reproduction; Emotion.

Everybody leaves

If they get the chance

And this is my chance

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List of Acronyms or Glossary

5-HT	Serotonin
ADHD	Attention Deficit/Hyperactivity Disorder
AFP	Alpha fetoprotein
AGD	Anogenital Distance
AGI	Anogenital Investigations
ANOVA	Analysis of Variance
AR	Androgen Receptor
AR45	Androgen Receptor variant 45
ARC	Arcuate Nucleus
AVPV	Anteroventral Periventricular Nucleus
BC/LA	Bulbocavernosus/Levator Ani
BNST	Bed Nucleus of the Stria Terminalis

BPA	Bisphenol A
BTB	Blood Testis Barrier
Ca	Cornu Ammon
Ca2+	Calcium
CAE	Closed Arm Entries
CAIS	Complete Androgen Insensitivity Syndrome
CAT	Closed Arm Time
CE	Copulatory Efficiency
CHMS	Canadian Health Measures Survey
CPP	Conditioned Place Preference
D1	Dopamine Receptor 1
D2	Dopamine Receptor 2
DA	Dopamine
dAMPH	dextro amphetamine
DAT	Dopamine Transporter
DES	Diethylstilbestrol
DEX	Dexamethasone
dHPC	Dorsal Hippocampus
DHT	Dihydrotestosterone
DHTP	Dihydrotestosterone Propionate
DLN	Dorsolateral Nucleus
DPN	diarylpropionitrile
E2	Estradiol
EB	Estradiol Benzoate
EDC	Endocrine Disrupting Compound
EE2	Ethinyl Estradiol
EL	Ejaculation Latency
EPA	Environmental Protection

	Agency
EPM	Elevated Plus Maze
ERRγ	Estrogen Related Receptor gamma
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
FST	Forced Swim Test
GD	Gestational Day
GluR2	Glutamate Receptor Subunit 2
GnRH	Gonadotropin Releasing Hormone
GPR30	G-protein Coupled Receptor 30
GPR54	G-protein Coupled Receptor 54
GR	Glucocorticoid Receptor
H2O2	Hydrogen Peroxide
hAR	human Androgen Receptor
HPC	Hippocampus
HPG	Hypothalamic Pituitary Gonadal Axis
IL	Intromission Latency
ir	Immunoreactive
KISS	Kisspeptin
KO	Knock-out
LC	Locus Coeruleus
LE	Long-Evans
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
LoF	List of Figures
LoT	List of Tables
MA	Monoamines

MeA	Medial Amygdala
MeApd	Medial Amygdala, posterodorsal division
METH	Methamphetamine
mg/kg	millogram per kilogram bodyweight per day
ML	Mount Latency
mPFC	Medial Prefrontal Cortex
MPN	Medial Preoptic Nucleus
mPOA	medial Preoptin Area
mRNA	messenger ribonucleic acid
MWM	Morris Water Maze
NE	Northeast
NE	Norepinephrine
NGS	Normal Goat Serum
NHANES	National Health and Nutrition Examination Survey
NMDA	N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartate Receptor
NOEL	No Observed Effect Level
NTP	National Toxicology Program
NW	Northwest
OAE	Open Arm Entries
OAT	Open Arm Time
OVX	Ovariectomized
P	Progesterone
PARA	Paraformaldehyde
PBS	Phosphate-buffered Saline
PBS-X	Phosphate-buffered saline with Triton X
PD	Postnatal Day
pd	Posterodorsal

PEI	Post-ejaculatory Interval
PIN	Prostatic Interstitial Neoplasias
POA	Preoptic Area
PPARγ	Peroxisome Proliferator Associated Receptor gamma
PPT	propyl pyrazole triol
PR	Progesterone Receptor
PSD	Post-synaptic Density
RAM	Radial Arm Maze
RDLN	Retrodorsolateral Nucleus
RfD	Reference Dose
RM	Repeated Measures
ROI	Region of Interest
RP3V	Rostral Periventricular Area of the 3rd Ventricle
SD	Sprague-Dawley
SE	Southeast
SN	Substantia Nigra
SNB	Spinal Nucleus of the Bulbocavernosus
SRC-1	Steroid Receptor Cofactor 1
SW	Southwest
T	Testosterone
TH	Tyrosine Hydroxylase
ToC	Table of Contents
TP	Testosterone Propionate
TR	Thyroid Receptor
TRβ	Thyroid Receptor beta
U.S.	United States

U.S.A.	United States of America
vHPC	Ventral Hippocampus
µg/kg	microgram per kilogram bodyweight per day

Chapter 1: Introduction

Bisphenol A (BPA) is a ubiquitous compound whose use began in the 1940s in the production of hard plastics such as polycarbonates. Since then, BPA has been a component in a host of other commercial and industrial products, including dental sealants, compact discs, medical tubing, polyvinyl chloride, thermal papers (e.g. those used in receipts) and in the lining of food and beverage tins. Originally synthesised in 1891 by Aleksandr Dianin, this compound was determined to possess weak estrogenic activity in 1936 by Dodds and Lawson and is now so common that over 90% of Americans and Canadians have detectable levels of urinary BPA (Calafat et al., 2008; Bushnik et al., 2010). In 1976, the United States (U.S.) government passed the Toxic Substances Control Act, and given the lack of any notable concern, grandfathered BPA in as an allowable compound, though little scientific study had been conducted on the physiological effects of BPA at that point. In 1982, the National Toxicology Program (NTP) of the U.S. Environmental Protection Agency (EPA) conducted a carcinogenesis assay and this study was used to determine the oral reference dose (RfD), the maximum allowable daily dose that should produce no physiological harm over a lifetime of exposure. On the basis of this study, the EPA set the RfD as 50 micrograms per kilogram of bodyweight per day (μg/kg) based on the perceived lack of harm in this study when using the 50

milligrams/kg (mg/kg) dose – referred to as the No Observed Effect Level (NOEL); and while the EPA noted that the carcinogenicity of BPA had not yet been properly assessed, the authors of the NTP study did conclude that BPA had no carcinogenic potential. However, aside from the finding that chronically dosing mice with 50 mg/kg BPA can lead to reduced adult bodyweight, this study found an increased (but non-significant) risk of leukemia (from <4% in controls to 18%) in mice receiving this dose, a significant increase in multinucleated giant hepatocytes in mice and a significant (>37%) increase in interstitial testicular tumours amongst rats receiving 50 mg/kg. It should be further noted that the laboratory that was hired to conduct this study, Litton Biotechnics, was later found to have a number of problems that may have altered the results of the study (Vogel, 2009). In 1993, however, one laboratory (Krishnan et al., 1993) identified BPA as a potential estrogenic contaminant leaching from polycarbonate water bottles, and noted that at low doses (25nM) it was able to induce the proliferation of MCF-7 cells, derived from human breast cancer. In 1997, a study by Nagel et al. found that exposure to small doses of BPA, 2 or 20 μg/kg, in the uterine environment, could increase the weights of the adult prostate gland of male mice, an effect also thought to be reducible to the known estrogenic activity of this compound. Given that BPA was able to exert dramatic physiological effects at a dose much lower than that previously determined to be safe for chronic human exposure, the “Low Dose Hypothesis” was formed. This hypothesis suggested that endocrine disrupting compounds may potentially exert harmful physiological effects at lower doses than those found at higher doses.

This issue has been somewhat contentious: the traditional maxim in toxicology has been that the dose makes the poison, a notion originally formulated in the 16th century by Phillippus Aureolus Theophrastus Bombastus von Hohenheim, better known as Paracelsus. Regardless, a burgeoning literature has since suggested that early life exposure to low doses of BPA can alter the typical hormone-sensitive development of the behaviour and neural physiology of the organism, as well as development of the reproductive tracts. Furthermore, there is now concern that BPA may be both a carcinogen and an obesogen, or an obesity-causing agent. In fact, the potential for such perturbations has led the Canadian government to list BPA as a toxic substance, a designation thus far not given this chemical in any other jurisdiction. As such, this review will examine the evidence surrounding the claims regarding the potential toxicity of BPA. I will discuss the known mechanisms of action of BPA and then examine the effects of this compound on development of hormone-sensitive physiological and behavioural parameters, as well as other potential harms produced by this substance. Finally, I will outline the research undertaken here. Specifically, using high and low (over and below the government-determined safe dose for daily exposure) doses of BPA administered to pups both pre- and postnatally through the mothers, I will examine the effect of BPA on learning, emotional and reproductive behaviours. I will further attempt to clarify the mechanisms of action of BPA *in vivo*, particularly surrounding the claims that BPA may act centrally via androgen receptors.

Bisphenol A Mechanisms of Action

As previously noted, BPA had originally been seen to possess weak estrogenic activity by Dodds and Lawson in 1936, and further work has since replicated this finding and determined that BPA is capable of exerting agonistic effects on both classical receptors, the α and β receptors (ERα & ERβ; Kuiper et al., 1998) and activates both classical receptors equally, despite its preference for ERβ binding (Matthews et al., 2001). In the presence of estrogen, doses of 1μM BPA (but not lower) can inhibit 17β-estradiol (E_2) at ERα, an effect not seen at ERβ *in vitro* (Hiroi, et al., 1999). Furthermore, it has been found that BPA can also cause the activation of membrane-bound estrogen receptors, including membrane bound α & β isoforms (Belcher et al., 2012) and the G-protein coupled receptor GPR30 (Thomas & Dong, 2006).

BPA has also been found to exert differing effects on the spectrum of steroid receptors. A number of *in vitro* studies using human androgen receptor (AR) transfected into various non-neural cell types have suggested that BPA is an antagonist at the AR (Sohoni & Sumpter, 1998; Sun et al., 2006), and is as effective at blocking the activity of this receptor as flutamide (Xu et al., 2005), though *in vivo* evidence for this mechanism of action is still lacking; no study has yet shown that BPA can alter androgen-sensitive behavioural or neurological parameters, and any indirect evidence for is explainable through the potential activity of other BPA-responsive targets.

BPA may also prevent activation of the thyroid receptor (TR; Moriyama et al., 2002; Sun et al., 2009), most likely the β-isoform (TRβ; Seiwa et al., 2004), perhaps partially due to enhancing the activity of the nuclear receptor co-repressor (Moriyama et al, 2002). Furthermore, BPA can stimulate glucocorticoid receptor (GR)-dependent gene expression (Sargis et al., 2010) and is an agonist at this receptor *in silico* (Prasanth et al., 2010) and the aryl hydrocarbon receptor (AhR; Bonefeld-Jørgenson et al., 2007), the receptor recognized by DDT and other known exogenous teratogens. BPA has also recently been found to bind the constitutively active orphan estrogen-related receptor gamma (ERRγ); however, while BPA neither enhances nor inhibits ERRγ-dependent gene expression, it does inhibit activity induced by the inverse agonist 4-hydroxytamoxifen (Takayanagi et al., 2006), which is also an antagonist at the classical ER. Finally, BPA induces accumulation of the peroxisome proliferator-activated receptor-gamma (PPAR-γ; Klintkiewicz et al., 2010) but is not a ligand for this receptor (Riu et al., 2011).

BPA effects on the reproductive tract.

The first study to show a perturbation of the reproductive tract from low dose bisphenol A exposure came from Nagel et al. (1997) in which the adult mouse prostate gland was found to be enlarged following prenatal exposure to either 2 or 20 μg/kg and since then a number of studies have examined the effects of this compound on the development of the Wolffian system. The

development of this system is sensitive to androgen receptors prenatally, as the administration of flutamide, the AR antagonist, from E15.5 to E17.5 in rats prevents normal prostate development, leading instead to the presence of a vaginal pouch in adulthood while reducing anogenital distance (AGD) and disrupting typical development of the external genitalia, epididymes and vas deferens (Welsh et al, 2007). Some studies utilizing rodent models have noted that prenatal exposure to BPA alters the male-typical development of Wolffian structures, including an enlargement of the adult prostate or other chemical changes in that organ or others, including the testes and epididymis (vom Saal et al., 1998; Welshons et al., 1999; Gupta, 2000; Ramos et al., 2001; Timms et al., 2005; Ogura et al., 2007; Arase et al., 2011; Nanjappa et al., 2012; Salian et al., 2009a; Salian et al., 2009b; Salian et al., 2009c; Wistuba et al;, 2003) as well as a shrinking of the testes (Kawai et al., 2003). However, not all studies have replicated such findings (Howdeshell et al., 2008; Tyl et al., 2008; Ramos et al., 2001). In humans, estimates of parental BPA exposure during pregnancy were inversely correlated with the AGD of children, measured years after birth (Miao et al., 2011), though the retrospective nature of this work poses issues, such as memory lapse or social desirability, which could be resolved with further study. A decrease in spermatogenesis in rodent models has also been noted (Salian et al., 2009a & b), potentially due to alterations of the blood-testis barrier (BTB; Cheng et al., 2011), while reduced sperm motility has been observed in rodents (Salian et al., 2009a; Thomas & Doughty, 2004; Aikawa et al., 2004) and the

presence of this problem has been inversely correlated with urine BPA concentration in human males as well (Li et al., 2011).

In both sexes, BPA administration can also alter the hormonal milieu, though this is dependent on the hormone and the developmental period in which BPA is introduced. With regard to males, one study found that BPA exposure increased testosterone (T) levels on P15, but not in adulthood (Ramos et al., 2003). Similarly, at P30 but not in adulthood, prolactin levels – which are associated with normal circulating levels of gonadal hormones in males and females – were also elevated, though no effect was seen at any point on luteinizing hormone (LH). Kawai et al. (2003) did not see any increase in serum T levels in young adult mice exposed to low doses (2 or 20 µg/kg) *in utero* and studies by Akingbemi and colleagues (2004) agreed with this, as they found that the perinatal administration of 2.4 µg/kg did not alter T or LH serum levels in adulthood, however T production in the testes was decreased. Transient (2 week) exposure to this dose in the pre-pubertal period decreased serum T & LH as well as reduced LHβ receptor mRNA and increased ERβ mRNA in the pituitary. Furthermore, chronic exposure from P21-P90 did result in decreased T production in the testes – but not serum T levels – while serum LH was decreased with this exposure. However, another study showed that adult male BPA exposure for 8 weeks, but not 4 weeks, at a low dose (50µg/kg) decreases T, but not corticosterone or LH (Takao et al., 1999), though this group did not expose their animals for a significant portion of the pre-pubertal period, as done

by Akingbemi and colleagues. A higher dose (1mg/kg) administered s.c. in adulthood also decreased T but increased LH titres (Tohei et al., 2001), though the differences here may be due to the dose or the different route of administration as the previous studies used the more environmentally relevant oral administration. A similar study using a higher dose (2 mg/kg) administered s.c. also noted a decrease in serum T levels and an increased LH secretion following injection of LH releasing hormone (LHRH; Herath et al., 2004). In humans, however, BPA concentrations in adulthood are negatively associated with levels of estradiol, thyroid stimulating hormone, and bioavailable androgens (Meeker et al., 2010). Overall, then, the animal research suggests that low dose BPA exposure perinatally does not seem to significantly alter the levels of bioavailable gonadal hormones in serum; levels of BPA in adulthood, though, do appear to be associated with altered hormonal levels in both rodents and humans.

Perinatal exposure to low doses of BPA can lead to decreased LH in the serum of adult females (Rubin et al., 2001; Fernandez et al., 2009) but increased LHRH mRNA (Monje et al., 2010) as well as earlier onset of puberty as measured by vaginal opening (Adewale et al., 2009; Fernandez et al., 2009) or time to first estrus (Howdeshell et al., 1999), while higher doses (50mg/kg), but not lower doses (50 μg/kg), can reduce estrous cyclicity following vaginal opening (Adewale et al., 2009). Perinatal BPA does not appear to alter serum

estradiol levels in either adulthood (Varayoud et al., 2011; Durando et al., 2011) or in the prepubescent period (Monje et al., 2007).

Structural changes in the female reproductive tract have also been noted, as postnatal (P1-P5) s.c. injections of BPA can lead to uterine polyps, ovarian cysts and even perturb development so much as to leave remnants of the Wolffian ducts in adult mice (Newbold et al., 2007). Rats exposed prenatally have seen decreases in progesterone receptor (PR) mRNA and protein in adulthood following 50μg/kg or 20 mg/kg postnatal BPA exposure, and decreases in ERalpha mRNA (in both doses) and protein in the 20 mg/kg, but not 50μg/kg, dose (Varayoud, 2011) in the uterus. This same study also found a trend towards more resorption sites in the uterine horns in pregnant females exposed to either dose, as well as fewer implantation sites in the 20mg/kg dose.

Overall, these data provide compelling evidence that perinatal exposure to BPA is able to perturb both the male- and female-typical developmental pattern, including steroidogenesis as well as the physiology of reproductive tissues. Given that many of these effects are noted at low doses, defined here as doses at or below the FDA-determined reference dose of 50μg/kg, the widespread exposure to this compound should be cause for concern amongst our nations' regulatory bodies. And given these data, it has also been suggested that the structural changes engendered by BPA may also provide risk for cancerous growths in these tissues in adulthood.

Cancer

As cancers in endocrine tissues are sensitive to the actions of various hormones, research has examined what relationship endocrine disruptors, including BPA, may have with the prevalence or treatment of these cancers particularly given the observation that diethylstilbestrol (DES) has been shown to increase the incidence of cancers in reproductive tissues, such as the mammary glands (Palmer et al., 2006). It has been difficult, however, to assess what role endocrine disruptors may play in causing or in the progression of reproductive cancers. As such, a great deal of work has been done using both human cancer cell lines, human tissue explants and animal models.

Breast Cancer

As a known estrogenic compound, a number of investigators have asked whether BPA may cause cancers or increase the proliferation of cancerous cells from breast tissue. Breast cancer primarily strikes women. It was initially more common in women in their 30s and 40s and decreasing around the time of menopause (Pike et al., 1983), though between 2005-2009, the median age of diagnosis was 61 (SEER, 2012). This progression suggests that estrogen exposure is involved in the incidence of breast cancer, and in fact, there was a decrease in breast cancer cases in 2003 when a large number of women stopped taking birth control following the widely-publicized finding that hormone replacement therapy may serve as a risk factor in developing this disease (Ravdin et al., 2007).

One of the first reports of the low dose effects of BPA came in 1993, when Krishnan and colleagues noted that BPA was able to induce the expression of progesterone receptors in an estrogen receptor-dependent manner and increase the proliferation of MCF-7 cells, a human breast cancer cell line. Prenatal administration of BPA to mice at low doses can alter the development of the mammary gland – including the epithelium, stroma and the fat pad – with perturbations seen as early as E18 (Vandenberg et al., 2007) These changes, lasted into adulthood (P30) and are of particular concern for tumorogenesis (Munoz-de-Toro et al., 2005). In rat, exposure to 2.5 µg/kg BPA prenatally led to ductal hyperplasias in P50 and P95 adults, effects noted on P50, but not P95, in higher doses (25, 250 & 1000 µg/kg; Murray et al., 2007); this increase in hyperplasic ducts was also noted by Durando and colleagues (2007) at P110 and P180 in rats exposed to 25 µg/kg BPA prenatally. Furthermore, they noted that when injected with a sub-carcinogenic dose of NMU, a known carcinogen, 2 of the 15 rats developed malignant tumours of the mammary glands, an effect not seen in controls exposed only to NMU. Similarly, in mice, prenatal exposure to BPA increased the risk of mammary cancers in adulthood in animals exposed to DMBA, another compound known to cause malignancies in mammary tissue (Weber-Lozada & Keri, 2011). This same study found that MCF-7 cells were also responsive to BPA, but not placebo, when grafted into ovariectomized (OVX) adult mice, though tumours were larger in mice treated with E_2. The effects of BPA appear to be estrogen-dependent, as tamoxifen treatment was able to reduce the volume of tumours when given after 7-weeks of tumour growth.

One further troubling possible mechanism of harm comes from the finding that BPA may decrease treatment responsiveness. In human cell lines, nanomolar concentrations of BPA were able to reduce the effectiveness of chemotherapeutic compounds in both ERα-positive and –negative cells, an effect not blocked by antagonists of ERα or ERβ (LaPensee et al., 2009). As these cell lines express both GPR30 and ERRγ, known targets of BPA, these receptors may confer this resistance.

Prostate Cancer

As noted above, one of the first studies on the low dose effects of bisphenol A determined that prenatal exposure in mice leads to an enlarged prostate (Nagel et al., 1997), suggesting that exposure to low doses of endocrine disrupting compounds early in life can alter the adult form of reproductive tissues. Also, in juvenile rats exposed to 25 or 250μg/kg prenatally, the prostate showed altered development of the prostatic stroma, decreased AR expression and decreased secretion of prostatic acid phosphatase (Ramos et al., 2001). These changes may be estrogenic in nature, as the there was no noted change to AGD, a marker of androgen function.

Wetherill and colleagues (2002) first noted that BPA was able to alter the fate of prostate-cancer cell lines *in vitro*. The LNCaP cell is a well-studied line derived from a metastasized prostate cancer from a human patient (Horoszewicz et al., 1983). Furthermore, these cells contain a mutant AR (T877A), found in approximately 12.5% of human cancers, which can use some anti-androgens or

even other endogenous steroids as ligands (Suzuki et al, 1993). Specifically, very low (1nM) concentrations of BPA were able to cause proliferation of LNCaP cells and induce AR-T877A-dependent gene expression, as was the potent androgen dihydrotestosterone (DHT; Wetherill, 2002). Higher doses, however, may in fact prevent the proliferative activity of AR activation (Wetherill et al., 2005). It was also found in this study that BPA may exert its effects on AR in a non-competitive manner, suggesting another binding site. This group also studied tumour growth in LNCaP cells grafted in to adult male mice (Wetherill et al., 2006). They found that castrated mice given BPA had larger tumour growth than controls and showed greater secretion of prostate specific antigen, a marker of prostate cancer, suggesting that BPA may increase the rate at which this form of malignancy is able to overcome androgen-ablation therapy.

Work by Gail Prins and her colleagues (Ho et al., 2006; Prins et al., 2011) has further shown that prostate lesions can develop *in vivo* subsequent to perinatal BPA exposure. Prostatic intraepithelial neoplasias (PIN) were shown to arise from post-natal exposure to a low dose (10μg/kg) of BPA when rats were give both T & estradiol benzoate (EB) implants in adulthood. Furthermore, this manipulation also led to hypomethylation and decreased expression of the gene encoding a phosphodiesterase, an event which preceded the incidence of PIN formation in these animals. Further analyses of the transcriptional effects of BPA have since been done, showing that LNCaP cells *in vitro* have altered expression of 88 different gene products, including a decrease in the expression of ERβ

(Hess-Wilson et al., 2007), which is known to have anti-proliferative effects in prostate (McPherson et al., 2007). This, and other perturbations in the development of BPA exposed tissues, may be accomplished by alterations to the methylation status of various genes, as Prins' lab also noted that post-natal BPA exposure can also alter methylation status and lead to the overexpression of proteins (like Dnmt3a & b) that are involved in methylation (Tang et al., 2012). Given the timing of exposure, however, it would be interesting to see if both pre- and postnatal exposure poses a greater tumourogenic risk.

Other Cancers and reproductive problems

Bisphenol A has also been linked to other cancers and health issues associated with reproductive tissues. For example, Newbold et al., (2007) found that postnatal exposure to BPA (100, but not 10 or 1000 µg/kg) led to an increase in cystic ovaries and hyperplasias of the cystic endometrium, while testicular seminoma proliferation of JKT-1 cells *in vitro* is induced by BPA acting on a membrane bound estrogen receptor (Bouskine et al., 2009), likely GPR30, which is overexpressed in these cells (Chevalier et al., 2012). BPA can also induce the proliferation of the OVCAR-3 (Ptak et al., 2011) and BG-1 ovarian cancer cells *in vitro* through a classical estrogen receptor pathway (Park et al., 2009).

Overall, then, the data support the possibility that BPA exposure early in life may increase the risk of cancer through alterations to the typical development of reproductive tissue, potentially by altering the sensitivity of these tissues to estrogens and androgens in adulthood, likely through alterations to the

methylation of DNA. Furthermore, continued exposure to BPA in adulthood may further enhance this risk by increasing the proliferation of tumours, as well as potentially reduce the effectiveness of treatment for these malignancies.

OBESITY

A number of environmental compounds have been discovered which have been termed obesogens, as exposure, particularly early in development, is thought to predispose individuals to obesity or other metabolic problems later in life. A number of different classes of compounds have been included in this category, including prenatal nicotine exposure, phthalates, pesticides, organotins and BPA (Thayer et al., 2012). In humans, serum BPA concentrations have been positively associated with insulin resistance, abdominal obesity and obesity in Chinese adults 40-years or older (Wang et al., 2012), Americans sampled in the National Health and Nutrition Examination Survey (NHANES) between 2003-2006 (Carwile & Michels, 2011) and Italian adults (Galloway et al., 2010). Furthermore, BPA levels have also been positively associated with diabetes and cardiovascular diseases (Melzer et al., 2010; Lang et al., 2008), and coronary artery disease, even amongst men and women who are otherwise healthy (Melzer et al., 2012). This may be due in part to decreased release of adiponectin from human tissue (Hugo et al., 2008), as this protein is negatively correlated with obesity and metabolic disorders (Nishida et al., 2007).

These findings are mirrored by results from rodent studies. For example, altered body weight has been noted in a number of experiments (Patisaul & Bateman, 2008; Rubin et al., 2001; Somm et al., 2009). It is likely that these effects are estrogenic in nature, given that both ERalpha, ERbeta and aromatase are found in human (Pedersen et al., 2001; Price & O'Brien, 1993) and animal adipose tissues (Heine et al., 2000; Foryst-Ludwig et al., 2008; Yamada & Harada, 1990), that mice lacking the aromatase gene have increased intra-abdominal adipose tissue and insulin levels (Jones et al., 2000) that postnatal exposure to DES, another estrogenic compound, is known to increase fat in adulthood in females (Newbold et al., 2005) and estrogen depletion, either through ovariectomy (Eckel, 2011) or menopause (Rachon & Teede, 2010), also produces the accumulation of fat.

The specific mechanisms by which BPA may exert these effects suggests that there may be altered expression of several genes involved in lipogenesis (Somm et al., 2009) as well as changes to pancreatic function (Alonso-Magdalena et al., 2005, 2006, 2010). However, not all of these effects are mediated by the classical estrogen receptors. Alonso-Magdalena et al. (2006) found that in adult tissue, non-genomic actions mediated through a membrane bound receptor were involved in altered pancreatic activity, while Sakurai et al. (2003) found that increases in expression of glucose transporter 4 and insulin-stimulated glucose uptake were not prevented by ICI, the estrogen receptor blocker. It may be that some of these adipose specific effects are due to ERRγ,

as this protein is expressed in human adipocytes at much higher levels than membrane-bound estrogen receptor GPR30 (Hugo et al., 2008). It has also been noted that BPA may increase adipogenesis via a glucocorticoid receptor mediated effect as well (Sargis et al., 2010). Finally, there has been some disagreement as to the interactive effects of diet, as 50ug/kg BPA only altered metabolic parameters in animals given a high-fat diet (Wei et al., 2011) while the lipogenic effects of BPA were found in both standard and high fat diets according to Somm et al. (2009). Interestingly, Dolinoy et al. (2007) found that maternal diet high in nutrients that serve as methyl donors –folic acid and genistein, the soy-derived phytoestrogen – could reverse some of the BPA-dependent changes to methylation status of the epigenome, though this prenatal administration used a much higher dose (10 mg/kg) than would be seen environmentally. As such, replication of this study with lower doses would be beneficial.

Together, these studies suggest that we should be concerned that continued exposure to BPA, even at environmentally relevant doses (Ben-Jonathan et al., 2009), may be acting to increase the collective weight of our nations, a serious issue, given that obesity costs Canada at least $4.3 billion in direct and indirect costs per year (Public Health Agency of Canada, 2009) and may cost the U.S. anywhere between $85.7 and $210 billion annually in health care costs alone (Cawley & Meyerhoefer, 2012).

Brain & Behaviour

Differentiation

Aside from their role in the development of reproductive tissues in the periphery, estrogens and androgens also play a role in the differentiation of the brain along male- and female-typical pathways in rodents. There are a large number of structures in the brain that exhibit such sexual dimorphisms, be those differences structural – such as the number of neurons in a nucleus or the number of synapses formed on a neuron – or chemical, such the expression of specific receptors. The male-typical developmental pathway is mediated by androgens (Phoenix et al., 1959), and in many regions, T is thought to masculinize the brain via its conversion to estradiol (Naftolin et al., 1975). Testosterone, derived from the testes, is able to cross the blood-brain barrier and access neural tissue, and where neurons express the cytochrome P450 enzyme aromatase (Roselli et al., 1985), T can be converted to E_2, which is then able to bind any of its receptors present in that cell. A large amount of work has accumulated to suggest that this aromatization is in fact required for the masculinization of the brain, though certainly not every region and behaviour requires only estrogen receptor activation; in certain regions, the activity of androgens is also required, for example, in the hippocampus (HPC; Jones & Watson, 2005). Females are also exposed to estrogens *in utero* but are not masculinized due to the presence of α-fetoprotein (AFP), a circulating estrogen binding protein that prevents estrogens in serum from being able to access the

brain (Vannier & Reynaud, 1975). AFP binds very weakly to BPA, however (Milligan et al., 1998; Teeguarden & Barton, 2004); as such, BPA has the ability to access the brain and potentially act at any of its receptors to alter development and to potentially prevent the full masculinization of specific neural regions and the behaviours those regions subsume. As a weak estrogen, BPA in significant concentrations may be sufficient to produce enough estrogenic activity to at least partially masculinize the female brain, however, as a partial agonist, it may prevent estrogens from properly activating their receptors in the brain of males, thus preventing the full masculinization of various neural systems.

In humans, though, androgen receptors play a strong role in the typical masculinization of the brain, as XY individuals with complete androgen insensitivity syndrome (CAIS) have female-typical sexual identity and sexual orientation (Bao & Swaab, 2011) and have female-typical cognitive abilities (Imperato-McGinley et al., 1991). Rats containing the testicular feminization mutation, homologues to human CAIS, still exhibit male-typical reproductive behaviours (Hamson et al., 2009) and are partially masculinized on spatial abilities (Jones & Watson, 2005). As a potential anti-androgen, BPA may then have the putative effect on the differentiation of the human brain.

Puberty

One area that has attracted a fair bit of attention has been in the neural systems that control reproductive behaviours and the onset of sexual maturity. For puberty to occur, a number of events must occur, including the accumulation

of a certain amount of body fat. Leptin, produced by white adipose tissue, is involved in puberty onset, presumably due to it's role in the activation of the kisspeptin (KISS) system in the hypothalamus (Elias, 2012), and in both rodents and humans, this system is involved in the onset of puberty of males and females (Clarkson et al., 2010) and the regulation of hormone titres as adults (Dedes et al., 2012). Humans with mutations to the gene encoding GPR54, the KISS receptor, show delayed puberty and decreased gonad function secondary to decreased gonadotropin secretion, while mice with a homozygous GPR54 knockout (GPR54 -/-) show a similar hormonal profile and gonadal cryptorchidism (Roseweir & Miller, 2008), and this protein is expressed by gonadotropin releasing hormone (GnRH) immunoreactive (-ir) neurons (Kallo et al., 2012). Specifically, KISS-ir neurons in the arcuate nucleus (ARC) are thought to mediate the hypothalamic-pituitary gonadal (HPG) system's negative feedback loop (Kauffman et al., 2007a) and this population of KISS-ir neurons is not sexually dimorphic (Kauffman et al., 200b). However, there is a much larger population of KISS-ir neurons within the female anteroventral periventricular (AVPV; Kauffman et al., 2007b) and the preoptic periventricular nuclei – together termed the rostral periventricular area of the third ventricle (RP3V) – and this structure in females controls the LH surge required for ovulation. The ability to produce this surge is prevented by aromatizable androgen exposure perinatally and more recent evidence has shown that the development of the KISS system is sensitive to estrogens, such that even a single injection of EB in low doses (10μg) on P1 can decrease the amount of KISS mRNA in both male and female

pre-pubescent rats of whole hypothalamus (Navarro et al., 2009) while neonatal testosterone proprionate (TP) decreases the number of KISS-positive (KISS+) neurons and the quantity of mRNA within those neurons in adult females (Kauffman, et al., 2007) and prepubertal castration (P20) can decrease the amount of KISS+ neurons in the RP3V of males, an effect reversed by either TP or EB treatment (Clarkson et al., 2012). This study also showed that the majority of these KISS-ir neurons expressed ERα and AR. In females, the ERβ-selective agonist diarylpropionitrile (DPN) can reduce adult KISS-ir in the AVPV, but neither DPN nor PPT – the ERα-selective agonist propyl pyrazole triol – mimicked the decreases in KISS-ir in the ARC (Patisaul et al., 2012), suggesting a non-classical pathway, perhaps mediated by GPR30.

BPA has been found to alter the pattern of expression of ERα & β mRNA in these regions. Cao et al (2012) found that there were changes to the expression of mRNA of both ERα & β, such that BPA was able to increase on P4 but reduce on P10 the ERα transcript in the AVPV and eliminate ERβ signalling in that region on P10 in females. In males, though, there were developmental and dose differences, as a high dose increased ERα mRNA on P4 only, while the low dose decreased this signal but only on P10. Neonatal estrogen exposure can also advance the day of vaginal opening in females (Bateman & Patisaul, 2008) an effect mimicked by BPA, yet, like PPT, dramatically reduce normal ovarian cyclicity and expression of FOS in GnRH-ir cells in adulthood. However, as

GnRH-ir cells do not express ERα early in development, this PPT-reducible effect must be mediated by ERα-containing afferent neurons, likely KISSergic cells.

Changes to ERα and KISS expression in the AVPV of females may explain the advanced puberty noted in BPA exposed females. Navarro et al. (2009) noted that high doses (100 or 500 µg/rat) of BPA given to males and females on P1-P5 were also able to decrease KISS mRNA. Patisaul and colleagues (2009) however, using 50 or 50000µg/kg BPA from P1-P5 found absolutely no effect on the KISS-ir in the AVPV or ARC of peripubertal males from BPA or EB (25µg), but they did find that the high dose did decrease staining in both structures in peripubertal females (though p=0.066 in the AVPV), a pattern roughly mimicked by administration of PPT. Using the same dosing regime, though with only 3 days of administration, further work from the Patisaul lab (Cao et al., 2012) noted that the high dose of BPA was able to increase KISS mRNA in the male ARC on P4, an effect that had disappeared by P10. Another study (Bai et al., 2011) giving 2µg/kg BPA both pre- and postnatally to the mother found that BPA exposure did increase KISS-ir neurons in the male AVPV on P30, P50 and P90, while the number of GnRH-ir neurons in the preoptic area (POA) was initially decreased relative to controls but then increased on P50 & P90. They also found that these males exhibited a P90 LH surge at the same time of day as occurred in wild-type female conspecifics, however, basal levels of LH and T at P90 were not significantly different, despite a LH decrease in P30 & P50 and an increase in T at P30 and P50. P90 males exposed to perinatal BPA did

have higher basal levels of E_2, however. These effects in males may help to explain the decrease in testicular production of T noted by Akingbemi and colleagues (2004) despite the agreement with Bai et al. (2011) that T levels in adulthood are not altered by postnatal BPA exposure. It may also explain the changes noted in LH levels in adult females (Rubin et al., 2001).

Reproductive Behaviour

Given that these structures and hormones play a role in sexual behaviour, a few studies have examined if BPA may alter sexual proficiency in rodent species. Kubo et al. (2003) found that 30 µg/kg administered pre- and postnatally decreased the intromissions of male rats on the day of sexual naïveté, but neither that dose nor 300 µg/kg had an inhibitory effect on reproduction in females while Farabollini et al. (2002) saw that male rats exposed to 40µg/kg postnatally, but not prenatally, exhibited both increased intromission latency and anogenital investigations (AGI). When collapsing both BPA groups in females, however, there was an increase in the frequency of lordosis. Pubertal (P23-P30) BPA exposure decreased intromission latency, though (Della-Seta et al., 2006).

Also found in the AVPV is a sexually dimorphic pool of tyrosine-hydroxylase (TH) containing neurons such that there is greater TH-ir in the female brain than the male. A high dose of BPA (~100mg/kg) administered twice daily from P1-P3 led to decreased TH-ir neurons in males, but not females (Patisaul et al., 2006), while pre- and postnatal administration to the dams of much lower doses (25 & 250 ng/kg) found that 250ng/kg led to decreased size of

the AVPV and a decrease in the number of TH-ir neurons in prepubertal female mice, eliminating the sex differences in those parameters (Rubin et al., 2006); no effects were found in the sexually monomorphic population of TH-ir neurons in the ARC, however.

Neurotransmitter Systems

Catecholamine systems outside of the hypothalamus also appear to be perturbed as a result of exposure to BPA. In 2003, Yoneda and colleagues noted that at concentrations of 50 μM and higher, BPA could cause the release of dopamine (DA) from PC-12 (adrenal medullary tumour) cells *in vitro*, and that this was done via a non-genomic pathway involving calcium (Ca^{2+}) release. In agreement, Yanagihara et al (2005) found that BPA at 10nM induced the synthesis of catecholamines from cultured bovine adrenal medullar cells. Intriguingly, the similar actions of E_2 were blocked by ICI administration, while this effect induced by *p*-nonylphenol, another environmental estrogen, was not prevented by ICI, suggesting again a non-genomic action for BPA. Indeed, both of these EDCs were found to increase the activity of TH. In the brain, cultured P1 mouse midbrain slices showed more glial fibrillary acidic protein (GFAP) staining following BPA exposure, an effect not seen with E_2 (Miyatake et al., 2006). Furthermore, low doses of BPA enhanced the DA-induced Ca^{2+} flux in astrocytes in these cultures. A high dose did increase expression of caspase-3, a marker of apoptosis, in these cultures however, an effect again not mimicked by E_2.

Within the brain, the catecholamine system can be perturbed due to BPA exposure as well, leading to a number of behavioural changes. For example, a number of studies have noted that BPA exposure can lead to hyperactivity (Xu et al., 2007). Specifically, one group (Masuo et al., 2004; Ishido et al., 2005) found that 87nM injected P5 increased movement of male rats prepubertally, and this was associated with decreased expression of the DA transporter (DAT) from midbrain of young rats. This effect was also seen in adulthood, but was specific to testing during the light – but not dark – phase (Kiguchi et al., 2008). BPA did not alter the activity-inducing effects of methylphenidate treatment, however.

Other changes to monoamines (MA) have also been noted. For example, low doses of perinatal BPA given to dams produced a decrease in TH-ir neurons in the substantia nigra (SN) of adult female, but not male, mice, but not due to cell death (Tando et al., 2007). Larger doses (5mg/kg) administered perinatally did affect males, however, in weeks 2 & 6 of life, leading to a decrease in TH-ir in the SN (Tanida et al., 2009), though it is difficult to say if the disagreement here is due to differences in dose, strain or developmental time point. In rats, postnatal exposure to low doses of BPA led to time-point dependent changes in MA levels in young males. On P7, 0.1 and 1 μg/kg caused an increase in 5-HT content in the HPC, while 1 μg/kg increased norepinephrine (NE) levels in that region; on P28, though, that effect was only seen for 5-HT with 1.0 μg/kg (Matsuda et al., 2010). DA levels in the striatum were also increased with 1.0 μg/kg on P28, but unfortunately, this region was not assayed on P7, so comparisons with the work

above are not possible. Pre- and postnatal BPA treatment was also found to increase DA levels in the caudate putamen and dorsal raphé nucleus of both male and female mice in adulthood, but not prepubescentally (Nakamura et al., 2010), and not in the POA at either time point; 5-HT levels were increased in both sexes, however, in the thalamus, SN and dorsal raphé. Furthermore, Kubo et al (2001; 2003) found that BPA in high or low doses can reverse the typical adult sex difference in the locus coeruleus (LC) such that exposure to doses as low as 30 μg/kg both pre- and postnatally caused the development of a larger LC in males with more cells in that structure. These lines of evidence are intriguing, and suggest the possibility that the changes found in the synthesis of brain MA could lead to alterations of behaviour beyond just motor issues and that BPA may be able to alter these parameters. Indeed, some of these behaviours have been studied, including various disordered psychological states, such as depression, drug abuse and externalizing issues like aggression (or, in humans, ADHD and oppositional defiance as well).

Drug Abuse

Given its effect on locomotion and DAergic systems, some have examined whether BPA may potentiate the effects of various drugs of abuse. Suzuki et al (2003) found that perinatal exposure to BPA in male mice could actually induce conditioned place preference (CPP) to a dose of methamphetamine (METH) too low to produce that reinforcement on its own. Furthermore, BPA potentiated the motor activity caused by METH and led to an increase in amount of DA1 receptor

(D1) and D1-induced G-protein activity in the limbic forebrain; no effect was seen on DAT expression. In a study of rats given 40 µg/kg perinatally, both sexes showed increased activity in a novel environment as compared to controls, though females, but not males, given BPA showed less time in this environment compared to control females (Adriani et al., 2003). Yet these males showed reduced locomotor activity in response to D-amphetamine (dAMPH), in disagreement with Suzuki and colleagues. This same group (Laviola et al., 2005) giving 10 µg/kg BPA to mice prenatally only, however, found that BPA did not alter dAMPH-induced locomotion in either males or females. Furthermore, CPP to dAMPH in females, but not males, was inhibited by BPA exposure. These differences are difficult to interpret, given different species, strains, doses and timing of exposure and highlight the need for a more standardized, and more environmentally relevant, approach to the study of endocrine disrupting compounds. But overall, BPA may alter, in some way, the behavioural response to monoaminergic compounds, which agrees with the physiological evidence that these chemical systems are altered in the brain.

Response to morphine administration has also been examined. Morphine and other opioids act through the endogenous opioid receptors, but are also known to activate the DAergic system, as is common for most drugs of abuse, though the nature of addiction to stimulants like METH is not the same as that to opiates (see Badiani et al., 2011 for a review). Using the same paradigm as their study with METH above, Mizuo and friends (2004) found that males perinatally

exposed to BPA showed CPP to a dose of morphine too low to induce CPP on its own and could potentiate morphine-induced locomotion. However, these effects were not due to alteration of μ-receptor expression or activity. It is interesting that this finding was replicated in a separate study, where it was further noted that E_2 did not alter CPP (Miyatake et al., 2006). They further discovered that this CPP-inducing effect of BPA exhibited a U-shaped dose response, with lower and higher doses only producing this effect; this non-monotonic response mapped well onto an assay of DA-induced G-protein activity in the limbic forebrain (Narita et al., 2006). It was later determined that these effects on CPP and DA receptor activity are reducible to BPA exposure during discrete time periods. Specifically, these effects were seen when BPA exposure occurred during organogenesis (E7-14) or lactation (P0-20), but not during the implanation (E1-7) or parturition (E14-20) phase (Narita et al., 2007). These data are important as prescription opiate use has risen in recent years, and is now considered to be the fastest growing drug abuse issue in the US (CDC, 2012). Unfortunately, most of the research on AMPH and morphine has examined only the male response; future research should seek to determine if these responses are also seen in females.

Aggression

The intruder test is a common paradigm for the testing of aggression and can measure offensive (attacking), defensive or ambivalent behaviour. Farabollini et al., (2002) noted that while females did not alter their activity in this test, males exposed prenatally to BPA (40μg/kg) showed more defensive posturing and less

ambivalency towards their intruder, but no difference in offensive activity. A study with mouse dams given either 2 or 20µg/kg also found no effect on offensive behaviours in this task in young adult males, nor did postnatal exposure to 50 µg/kg in rats (Patisaul & Bateman, 2008). Externalizing behaviours were not associated with prenatal BPA exposure in young boys either, though there was a significant relationship between mothers' week 16 serum BPA concentration and externalizing behaviour in girls aged ~2 years (Braun et al., 2009), a finding which dovetails nicely with the data regarding the timing of BPA exposure perinatally and response to drug administration and DA function (Narita et al., 2007). Further analysis of human children found again that BPA exposure *in utero* was associated with hyperactivity as well as anxiety and depression in 3-year old girls, but not boys (Braun et al., 2011).

Emotional Behaviour

Emotionality, particularly anxiety and depressive-like behaviour, has also been studied, though the results here are much more contradictory. In the adult mouse, Gioiosa et al (2007) found an elimination of the sex difference in the elevated plus maze (EPM) following 10 µg/kg perinatal exposure, while other mouse studies using comparable doses and timings of exposure found no effect in females (Ryan and Vandenburgh, 2006) or males (Miyagawa et al., 2007) on this task. In rats, a similar discordance has emerged using the EPM, with both Fujimoto et al. (2006) and Negishi et al. (2004) finding no anxiogenic or anxiolytic effects following perinatal exposure to 15 µg/kg or 100 µg/kg, respectively, while

Farabollini et al. (1999) noted an anxiolytic response in males. Fujimoto also found that BPA exposure led to an increase of depressive-like behaviour in male rats in the Porsolt chamber, or forced-swim test (FST). This area of research has otherwise been untouched, despite epidemiological data in humans suggesting that women exposed to DES *in utero* have a greater history of depression later in life (O'Reilly et al., 2010).

Learning

The hippocampus is a large structure in the allocortex and has well documented roles in both learning and stress (Fanselow & Dong, 2010). Its role in learning has been heavily studied in humans and rats (Squire, 1992), and plays a role in the acquisition and retention of information (Morris et al., 1990). One memory task that has been particularly well studied is the Morris water maze (MWM), a test of spatial reference (long-term) memory. Sexual dimorphisms have been noted in this task (Jonasson, 2005) and both estrogen receptors and androgen receptors are known to be involved (Isgor & Sengelaub, 1998). A number of studies have examined the effects of BPA on both learning, including MWM performance, and developmental changes to hippocampal physiology. Carr et al. (2003) found that juvenile rats exposed to 100μg/kg BPA postnatally eliminated sex differences on this task, while a higher dose of 250μg/kg inhibited female acquisition of this task. Xu et al. (2007) noted a decrement in performance at the 50μg/kg dose in adult males, but not females, while Ryan and Vandenburgh, using other paradigms, noted subtle

improvements in adult females dosed perinatally with 2 or 200μg/kg. It is possible that these results may be due to changes in synaptogenesis. MacLusky et al. (2005) found that acute BPA (80-300μg/kg) exposure in adulthood could inhibit the estrogenic increase in HPC spine density in OVX female rats. Likewise, Leranth et al. (2008) noted that acute adult BPA (300μg/kg) exposure decreased spine density in the HPC and mPFC in male rats and prevented the T-induced increase in spines in these regions following castration. Likewise, Eilam-Stock et al. (2012) found that acute BPA (40 μg/kg) could reduce spine density in males but only after prior exposure to a memory task (object recognition, in which BPA increased time spent with the old object); there was also a concomitant decrease in PSD-95 – but not GluR2 - in this group. However, Tanabe et al. (2012) found that acute exposure (2hr) to 10nM BPA in HPC slices taken from adult male rats promoted spinogenesis, and that this was blocked by hydroxy-tamoxifen, but not ICI, suggesting the role of ERRγ in this rapid, non-genomic process. Removal of Ca^{2+} and blocking NMDAR also blocked this effect. Likewise, after noting that a higher (500μg/kg or above) but not a low (50μg/kg) dose of perinatal BPA to male mice inhibited MWM performance in juveniles and young adults, Xu et al., (2010a) noted a decrease in NMDAR subunits 1, 2A & 2B and ERβ. Further work from this lab also found an increase in aromatase expression in the early postnatal period as well (Xu et al., 2010b). However Xu et al. (2010c) found that P1 HPC exposed acutely to 100nM BPA show an increase in filopodia, an effect mimicked by estradiol and blocked by ICI. BPA had no effect on the expression of NMDAR subunits, however, but through an estrogen-dependent mechanism,

did increase phosphorylation of those receptors. Estradiol exerted the same actions as BPA, however co-administration of BPA reduced the effectiveness of estradiol. Thus, acute BPA seems to have an enhancing effect on spine growth, through a rapid ERRγ- & NMDAR-mediated pathway, but prolonged exposure may inhibit learning. However, the effects of this EDC on spatial learning are unclear.

CURRENT STUDY

The review above has shown that exposure to low doses of BPA in the perinatal period can alter a number of physiological and behavioural parameters in adulthood, including problems with fat accumulation, reproductive tracts and behaviour, emotionality and learning as well as the neural substrates that subsume them. However, there has often been disagreement or an incomplete analysis of these behaviours. Some investigators may dose only during the prenatal or postnatal period, some during both; some use one or two doses of BPA; some give the drug directly to the pup, others only administer BPA to the dam; some test only males or females. While some methodological differences are beneficial (say, rats vs. mice or SD vs. LE), some of the aforementioned differences reflect exposure patterns that are not environmentally valid. As such, the research described in this dissertation attempts to use a variety of doses of BPA administered to the mother during the gestational and lactational periods, in an attempt to determine any dose-specific effects that mimic the likely exposure

to this chemical during the development of the human child. The first chapter will look at reproductive behaviours in adult rats, but will extend the literature by examining the effects of BPA on sexually experienced animals as well as those that are sexually naïve. The second chapter will examine emotionality using the EPM and the FST. The third chapter will examine the expression of steroid receptors in areas known to be involved in the expression of these behaviours.

Finally, while *in vitro* work has strongly suggested that BPA is a potent anti-androgen, the research looking at the developmental effects of BPA *in vivo* simply do not support that claim. The final chapter of this dissertation will examine an androgen-sensitive pool of motor neurons in the spinal cord. However, the developmental actions of BPA will be compared to chronic exposure to this compound in adulthood.

Chapter 2. Pre- and postnatal Bisphenol A treatment results in persistent deficits in the sexual behaviour of male rats, but not female rats, in adulthood.[1]

Introduction

Bisphenol A (BPA) is a known endocrine disrupting compound used in the production of hard plastics as well as in the epoxy resin that lines the insides of food and beverage cans. BPA has long been known to have weak estrogenic activity (Dodds & Lawson 1936). However, recent reports have suggested that, paradoxically, some of BPA's most pronounced physiological effects may actually be more evident at lower doses. In 1997, Nagel and colleagues noted an increased prostate gland weight in males whose mothers had been given low (2 or 20 ug/kg bw/day) doses of BPA during the gestational period. Since that time, there has been concern that early developmental exposure to low doses of BPA might also have a negative effect on a number of sex-steroid mediated behaviours, including reproductive function in males (Farabollini et al., 2002). However, Farabollini and colleagues fed only a single dose of BPA to dams

[1] This chapter represents work previously published in Hormones & Behavior, 59, 246-251, 2011.

either prenatally or postnatally, but not both, and only examined the behaviours of sexually naïve animals. Given that (1) the organization of sexually dimorphic behaviours is critically dependent on the perinatal actions of testosterone and its estrogenic metabolites in the developing brain (McEwen et al., 1977), (2) that there are two separate surges of testosterone perinatally, one prior to parturition (Ward & Weisz, 1980) and one immediately following (Corbier et al., 1978), and (3) that human exposure to BPA through the mother will also span both of these periods, we suggest that it is important to administer BPA to experimental animals across both the prenatal and postnatal critical periods in development. It is also known that males improve their sexual performance with experience (Rabedeau & Whalen, 1959; Lopez et al., 1999), thus it is possible that repeated sexual encounters may be able to mitigate negative effects of perinatal BPA exposure on adult sexual behaviour.

The current study is intended to address three questions. First, does chronic BPA treatment during the perinatal period alter sexual behaviour in adulthood? Second, is this effect dose-dependent, and third, does sexual experience mitigate any initial deficits in sexual performance?

Materials & Methods

Fifteen 60-day old Long-Evans (LE) females from Charles River served as dams for the BPA treatment manipulation. These females were maintained in a colony room in the Animal Resource Centre at Simon Fraser University, and had access to standard rat chow (Purina) and water *ad libitum*. All procedures conformed to the guidelines of the Canadian Council on Animal Care, and were reviewed and approved by the University Animal Care Committee prior to the study.

BPA was administered orally, dissolved in corn oil. During a pre-experimental phase, all females were trained to drink corn oil from a syringe, to ensure proper administration of the vehicle and also to reduce stress associated with handling and exposure to a new stimulus. Following this training period, the females were paired with LE stud males in segregated cages, for up to a week, in order for mating to occur. The appearance of vaginal plugs was taken as evidence of successful insemination, at which time the females were separated from the studs and placed in single housing in polysulfone cages with BPA-free water sacks. Each female was randomly assigned to one of 5 BPA (Sigma, Oakville, ON; >99% pure) dose groups: Oil vehicle, 5 µg/kg bw/day, 50 µg/kg bw/day, 500 µg/kg bw/day, or 5 mg/kg bw/day, such that there were 3 females in each group;

this was done to ensure that potential dose effects could not be confounded with individual variation associated with a single dam. Although plugs were found for each female, one female assigned to the 500 µg/kg bw/day group did not become pregnant. Thus, there were only two pregnant females in that group. The day that the plugs were found was considered gestational day 1 (GD1), and BPA dosing began on GD7, continued through the remainder of gestation, and after parturition continued through post-natal day 14 (P14), with the day of birth being considered P0. Litters were culled to 4 males and 4 females each, except in the 500 µg/kg bw/day group, in which litters were culled to 6 males & 6 females each; thus, each dose group had a total of 12 males and 12 females. Because of the small litters by the 2 dams in the 500 µg/kg bw/day group, that dose group had only 7 females. Upon weaning (P21), pups were group housed in polysulfone cages with same-sex littermates, and access to tap water and rat chow *ad libitum*.

The sexual behaviour of the experimental male and female animals was tested after they reached adulthood, at between 90 and 120 days of age. Males were paired with stimulus females that had been gonadectomized and given silastic capsules (1.57 mm inner diameter, 3.18 mm outer diameter; Dow Corning, Midland, MI) containing estradiol benzoate (Steraloids, Newport, RI; 1 X 10mm capsule) and injected with progesterone (P) subcutaneously four hours prior to the onset of testing, in order to induce sexual receptivity. Female experimental animals were paired with stimulus males that had been

gonadectomized and given 2 X 20mm silastic capsules (1.57 mm inner diameter, 3.18 mm outer diameter) containing testosterone propionate (Steraloids). All stimulus animals were previously sexually experienced, ensuring competence across all testing sessions.

Males were given sexual experience on four separate occasions, with performance evaluated on day 1, the day of sexual naivete, and day 4, when they were sexually experienced. Briefly, the experimental males were placed in clear glass aquariums (30.5cm x 25.5cm x 51cm) with receptive stimulus females and allowed to interact. Each session ended following the first mount that occurred following ejaculation; the session was terminated after 30 minutes if the male failed to ejaculate. All testing took place within the first four hours of the dark phase, and the second and third days of reproductive experience – during which data were not collected – occurred on consecutive days following day 1. The final day of testing occurred seven days following the first test. For each male, the number of anogenital investigations (AGI) of the stimulus female was quantified, as were the numbers of mounts and intromissions, and the latencies to mount, intromit, ejaculate and the post-ejaculatory interval (PEI; latency to first mount of the next bout of copulation). An index of copulatory efficiency (CE: # intromissions / # of mounts) was also created. A maximum of 1800 seconds (30 minutes) was recorded for the latency measures.

Prior to the experiment, females were briefly placed with males and observed for the presence of proceptive behaviours as a measure of ovarian

cyclicity. Once the 4- or 5-day cycle was determined, their sexual behaviour was assessed in two testing sessions, separated by four days (i.e. the duration of one estrous cycle). Briefly, on the day of behavioural estrous, the experimental females were placed in clear glass aquariums (30.5cm x 25.5cm x 51cm) with stimulus males and allowed to interact. Proceptive behaviours (ear wiggling and hop-darting behaviour) were quantified until each female had received 10 mounts with pelvic thrusting by the male. The total number of proceptive behaviours as well as the lordosis quotient (the number of lordoses shown per 10 mounts X 100) were tabulated for each female on each occasion. All behaviours were scored by an investigator blind to experimental condition.

Statistical Analysis

For both males and females, only the first and final days of testing were analyzed, as we were interested in any deficits that may be present when the animals were sexually naïve, contrasted with deficits persisting after the accumulation of sexual experience. A one-way ANOVA, with post-hoc Tukey's analyses, was used to test for specific group differences. We further sought to determine, as a post-hoc analysis with the one-way ANOVA, if the obvious non-linear dose responses noted in the male sexual behaviours exhibited a significant quadratic polynomial component.

In order to gain a general perspective on male performance, we also created a sexual behaviour composite score for each animal on both Day 1 (sexually naive condition) and Day 4 (sexually experienced condition). This composite was created by calculating z-scores for each animal on each parameter – z-scores express data in units of standard deviation, thus providing a standard measurement scale across the various measures – which were then averaged, thereby giving each animal a single composite score indicating their average performance relative to the other animals in the study on that test day.

Finally, to ensure that the individual characteristics of dams were not significantly affecting these parameters, we used ANOVA and subsequent Tukey's post-hoc tests within each dose group to determine if litter membership had any significant effects on these behaviours.

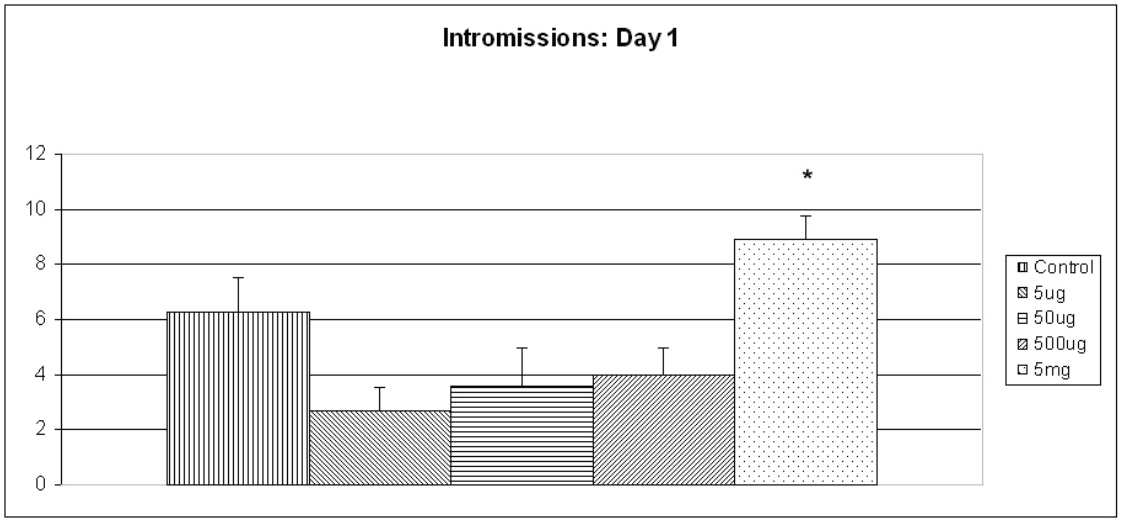
Results

Overall, our results indicated that males receiving low doses of Bisphenol A had impaired sexual performance as compared to controls, particularly for animals that were sexually experienced. Furthermore the dose-response relationship between perinatal BPA and adult male rat sexual behaviour was significantly non-monotonic for many of the parameters of male sexual behaviour. The adult sexual behaviour of females, in contrast, did not appear to be affected at any exposure level.

Male Sexual Behaviour

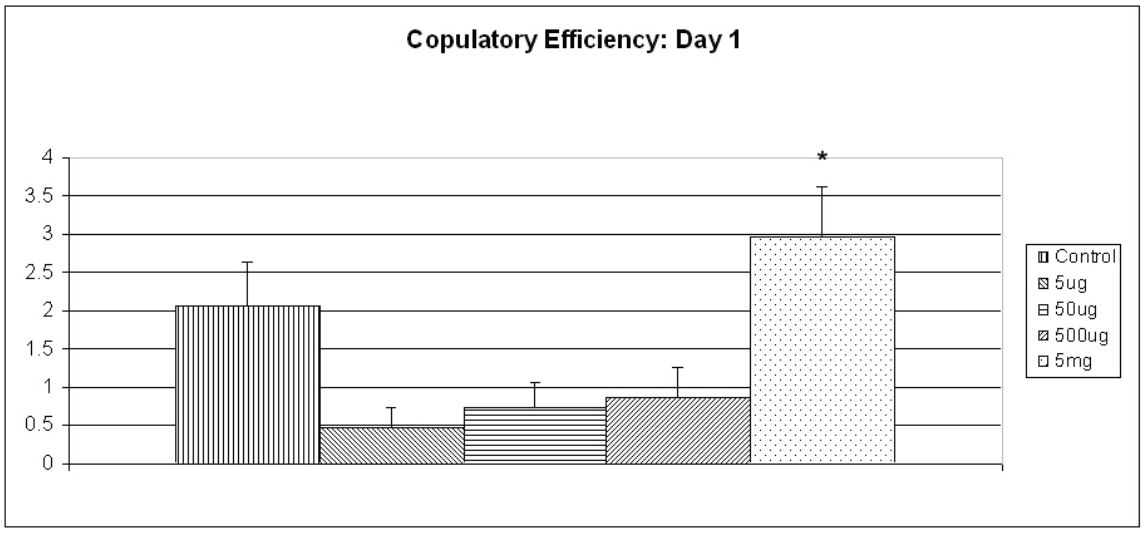
On the first day of testing, overall ANOVA revealed significant main effects for the number of intromissions ($F_{4,55}$= 5.28, p = 0.001; Figure 1), copulatory efficiency ($F_{4,55}$ = 4.87, p = 0.002; Figure 2), and PEI ($F_{4,55}$ = 4.79, p = 0.002; Figure 3). Mount latency (ML; $F_{4,55}$ = 3.49, p = 0.013), intromission latency (IL; $F_{4,55}$ = 4.47, p= 0.003) and ejaculation latency (EL; $F_{4,55}$ = 6.05, p < 0.001) were also significant.

Figure 1. Intromission Day 1



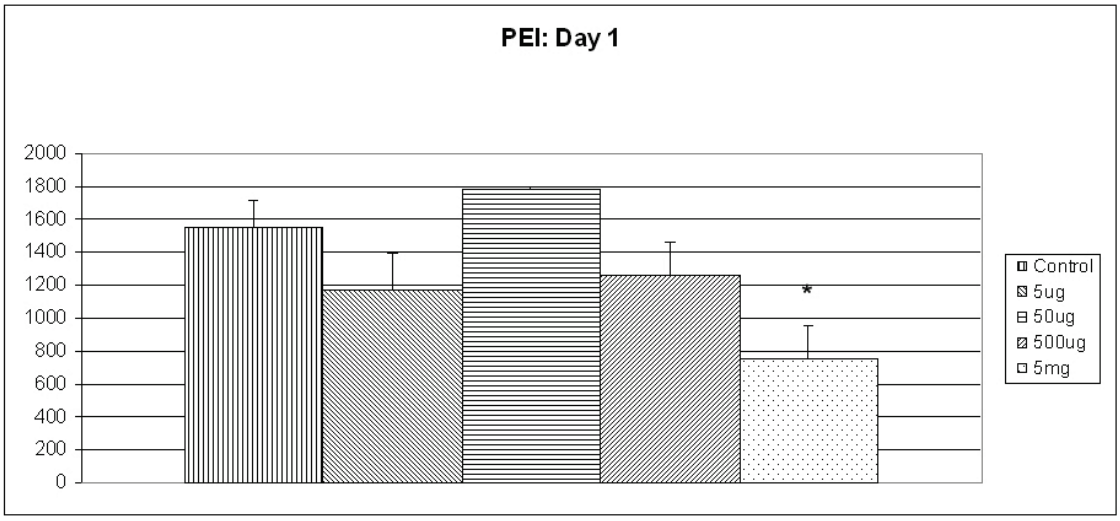
* $p < 0.05$ vs. other BPA groups

Figure 2. Copulatory Efficiency Day 1



* $p < 0.05$ vs other BPA groups

Figure 3. Post-Ejaculatory Interval Day 1

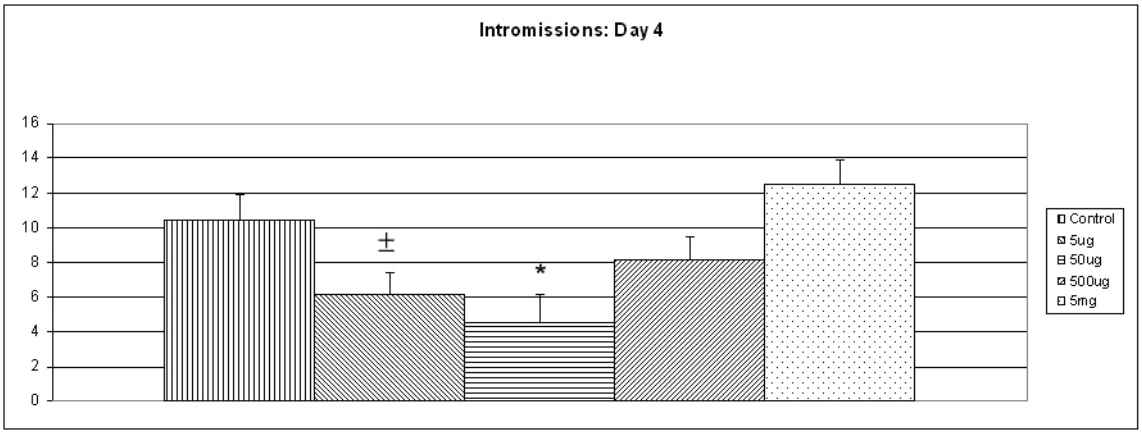


* $p < 0.05$ vs 50µg group & controls

Overall, our results suggest that sexual behaviour exhibits a non-linear dose response relationship with BPA, and that the highest dose group shows no impairment (in fact, they showed increased appetitive behaviour compared to all other groups). Post hoc testing revealed that the 5mg/kg group had a greater number of intromissions than the three other BPA groups ($p = 0.002$ vs 5 µg/kg, $p = 0.009$ vs. 50 µg/kg & $p = 0.02$ vs 500 µg/kg), though controls did not differ from any of the BPA groups. For PEI, the 5 mg/kg group was significantly different than both the controls and the 50 µg/kg groups ($p = 0.02$ & 0.001, respectively). Like the number of intromissions, the CE showed an inverted U-shaped dose response curve, with the 5 mg/kg group performing significantly better than the other 3 BPA-treatment groups ($p = 0.005$ vs. 5 µg/kg $p = 0.015$ vs. 50 µg/kg & $p = 0.024$ vs 500 µg/kg); controls were not significantly different from any of the BPA groups. Finally, mount, intromission and ejaculation latency all exhibited similar patterns with one another. In all three cases, the 5 mg/kg group showed significantly faster latencies than the 50 µg/kg, the 5 µg/kg and the control groups, but not the 500 ug/kg group (data not shown).

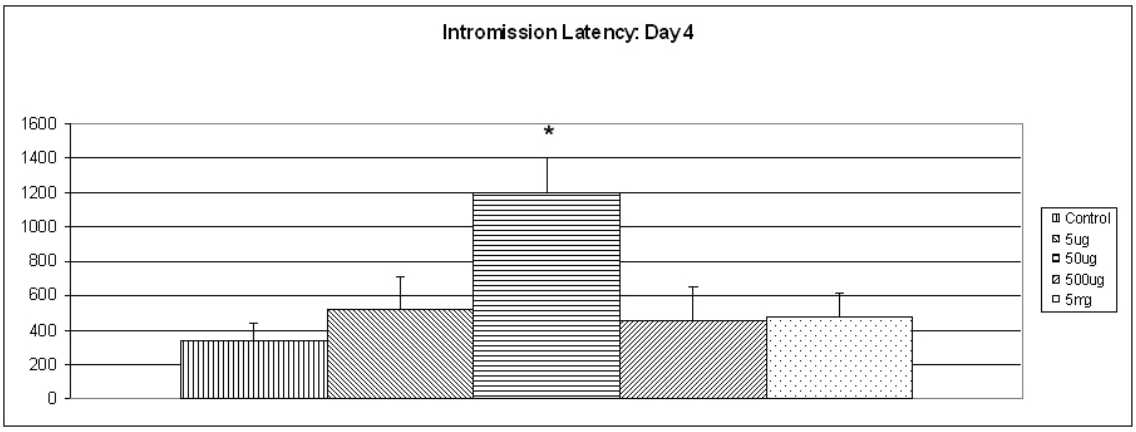
On day 4, ANOVA was significant for the number of intromissions ($F_{4,55} = 5.36$, $p = 0.001$; Figure 4), intromission latency ($F_{4,55} = 4.02$, $p = 0.006$; Figure 5), ejaculation latency ($F_{4,55} = 3.93$, $p = 0.007$; Figure 6), PEI ($F_{4,55} = 3.02$, $p = 0.025$; Figure 7) and CE ($F_{4,55} = 4.93$, $p = 0.002$; Figure 8).

Figure 4. Intromissions Day 4



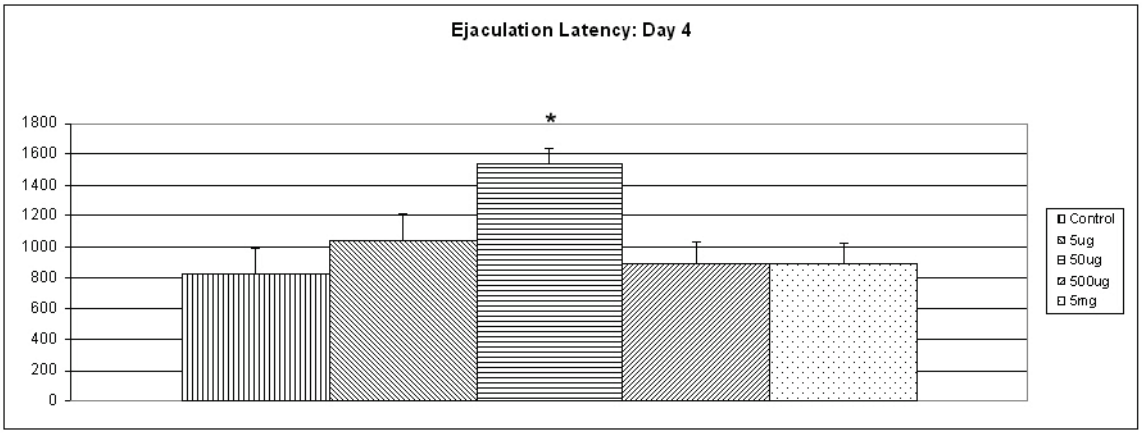
*
p < 0.05 vs controls and 5mg group ± p < 0.05 vs. 5 mg group

Figure 5. Intromission Latency Day 4



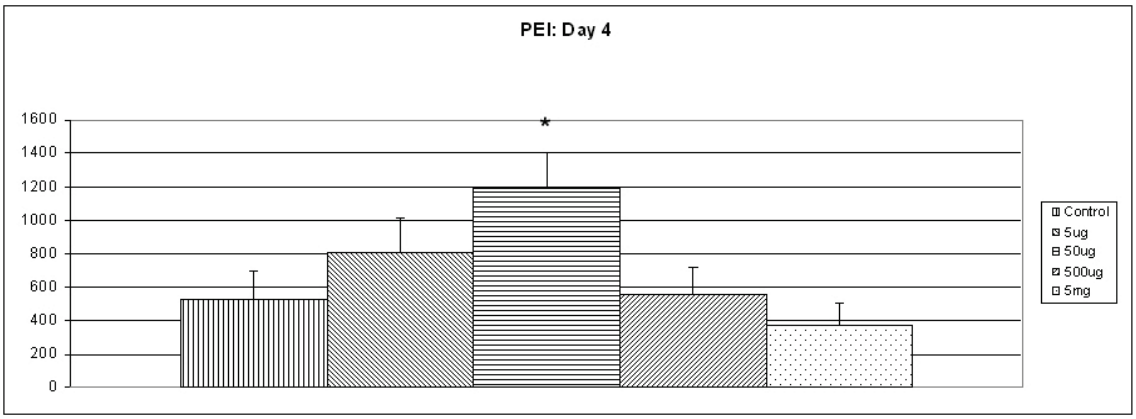
*
p < 0.05 vs controls, 500µg group & 5mg group

Figure 6. Ejaculation Latency Day 4



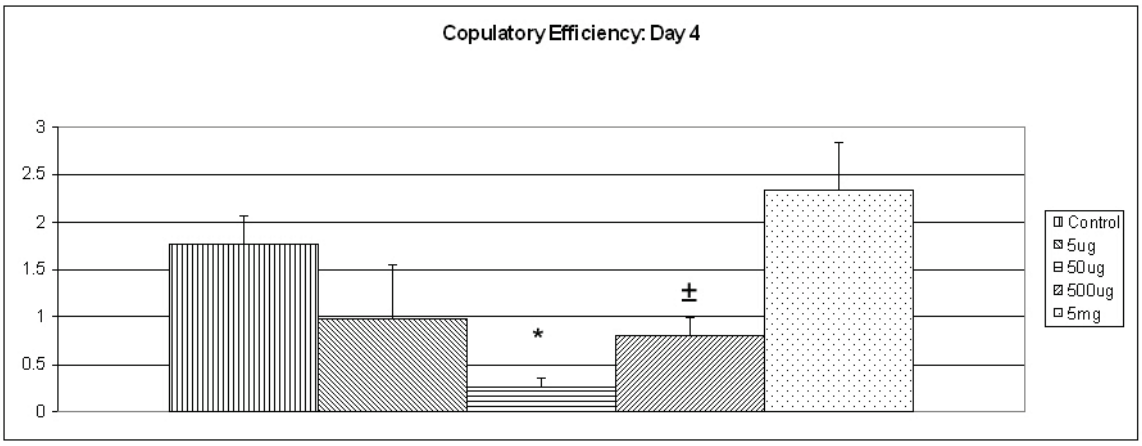
*
p < 0.05 vs controls, 500µg group & 5mg group

Figure 7. Post-Ejaculatory Interval Day 4



*
p < 0.05 vs. 5 mg group

Figure 8. Copulatory Efficiency Day 4



* $p < 0.05$ vs controls and 5mg group ± $p < 0.05$ vs. 5 mg group

Post-hoc analysis revealed that both the control and the 5 mg/kg groups had a higher number of intromissions than the 50 µg/kg group ($p = 0.029$ & 0.001, respectively), and that the 5 mg/kg group also performed better than the 5 µg/kg group ($p = 0.016$). With respect to intromission latency, the Tukey's post-hoc tests showed that the 50 µg/kg group performed worse than controls ($p = 0.007$), the 500 µg/kg group ($p = 0.028$), the 5 mg/kg group ($p = 0.031$), but not the 5 µg/kg group ($p > 0.05$). The post-hocs for ejaculation latency again revealed that the 50 µg/kg group had an increased latency compared to all groups ($p = 0.01$ vs. controls, $p = 0.023$ vs. 500 µg/kg & $p = 0.022$ vs. 5 mg/kg) except for the 5 µg/kg group ($p > 0.1$). With respect to PEI, the post hoc revealed only that the 5mg/kg group was quicker to resume sexual activity than the 50 µg/kg group ($p = 0.021$). Finally, the post-hoc analysis showed that the copulatory efficiency of both the controls and the 5mg/kg group was better than that of the 50 µg/kg group ($p = 0.042$ & $p = 0.002$, respectively), while the 5mg/kg group was also better than the 500 µg/kg group ($p = 0.038$).

The ANOVA revealed that there were significant, non-monotonic dose responses on both days. On day 1, AGI ($F = 7.99$, $p = 0.007$), number of intromissions ($F = 16.231$, $p < 0.001$), mount latency ($F = 4.181$, $p = 0.046$), intromission latency (8.047, $p = 0.006$), ejaculation latency ($F = 3.352$, $p = 0.024$), post-ejaculatory interval ($F = 4.257$, $p = 0.044$), and copulatory efficiency ($F = 16.318$, $p < 0.001$) all exhibited significant quadratic components. The pattern of results on Day 4 was similar, with mounts ($F = 6.541$, $p = 0.013$), intromissions (F

= 19.12, $p < 0.001$), intromission latency ($F = 7.586$, $p = 0.008$), ejaculation latency ($F = 8.18$, $p = 0.006$), post-ejaculatory interval ($F = 7.958$, $p = 0.007$), and copulatory efficiency ($F = 18.248$, $p < 0.001$) all showing significant quadratic components.

The composite scores for both day 1 and day 4 were significant on overall ANOVA (Day 1, $F = 5.375$, $p = 0.001$; Day 4, $F = 3.128$, $p = 0.022$). The one-way ANOVA showed that there were significant quadratic trends on both day 1 ($F = 8.803$, $p = 0.004$) and day 4 ($F = 9.228$, $p = 0.004$) while the Tukey's post-hoc analysis revealed that the 5 mg/kg group performed better than all other groups on day 1, while the 50 µg/kg group performed worse than controls, as well as the 5 mg/kg group ($p = 0.047$ and 0.019, respectively) on day 4.

Finally, we examined the litters within each BPA dose group to determine if there were any maternal effects on the specific parameters included in this study. For the 500 µg/kg group, there were litter differences in anogenital investigations on day 1 ($F = 5.795$, $p = .037$) and on the number of mounts made on Day 4 ($F = 8.147$, $p = 0.017$). However, neither of these parameters was altered by BPA on either day of analysis. No other litter effects were seen in any of the other BPA groups.

Female Sexual Behaviour

The ANOVA did not reveal any effect of BPA on either proceptive behaviours or receptivity on the first ($F = 0.589$, $p = 0.672$; $F = 0.117$, $p = 0.976$, respectively) or second ($F = 0.726$, $p = 0.579$; $F = 0.117$, $p = 0.976$, respectively) day of testing.

Discussion

The data presented above show that perinatal administration of low levels of BPA (50 µg/kg bw/day) impairs adult sexual performance in experienced male LE rats. Those animals receiving high doses (5 mg/kg bw/day) performed as well as controls on consummatory behaviours (eg. intromissions, copulatory efficiency) across all days of testing; however, their improved appetitive behaviour (mount, intromission and ejaculation latency and PEI) on the day of sexual naïveté disappeared on day 4 of testing, due to improvements amongst the controls. The sexual performance of female rats was not altered by perinatal exposure to BPA at any dose level. Furthermore, in males, BPA produces a significant non-monotonic dose response curve, which persists from sexual naïveté through multiple sexual experiences.

Male Reproductive Behaviour

With respect to sexually naïve animals, a significant non-monotonic dose response was apparent across a number of the parameters investigated, indicating that BPA is able to produce greater deficits at lower doses. Overall, the general pattern of results on the initial day of testing did not reveal any significant

differences between the controls and the BPA groups, as might be expected due to the large variances associated with sexual inexperience. However, the 5 mg/kg group outperformed the other BPA dose groups with respect to intromissions and copulatory efficiency, and was quicker to mount, intromit, ejaculate and resume mounting following ejaculation than all other groups except the 500 μg/kg group. The pattern of results on this day is quite intriguing: while the 5 mg/kg group outperformed only the other BPA, but not control, groups on most measures of sexual behaviour, on the so-called appetitive measures (ML, IL, EL & PEI) – behaviours that may be mediated by different regions of the medial preoptic area than consummatory parameters (Balthazart et al., 1998) – the 5 mg/kg group outperformed even the controls, but not the next highest dose group. The specific mechanisms by which a high dose of BPA could affect only these parameters is unclear, though it may be mediated by estrogen receptor alpha (ERα), given the role that ERα plays in the development of sexual behaviour (discussed below). It is also possible that the dopamine (DA) system may be one of the downstream pathways involved in the alteration of appetitive behaviours. Tando and colleagues (2007) have found that mice given BPA orally during both the pre-and post-natal period show an increase in DA2 receptor (D2) binding and a concomitant decrease in DA transporter binding, indicative of potentiated DAergic activity. Previous work by Pfaus and Phillips (1991) has shown that D2 receptor antagonists can inhibit appetitive sexual behaviours, including mount and intromission latency, while consummatory behaviours were not readily affected by these drugs. Furthermore, BPA has been found to

increase tyrosine hydroxylase-immunoreactive neurons in the anteroventral periventricular hypothalamus (Patisaul et al., 2006). Together, this is suggestive of a potential mechanism for BPA to alter these specific aspects of sexual performance in males.

The differences in performance were more dramatic when the animals were sexually experienced, however. Males whose mothers received 50 μg/kg bw/day showed fewer intromissions in adulthood, took longer to intromit and ejaculate, required more time to resume mounting following ejaculation, and demonstrated a lower copulatory efficiency than males in other groups. The differences on appetitive measures between the control and 5 mg/kg groups were eliminated with experience.

The significant difference noted with the composite sexual behaviour scores further indicates that the 50 μg/kg dose group is generally impaired with respect to their sexual performance as a group. The observation of significant non-linear trends is evidence, at least for the doses we studied, that the dose-response relationship of BPA and these parameters is non-monotonic, suggesting that BPA is likely to exert its maximal physiological effects at lower doses. This finding is not surprising; previous research on steroid-sensitive behaviours (Roof, 1993) has found similar patterns, while BPA is known to exert greater effects at lower (environmentally relevant) doses than at the higher doses typically examined in standard toxicological tests (see Welshons et al., 2003). And, as these trends are apparent on variables that are indicative of both appetitive and

consummatory aspects of copulation (e.g. PEI vs. # of intromissions), it would appear that the activity of low doses of BPA on sexual performance spans both aspects of this complex behaviour.

BPA has been found to exert its effects on a number of different systems. It can weakly bind both α and β isoforms of the classical estrogen receptor (ER; Kuiper et al., 1997), has been found *in vitro* to be a potent blocker of the androgen receptor (AR; Sohoni and Sumpter, 1998), may block the activity of the thyroid hormone receptors (Moriyama et al., 2002), and has been found to have epigenetic activity, such as altering the methylation of various genes (Dolinoy et al., 2007). However, given that male sexual behaviour is critically dependent upon the activity of ERα (see Rissman et al., 1997), it is tempting to speculate that this must be at least one of the sites of action leading to the reduced sexual performance of males receiving low doses of BPA in early development. The fact that lower doses had a greater effect in demasculinizing sexual behaviour, is consistent with a partial agonist action of BPA (Gould et al., 1998); at low doses, BPA might be preventing normal binding of the testosterone metabolite 17β-estradiol to the ERα, while being insufficient in its own activity to compensate for that loss of function. However, BPA has been found to decrease steroid receptor-cofactor (SRC-1) expression and increase repressor of estrogen receptor expression in female rats (Monje, 2009), suggesting other indirect mechanisms related to ERα activity that may account for the demasculinizing effect noted here. While previous work has found that reducing SRC-1 via antisense

oligonucleotides can feminize sexual behaviour in males, no effect was found with respect to the number of intromissions or other indices of normal masculine sexual behaviour (Auger et al., 2000).

Female Sexual Behaviour

The current findings indicate that perinatal BPA administration has little effect on adult female sexual behaviour, either on the initial day of sexual experience or upon subsequent encounters. Reports to date disagree somewhat on this issue; for example, Farabollini et al (2002) found that BPA increased the expression of lordosis and sexual motivation, as determined by exit latencies. However, they used only one dose of BPA (40 μg/kg) administered orally to dams (Sprague-Dawley) during either gestation or following parturition, but not both, so methodological differences may account for discrepancies between that paper and the present report. Regardless, both lines of evidence suggest that oral administration of BPA to dams does not have a negative effect on the sexual behaviour of females in adulthood. That conclusion conflicts with results of a study by Monje and colleagues (2009), who noted a decrease in the proceptive behaviour of females exposed to BPA postnatally. In that study, Wistar pups were directly injected (s.c.) with BPA, so methodological differences may again account for differing results, though Adewale et al. (2011) using postnatal s.c. injections from P0 to P4 found that BPA did not alter adult proceptivity. Ryan et al (2010) have noted, using a design similar to the current study, that lordosis is

unaffected by pre- and postnatal BPA exposure through the mother. The literature thus far generally indicates that female sexual behaviour is relatively insensitive to perturbation by Bisphenol A exposure during the perinatal period, regardless of dose range. It should be noted, however, that there are differences in the pattern of female sexual behaviours between those receiving BPA and either estradiol benzoate (EB) or ethinylestradiol (EE2). For example, Della Seta and colleagues (2008) found that environmentally revelant doses of EE2 administered during gestation and postnatally through to puberty reduced both proceptive and receptive behaviours in adulthood, while Henley et al. (2009) found a decrease in adult proceptive and receptive behaviours and an increase in partner preference for estrous females over males when postnatally exposed to EB. Together, these lines of evidence suggest that there are differing effects of ER activation in females based on compound, which may be explained by the differences in the degree of ER activation by these compounds.

One limitation in our study is that we only examined the lordosis quotient as a measure of receptivity, and did not examine any male-typical behaviours (e.g. partner preference). Previous studies have used exits and latencies to return to males as measures, though lordosis quotient – as measured here – was inhibited by EB in the Henley study.

Conclusion

The present data support the conclusion that adult male sexual behaviour is impaired by pre- and postnatal administration of Bisphenol A and further indicate that this impairment persists despite multiple sexual encounters. On day 1, an obvious non-monotonic trend is suggestive of impairment at low doses, though there were no differences between these groups and the controls. However, performance amongst the low dose BPA groups is clearly inhibited on day 4, when the animals are sexually experienced. To our knowledge, this is the first report indicating that sexual performance is impaired in sexually experienced animals following neonatal exposure to Bisphenol A. Female sexual behaviour was not impaired following BPA administration.

Evidence increasingly shows that environmentally relevant doses of Bisphenol A can have a detrimental effect on various reproductive parameters. Male sexual performance (Farabollini et al, 2002; current report), hormone levels (Akingbemi et al., 2004; Salian et al., 2009a), sperm counts (Salian et al., 2009a & b; Herath et al, 2004), and sperm motility (Salian et al., 2009a; Aikawa et al., 2004) have all been found to decrease following low dose BPA exposure, with only a few exceptions (Ema et al, 2001; Nagao et al., 2002). Furthermore, Salian and colleagues (2009a) have noted decreased concentrations of androgen and

estrogen receptors in testes of BPA-exposed males, along with reductions in litter sizes, effects transmitted (presumably epigenetically) through several generations.

With annual production in the USA alone exceeding 2.3 billion pounds annually (NTP-CERHR, 2008), BPA has become a ubiquitous environmental contaminant that is detectable in the blood of more than 90% of Americans (Calafat et al., 2008). Recently, Health Canada has taken the precautionary step of requiring baby-bottles to be free of BPA. However, this study adds to the growing evidence that exposure through the mother also can have detrimental effects on the adult behaviour of affected individuals. While the literature has suggested that BPA-treated males are still capable of fertilising receptive conspecifics, their sexual performance is inhibited as is their reproductive capacity and that of their offspring; at the population level, it is possible that mass exposure to BPA developmentally may have negative effects on sexual health and fecundity in a variety of species.

Chapter 3: Perinatal BPA exposure demasculinises males in measures of affect but has no effect on water maze learning in adulthood.[2]

Introduction

Bisphenol A (BPA) is a ubiquitous endocrine disrupting compound, originally found to have weak estrogenic activity (Dodds & Lawson, 1936). BPA is now known to act as an agonist at both classical estrogen receptors (Kuiper et al., 1997) and the membrane-bound estrogen receptor GPR30 (Thomas and Dong, 2006), and as an antagonist at the androgen receptor (AR; Sohoni and Sumpter, 1998) and the thyroid receptor (TR; Moriyama et al., 2002). A monomer used in the production of hard plastics, BPA is found in a wide variety of consumer goods, including many types of food and beverage containers. Given widespread human and animal exposure to BPA -- it is detectable in more than 90% of Americans (Calafat et al., 2008) and Canadians (Bushnik et al., 2010) – population-level effects, particularly on reproduction, may be cause for concern. Indeed, we have previously reported that BPA impairs sexual performance

[2] The chapter represents work previously published in Hormones & Behavior, 61, 605-610, 2012.

(Jones et al., 2011), and others have noted decreased sperm counts and motility (Salian et al., 2009a & b; Herath et al, 2004) and decreased fertility, possibly descending through multiple generations via epigenetic mechanisms (Salian et al., 2010).

Because BPA acts via sex steroid-mediated signaling pathways to affect reproductive functions, it is plausible that BPA may similarly alter other sexually-dimorphic systems, especially sex differences in cognitive and affective domains. The limited number of studies on this topic have thus far yielded conflicting results.

Sex differences in anxiety behaviour are reliably observed in rats and mice, with males typically showing greater anxiety on various measures, including the elevated plus maze (EPM). This test, which exploits a natural aversion to open spaces, consists of an elevated platform with a small central hub from which four arms emanate; two of these arms are enclosed by surrounding walls, while the other two are open. Lower levels of anxiety are inferred from increased exploration of the open arms, and conversely, increased time hiding in the closed arms reflects heightened anxiety (Rodgers and Dalvi, 1997). In mice, Ryan & Vandenbergh (2006) and Miyagawa and colleagues (2007) found no significant effects in adulthood on any EPM measure following pre- and postnatal administration of BPA. However, Gioiosa et al. (2007) reported that perinatal BPA treatment resulted in increased central hub time in adult male mice, and abolished normal sex differences in closed arm time (CAT)

and the total number of open arm entries (OAE). Research examining rats likewise has produced equivocal results. In two prior studies, perinatal BPA administration had no effect on adult EPM behaviour in males (Fujimoto et al, 2006; Negishi et al, 2004), while one study reported that in males, perinatal BPA administration increased the percentage of both open arm entries (%OAE) and open arm time (%OAT) in adulthood (Farabollini et al., 1999). However, an additional study administering BPA postnatally reported a *decrease* in both OAE & OAT with a corresponding increase in CAT. Only two studies have included females (Fujimoto, et al., 2006; Farabollini, et al., 1999): while both studies noted a decrease in overall activity, Fujimoto and associates (2006) saw this at a low dose (15ug/kg bw/day) while Farabollini et al., (1999) observed this effect at a high dose (400ug/kg) but not a low dose (40 ug/kg). Overall, the pattern of results from the few studies to date is difficult to assess, particularly given the diversity of species, strains and methods employed.

Very little is known about changes in other measures of affect. Some work (Pare & Redei, 1993) has found females to exert more depressive-like behaviours in the forced-swim test (FST) – a measure of learned helplessness believed to reflect depression-like behaviour in rats FST – but this has not always been the case. Some studies have found no difference, or a difference such that males show greater learned-helplessness (Fujimoto et al., 2006). One study (Fujimoto, et al., 2006) reported on the effects of early BPA on adult behaviour in the FST. Perinatal BPA administration reportedly eliminated the noted sex

difference in struggling behaviours observed in controls, due to a feminization of male behaviours. Furthermore, BPA increased total immobility in females, but not males.

Learning and memory may also be sensitive to perinatal BPA exposure. A number of different tasks measuring spatial reference memory, including the Morris water maze (MWM), show a male-typical advantage (Jonasson, 2005). Xu and colleagues used the MWM to examine both rats (2007) and mice (2010) while Miyagawa et al. (2007) studied mice using the step-through passive avoidance paradigm; in each case an impairment was noted in adult males exposed to BPA both pre- and postnatally. Carr et al. (2003) did not see this effect, though that study examined juvenile rats, not adults. Female learning and memory has also been affected. Carr et al. (2003) observed that BPA-exposed juvenile females spent less time in the target quadrant of the MWM during a post-acquisition probe trial, while Ryan and Vandenbergh (2006) noted an improvement in ovariectomized adult female mice in the radial arm maze (RAM), a measure of working memory.

While anxiety and depression are known to be estrogen-sensitive behavioural parameters (ter Horst, 2010), spatial learning is dependent on androgen receptor (AR) function for normal sexually dimorphic organization (Isgor & Sengelaub, 1998; Jones & Watson, 2005). BPA reportedly antagonizes AR *in vitro* (Sohoni & Sumpter, 1998), but the National Toxicology Program report on BPA (2008) has stated that the weight of evidence does not support the

claim that BPA is an AR-antagonist *in vivo*. However, the impairment of performance noted above, as well as the inhibition of AR-dependent synaptogenesis in the prefrontal cortex (Leranth et al., 2008) would seem to contradict this conclusion. In order to resolve some of the ambiguity regarding effects of BPA on sexual differentiation of affective and cognitive behaviours, we treated developing male and female rats with one of five different doses of BPA – whereas most studies to date have examined only one or two – throughout the perinatal organizational critical period. We then evaluated their behaviour, in adulthood, in models of depression (forced swim test), anxiety (EPM), and spatial cognition (MWM).

This methodology was designed to ensure that animals were exposed to BPA during the entire critical period of development for those structures involved in the behaviours examined here. Furthermore, there has been some controversy surrounding the specific level of human exposure. Some models have suggested intake lower than 1-5 μg/kg bw/day (Vandenberg et al., 2007; Lakind & Naiman, 2011) with recent research suggesting a dosage as high as 400 μg/kg bw/day (Taylor et al., 2010); the United States Food and Drug Administration, however, has suggested that 50 μg/kg bw/day is a safe dosage for humans. Thus, our dosing regimen should not only determine any non-linear effects produced by BPA on these behaviours, but also indicate the level of disruption, if any, to these behaviours at whichever dose is ultimately closest to that of typical human exposure levels in North America.

Methods

Experimental Design

Fifteen 60-day old Long-Evans (LE) females from Charles River Laboratories (Quebec) served as dams for the BPA treatment manipulation. These females were maintained in a colony room in the Animal Resource Center at Simon Fraser University, and had access to standard rat chow (Purina) and water *ad libitum*. All procedures conformed to the guidelines of the Canadian Council on Animal Care, and were subject to prior approval by the University Animal Care Committee.

The females were trained to drink corn oil from a syringe, to ensure proper administration of the vehicle and also to reduce stress associated with handling and exposure to a new stimulus. They were then paired with LE stud males and, when vaginal plugs were found in the cages (denoting copulation), they were moved to single housing in polysulfone cages with BPA-free water sacks. The pregnant females were randomly assigned to one of 5 BPA dose groups: oil vehicle, 5 µg/kg bw/day, 50 µg/kg bw/day, 500 µg/kg bw/day, or 5000 µg/kg bw/day, such that there were 3 gestating females in each group. Although copulatory plugs were found for each female, one female assigned to the 500

µg/kg bw/day group did not become pregnant. As such, there were only two females in that group. The day that the plugs were found was considered gestational day 1 (GD1), and BPA dosing began on GD7. Dosing continued through post-natal day 14 (P14), with the day of birth being considered P0. Resultant litters were culled to 4 males and 4 females each, except in the 500 µg/kg bw/day group, in which litters were culled to 6 males & 6 females each; thus, each dose group had a total of 12 males and 12 females. Upon weaning (P21), pups were group housed in polysulfone cages with same-sex littermates, and access to tap water and standard Purina rat chow *ad libitum*. Behavioural tests were conducted between the ages of 90 & 150 days, as described next.

Morris Water Maze

All animals were handled for a total of five minutes per day, for the seven days preceding water maze testing. Animals were removed from their home cages, and placed on an elevated platform (Perrot-Sinal et. al., 1996), similar to the one on which they would be placed during testing, to reduce anxiety during the testing procedure. On the last day of handling, tails were marked (fabric marker) for individual identification.

A 150 cm diameter pool was centered within a rectangular room measuring 470 cm X 400 cm, with a video camera (Sony DCR-SR85) centered overhead; behaviour in the pool was tracked and quantified from the video feed

using AnyMaze software (Stoelting, Wood Dale IL) running on a Windows PC. The pool was filled with water at 23± 1˚C, made opaque with non-toxic acrylic white paint. For analysis purposes the pool was divided in the software into 4 equal imaginary quadrants, arbitrarily identified as northwest (NW), northeast (NE), southwest (SW) and southeast (SE). An escape platform, 2.5 cm below the water surface and thus hidden from view, was randomly placed in the middle of the SE quadrant, and left in that location for the entirety of the testing period. For other analyses (described below), the maze was divided into 3 imaginary concentric rings: an inner ring, a middle ring – which contained the platform – and an outer ring along the wall.

Each animal was tested four times per day for five days. The animals were released into the pool from each of 4 starting locations every day in a pattern that was randomly determined prior to testing. For every trial, the animal was placed in the pool facing the wall. Animals were then allowed 60 seconds to find the platform. If they were unable to find the platform in that time, they were guided to it by hand. They were allowed to remain upon the platform for 15 seconds, and were then removed. A minimum of 5 minutes elapsed between trials, during which time the animal was placed on an elevated platform in the testing room. A heat lamp was affixed above the platform. All testing started at noon, and the order in which the animals were tested was randomly changed, to prevent any time of day effects. For each trial, data was collected for total time spent in each quadrant or ring, distance covered, and swim speed..

Forced Swim Test

All animals were tested for depressive-like symptoms using the Porsolt Forced Swim Test. Using a version of the test modified for use with rats (Cryan et al., 2005), animals were placed in a clear plastic cylindrical tank (20cm diameter, 45cm high; Stoelting) filled with 25±1˚C water to a depth of 30cm. Each rat remained in the tank for 15 minutes and was then returned to its home cage and kept under a heat lamp temporarily to ensure a quick return to baseline temperature. The rat was returned to the tank 24 hours later for 5 minutes. All trials were recorded with a Sony digital camcorder for later analysis, at which time the following measures were quantified: number of rotations, time until immobility, total time immobile, and the number of mobile and immobile episodes.

Elevated Plus Maze

The elevated plus maze apparatus consists of a cross of 4 arms (50 X 10cm), of which two are open platforms and two are enclosed by 40cm - high walls, plus an open central hub, all elevated 70cm above the floor. Each animal was placed in the central hub of the maze and allowed to freely explore for a total of 5 minutes. The trials were recorded by a digital videocamera (Sony DCR-SR85) connected to a Windows PC running AnyMaze analytic software (Stoelting). The software was used to compute the following measures: time in the central hub, time in

closed arms and open arms, number of entries (all 4 paws) into the arms and hub. The quantity of fecal boli deposited, an additional indicator of anxiety, was also noted.

Statistical Analysis

All statistical procedures were carried out using SPSS v.17. Data from the MWM was analyzed using repeated measures analysis of variance (RM-ANOVA), with post-hoc Tukey's HSD tests to examine specific group differences. For the EPM, effects of BPA dose within sexes were assessed using 2 X 5 ANOVA, with follow-up post-hoc Tukey HSD tests used to examine specific group differences. Also, t-tests were used to perform *a priori* planned comparisons of sex differences. Lastly, for the FST, following established procedures (Cryan, et al., 2005) Day 2 data were evaluated using RM-ANOVA, with follow-up Tukey tests of group differences.

Results

Morris Water Maze

The RM-ANOVA did not reveal any effects of dose within any of the male or female groups on either latency to find the platform, distance travelled in the pool or the speed of movement, nor were there any sex differences for latency or distance. However, there was a significant difference on speed, with females swimming faster than males ($F_{1,22} = 6.31$, $p = 0.02$). While this sex difference was also apparent at the highest dose, it disappeared with the 5, 50 and 500 µg/kg groups. Furthermore, amongst the males, the 5 µg/kg group was significantly faster than the controls. Amongst the females, the 5000 µg/kg group was faster than the controls and all other dose groups except the 5 µg/kg group. No differences were found with respect to time spent in the outer ring or in any of the quadrants of the maze.

Elevated Plus Maze

Overall analyses in females indicated a significant change in the number of fecal boli deposited during the testing session ($F4,50 = 4.44$, $p = 0.004$) with

the 500 µg/kg group producing more boli than all other groups. A difference in the percentage of open arm entries (%OAE) was also observed ($F_{4,50} = 2.99$; $p = 0.027$), with the low dose group showing an increase over controls ($p = 0.017$). Amongst the male groups, the ANOVA revealed a significant difference in distance travelled in the EPM ($F_{4,54} = 3.45$, $p = 0.014$), with the lowest dose group travelling more in the maze than controls ($p = 0.011$).

Normal sex differences in the EPM were confirmed on a number of measures, looking at the untreated control animals. Control females travelled further ($p = 0.027$), spent more time being mobile within the maze ($p = 0.019$; Figure 9), and had a greater number of open ($p = 0.041$) and hub ($p = 0.004$; Figure 10) entries than males, with males producing more boli than females ($p = 0.0001$) and making a greater percentage of closed arm entries (%CAE; $p = 0.008$). However these sex differences were almost entirely abolished by the lowest dose of BPA, 5 µg/kg bw/day, with only a sex difference in defecation surviving ($p = 0.002$). The elimination of these differences appears to be largely due to a feminization of male anxiety-like behaviours. In keeping with the paradoxical “low dose” effects of BPA that have been reported elsewhere (eg. Welshons et al., 2003; Jones, et al., 2011), higher doses of BPA had less effect on sex differences. At the 50 µg/kg dose only %CAE failed to reach significance significant at the $\alpha = 0.05$ level, and even that measure showed a strong trend ($p = 0.071$). At the 500µg dose, both distance ($p = 0.028$) and mobile time ($p = 0.01$) were significantly different, as were the number of entries into the closed arms

(p= 0.005) and hub (p = 0.003), with females having greater values on all of those parameters; this was the only dose at which the sex difference in boli deposition was not observed. At the highest dose, only boli ($p < 0.001$) and mobile time (p = 0.001) were significant, with females being more mobile than males (Table 1).

Figure 9. Total Time Mobile in the EPM

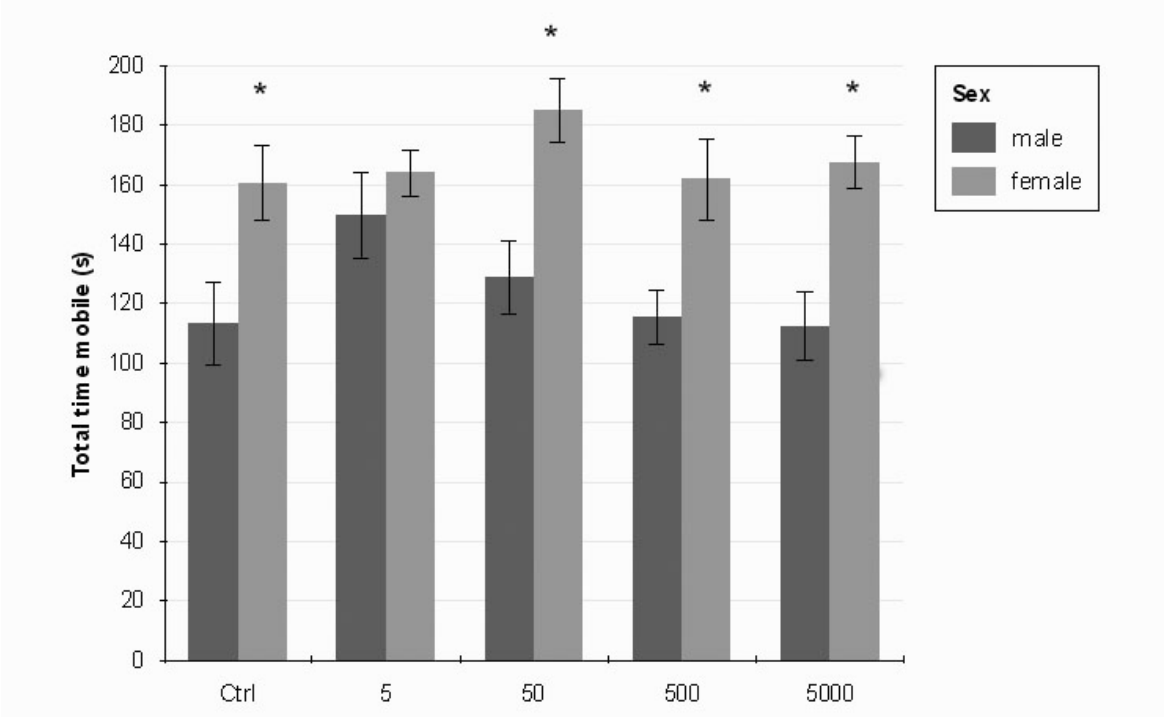
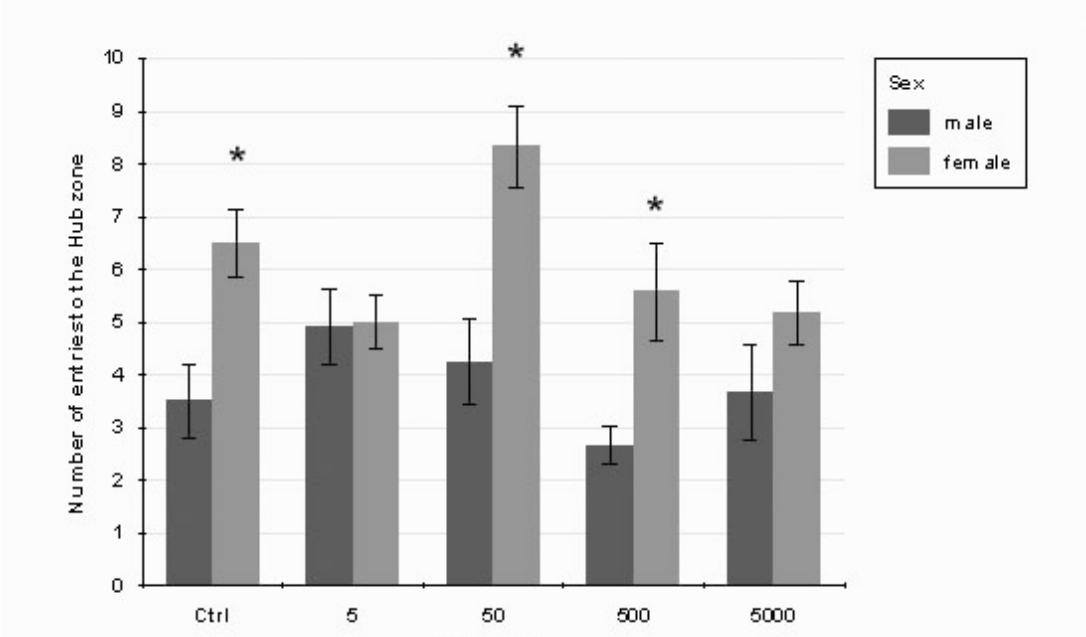


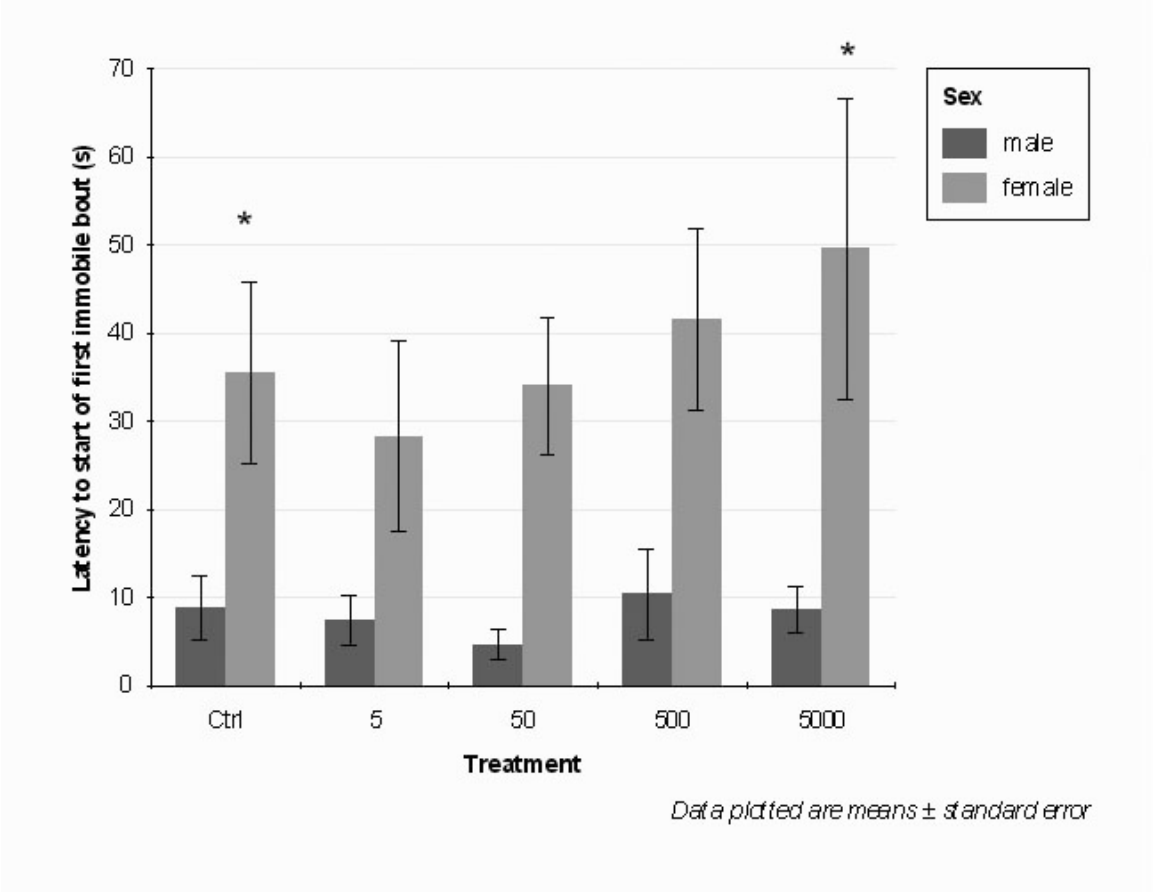
Figure 10. Total Number of Hub entries in the EPM



Forced Swim Test

ANOVA did not reveal any significant differences across dose within the male groups, nor were any of the female BPA groups significantly different from the controls on any parameter. There were a number of sex differences that were observed between the control groups. Females spent less time immobile ($p < 0.001$), made more rotations ($p = 0.009$) and took longer until the first period of immobility ($p = 0.023$; Figure 11) than males. In the 5μg group, immobile time (female < male; $p < 0.001$) and the number of mobile and immobile episodes were significant (male > female for both; $p = 0.014$ & 0.01, respectively), while latency to immobility failed to reach significance ($p = 0.055$). In the 50μg group, females spent less time immobile than males ($p = 0.002$) and made more rotations ($p = 0.001$), a pattern that held for the 500μg group as well ($p = 0.007$ & 0.008, respectively). In the 5000μg group, females spent less time immobile ($p < 0.001$), had fewer mobile ($p = 0.003$) and immobile ($p = 0.002$) episodes, made more rotations ($p = 0.001$) and took longer until their first immobile period ($p = 0.027$; Table 2).

Figure 11. Latency to Immobility in the FST



Discussion

Taken together, our data show that BPA has the potential to demasculinize males on a number of estrogen-sensitive parameters, but does not alter AR-sensitive behaviours.

EPM & FST

As noted earlier, sex differences are reliably found in the EPM, such that males show greater anxiety-like behaviour than females. Here, we found that the lowest dose of BPA, 5 μg/kg, reduced anxiety in females, increased activity levels in males and eliminated all sex differences on both anxiety and activity levels through an apparent demasculinization of males. The highest dose (5000 μg/kg) also eliminated most of these differences. Most of the sex differences were still apparent at the 500 μg/kg dose, with all sex differences noted between controls still seen between males and females receiving the 50 μg/kg dose. This pattern of results is indicative of a non-monotonic dose response in the ability to eliminate these sex differences, a pattern that has been observed before in hormonal systems generally and with BPA specifically, with

respect to both physiological (Welshons et al., 2003) and behavioural systems (Jones et al., 2011).

The pattern of results in the FST likewise indicates that the 5 μg/kg dose had the greatest effect on sexual differentiation. While the finding that females spend less time immobile than males was fixed across all doses, the 5 μg/kg dose did eliminate the sex difference in the latency to immobility as well as the number of rotations, and produced sex differences in the number of mobile and immobile episodes. The 50 and 500 μg/kg doses produced minimal changes only eliminating the sex difference in the latency to immobility, while the 5000 μg/kg dose did not eliminate sex differences, but did produce sex differences such that males had more mobile and immobile episodes than females. So once again, as observed in for EPM testing, BPA exhibited a non-monotonic dose-response relationship with FST, with the lowest dose of BPA exerting the greatest effects on FST behaviours.

Morris Water Maze.

Previous work has shown that BPA can alter spatial learning, but we did not see any impairment in the learning strategy of males and females exposed to BPA, or their ability to solve the maze.

While there is ample evidence that the sex differences in spatial learning are dependent on androgens acting perinatally (Isgor & Sengelaub,

1998; 2003), research from our lab examining the androgen-insensitive rat shows that the AR, while required for full masculinization of this behaviour, does not fully explain the observation of a male-typical advantage on this task (Jones & Watson, 2005). Indeed, Isgor & Sengalaub (1998) noted that prenatal exposure to estradiol benzoate improved female MWM performance on the last (7^{th}) day of training, though in that study, animals were given a total of 42 trials, twice what they were given in the current study.

Previous research has found BPA to be an antagonist at the AR (Sohoni & Sumpter, 1998), to have anti-androgenic activity in the prefrontal cortex (Leranth et al., 2008) and to inhibit the cognitive abilities required to solve the MWM (Xu et al, 2008), but we found no impact of perinatal BPA, administered throughout the critical period for steroid-dependent sexual differentiation, on androgen-sensitive spatial-navigational abilities. This is consistent, however, with the National Toxicology Program's report on BPA (2008), which states that BPA does not exhibit any effects on the AR. It is unclear if the discrepancies in the research literature are due to differences in methodologies, but as we administered repeated doses of BPA both perinatally and postnatally through to P14, we conclude that perinatal BPA does not appear to alter androgen-sensitive (AR-mediated) cognitive behaviours as assessed by the MWM. Consistent with the EPM and FST, however, activity levels – as measured by swim speed – were altered by BPA. Again, the low dose of BPA was able to demasculinize males in some cases, an effect not seen at higher doses.

Conclusion

Overall, our results here add to the growing body of evidence indicating that perinatal BPA treatment has a non-monotonic, low dose effect on various physiological and behavioural parameters that are normally sexually-dimorphic and organized through estrogenic actions; however, there is sparse evidence that BPA can alter androgenic systems *in vivo*. The estrogenic, affective parameters, however, were more uniformly altered by the lowest dose of BPA; specifically, we found a demasculinization of affect in males and a further decrease in anxiety in females.

To mimic the likely pattern of human exposure to BPA, both pre- and post-natally, we chose to administer BPA to our animals throughout the perinatal period. Unfortunately, this prevents the analysis of maternal effects on adult behaviour or the effect of BPA on maternal behaviour itself; however, we felt that the mimicking of human developmental exposure patterns would increase the ecological validity in the study of the potential teratogenic effects of this compound. As such, we cannot claim the sum total of all BPA-derived effects noted here are due to direct physiological effects on the developing organism. Indeed, some work has noted subtle but significant effects of BPA on maternal behaviour (Della-Seta et al., 2005; Cox et al., 2010). As such, while there exists

the possibility that some of the effects of BPA on adult behaviour may be mediated indirectly through the behaviour of the mother, we believe that it is more important to note the cumulative effects of this compound following exposure during the entire perinatal period.

Sex differences in the development of anxiety have received increased attention recently, and are widely thought to be influenced by the activity of estrogens acting at estrogen receptor β (ERβ; Imwalle et al., 2005). Likewise, a growing literature has suggested that ERβ, not ERα, is involved in the antidepressant effects of estrogens in adults (see Osterlund, 2010). Because BPA activates this receptor *in vitro* (Matthews et al., 2001), it seems likely that at least some of BPA's effects on the measures of anxiety- and depression-like behaviour may be attributable to alteration of the normal development of ERβ-mediated neural systems.

In addition, others have noted that there are two primary components being measured in the EPM: anxiety and activity (Doremus et al., 2006). Some of the effects that we have noticed here, such as closed arm entries (Doremus et al., 2006), distance travelled and mobile time, are clearly related to activity levels. Our results show a feminization of activity by BPA, such that males, particularly at the lowest dose analyzed here, were more active than controls on this apparatus. This is consistent with other work showing motor hyperactivity in rats exposed to BPA perinatally (Kiguchi et al., 2008; Masuo et al., 2004a & b).

Generally, the pattern of results suggests that maternally delivered Bisphenol A has the potential to alter the normal development of affective behaviours, eliminating the normal sex differences in these domains and altering learning strategy in both males and females, though this only occurred at an environmentally-relevant dose in males. Certainly, this further adds to the growing concern about BPA consumption amongst the general population. While Health Canada has taken the step of banning BPA in baby bottles, this has not yet been done in either Europe or the USA; maternal exposure to BPA, however, may also serve to alter the normal development of the child.

Chapter 4: Perinatal Bisphenol A exposure alters Estrogen Receptor-alpha expression in the medial amgdala of male rats.

Introduction

Bisphenol A (BPA) is a weakly estrogenic compound (Dodds & Lawson, 1936) used in the production of a host of commercial and industrial products, including hard plastics, the lining of food and beverage tins, dental sealants, medical tubing, carbon paper, polyvinyl chloride, compact discs and more. Given the wide variety of products in which BPA can be found, it has been estimated that over 90% of all Americans (Calafat et al., 2008) and Canadians (Bushnik et al., 2010) have detectable levels of BPA in their bodies. Health Canada has set the tolerable daily intake for human exposure at 25 μg/kg bodyweight/day (μg/kg), while the Food and Drug Administration in the US allows twice as much. However, the ubiquity of this compound poses a potential danger for humans as a number of studies have found that exposure to BPA is associated with a number of physiological and behavioural changes. One of the earliest studies examining the effects BPA noted that exposure to low doses of BPA could alter the development of the prostate gland in adult mice (Nagel et al., 1997). Since then, a host of studies have noted other perturbations in the reproductive system, including decreased sperm counts and motility, altered testicular expression of

steroid receptors (Salian et al., 2009) and reduced epididymal weights (vom Saal et al., 1998). These physiological issues are mirrored by concomitant behavioural perturbations. For example, male deer mice exposed to a low dose of BPA both pre- and post-natally attract less attention from female conspecifics in adulthood (Jašarević et al., 2011), while male rats exposed to BPA perinatally have also been found to exhibit deficits in sexual performance as adults (Farabollini et al., 2002), deficits that persist across multiple sexual encounters (Jones et al., 2011).

Numerous studies have shown that expression of the estrogen receptor alpha (ERα) is required for typical male sexual behaviour. For example, Wersinger and colleagues (1997) found that male homozygous ERα-knockout (ERαKO) mice displayed fewer mounts, thrusts, intromissions and ejaculations, and that their latencies to express these behaviours were significantly increased; this data converges nicely with findings from Ogawa et al. (1998) that shows an almost complete elimination of male-typical sexual behaviours in ERαKO male mice, though these behaviours could be partially rescued with the administration of either testosterone proprionate (TP) or dihydrotestosterone proprionate (DHTP). Furthermore, the activity of ERα in the expression of male sexual behaviour appears to be mediated specifically by the classical genomic activities of that receptor, as mutant mice expressing only a form of ERα that cannot bind the estrogen response element also exhibit a severe decrement in male-typical performance (McDevitt et al., 2007). Conversely, knockout of the beta isoform of

the estrogen receptor (ERβ) does not alter male sexual behaviour in adulthood (Ogawa et al., 1999).

The medial preoptic nucleus (MPN) of the medial preoptic area (mPOA), the posterodorsal bed nucleus of the stria terminalis (BNSTpd) and the posterodorsal medial amygdala (MeApd) have all been implicated in the sexual behaviour of males (Tsutsui et al., 1994; Kondo and Arai, 1995; Paredes et al., 1993) as lesioning of these structures can reduce or even eliminate male typical copulatory behaviours. As such, the adult expression of ERα in sexually experienced adult male rats was assayed in these regions following exposure to one of several doses of bisphenol A during the pre- and postnatal period.

BPA is also known to stimulate glucocorticoid receptor (GR)-dependent gene expression (Sargis et al., 2010) and is an agonist at this receptor *in silico* (Prasanth et al., 2010). Neurons throughout the hippocampus (HPC) heavily express this receptor and glucocorticoids have been found to play a role in the myriad behaviours and physiological processes influenced by this structure. The HPC is well known to play a role in learning and memory (Fanselow & Dong, 2010), and research has indicated that the dorsal HPC (dHPC) in rodents – the posterior HPC in humans – is critical for learning to navigate through spatial environments though lesions of the ventral HPC (vHPC) do not dramatically impair this form of learning (Moser et al., 1995), instead, the vHPC has been hypothesized to alter emotional behaviours, including anxiety, as lesioning this region has an anxiolytic effect in the elevated plus maze (EPM; Kjelstrup et al.,

2002) as well as other stress-related parameters (Henke, 1990). The GR are known to play a role in this as well as dexamethasone (DEX) administration prenatally alters anxiety in adulthood (Nagano et al., 2010). Furthermore, the vHPC is known, through direct connections to the hypothalamus (Canteras & Swanson, 1992), to mediate in part the hypothalamic-pituitary-adrenal (HPA) negative feedback loop. Disruptions to this HPA negative feedback loop can be measured with the DEX suppression test in which DEX administration suppresses cortisol production in healthy subjects but not in the majority of those with a diagnosis of depression (Fountoulakis et al., 2008). Furthermore, anti-depressants increase neurogenesis in the HPC (Duman et al., 2001) and this is accomplished through a GR-dependent mechanism (Anacker et al., 2011). Given the role of hippocampal GR in anxiety and depression, the expression of that receptor was measured in the CA1 of the vHPC.

Methods

Fifteen 60-day old Long-Evans (LE) females from Charles River served as dams for the BPA treatment manipulation. These females were maintained in a colony room in the Animal Resource Centre at Simon Fraser University, and had access to standard rat chow (Purina) and water *ad libitum*. All procedures conformed to the guidelines of the Canadian Council on Animal Care, and were reviewed and approved by the University Animal Care Committee prior to the study.

BPA was administered orally, dissolved in corn oil. During a pre-experimental phase, all females were trained to drink corn oil from a syringe, to ensure proper administration of the vehicle and also to reduce stress associated with handling and exposure to a new stimulus. Following this training period, the females were paired with LE stud males in segregated cages, for up to a week, in order for mating to occur. The appearance of vaginal plugs was taken as evidence of successful insemination, at which time the females were separated from the studs and placed in single housing in polysulfone cages with BPA-free water sacks. Each female was randomly assigned to one of 5 BPA (Sigma, Oakville, ON; >99% pure) dose groups: Oil vehicle, 5 µg/kg bw/day, 50 µg/kg bw/day, 500 µg/kg bw/day, or 5 mg/kg bw/day, such that there were 3 females in

each group; this was done to ensure that potential dose effects could not be confounded with individual variation associated with a single dam. Although plugs were found for each female, one female assigned to the 500 µg/kg bw/day group did not become pregnant. Thus, there were only two pregnant females in that group. The day that the plugs were found was considered gestational day 1 (GD1), and BPA dosing began on GD7, continued through the remainder of gestation, and after parturition continued through post-natal day 14 (P14), with the day of birth being considered P0. Litters were culled to 4 males and 4 females each, except in the 500 µg/kg bw/day group, in which litters were culled to 6 males & 6 females each; thus, each dose group had a total of 12 males and 12 females. Because of the small litters by the 2 dams in the 500 µg/kg bw/day group, that dose group had only 7 females. Upon weaning (P21), pups were group housed in polysulfone cages with same-sex littermates, and access to tap water and rat chow *ad libitum*.

Subjects were tested on a variety of behavioural measures in adulthood, as has been reported previously (Jones et al., 2011; Jones & Watson, 2012; better known here as chapters 2 & 3). Following these tests, animals were killed with carbon dioxide and then perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PARA). Brains and spinal cords were removed and placed in PARA for 2 hours and then transferred to 30% sucrose in PBS as a cryoprotectant. The tissue was then frozen on a sliding microtome and sliced in serial sections of 6 at a thickness of 50µm and then

transferred into de Olmos solution for storage at -20°C. One series was used for immunohistochemistry. Some tissue was unusable, due to technical difficulties. However, group size was 9 or above in every case.

Immunohistochemistry

At room temperature, tissue was rinsed in PBS with 0.1% Triton-X (PBS-X) and then treated for 15 minutes with hydrogen peroxide in PBS-X to eliminate endogenous peroxide (H_20_2) activity, rinsed again in PBS, blocked with 4% normal goat serum (NGS) and then incubated for 48 hours with anti-ERα C1355 (1:10000; Upstate, Lake Placid, NY) or overnight with anti-GR M-20 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-X with 2% NGS at 4°C. Following this incubation, tissue was rinsed in PBS-X, and then incubated in biotinylated goat-derived anti-rabbit secondary (1:500; Vector Laboratories, Burlington, ONT.) in PBS-X for 2 hours. Following another rinse in PBS-X, tissue was incubated for 1 hour in avidin-biotin complex (Vector Laboratories) and then treated for 3 minutes with diaminobenzidine in Tris containing H_20_2 and nickel chloride. Tissue was then mounted on gelatin-coated slides, dried and treated with a series of graded alcohols and xylene, and coverslipped with Permount (Fisher Scientific, Ottawa, ONT.) immediately.

Microscopy

Slides were examined on a Nikon Eclipse E600 optical microscope. Pictures were taken with Nikon Elements F (Nikon Canada, Mississauga, ONT.) on a desktop PC running Windows 7 (Microsoft) using a DS-Fi1 digital camera (Nikon Canada) with all analyses done using ImageJ (National Institutes of Health).

All brain regions were identified through the use of a rat brain atlas (Swanson, 2004). For analysis of ERα, pictures were taken from both hemispheres in two different slices. A region of interest (ROI) was defined for each of the three nuclei. For the MPN (atlas level 20 & 21), this ROI encompassed the central nucleus (MPNc) and the area immediate surrounding it. The second ROI, for the BNSTpd (atlas level 20 – 22), was applied to the dorsal-most aspect at atlas level 22. For the MeApd, a third ROI was applied to atlas levels 28 – 30. Counting of neurons was done using the ITCN plug-in for ImageJ.

For analysis of GR, pictures were taken from CA1 in the ventral poles in slices posterior to bregma -4.4 mm. Three pictures were taken from each animal and 25 darkly stained neurons were used for analysis. Using ImageJ, a tissue punch was taken within each neuron, and the mean grey value was taken. A ratio was then calculated of the mean grey values from the neuron to the mean grey value of a punch from the neighbouring stratum in which no stained neurons were visible.

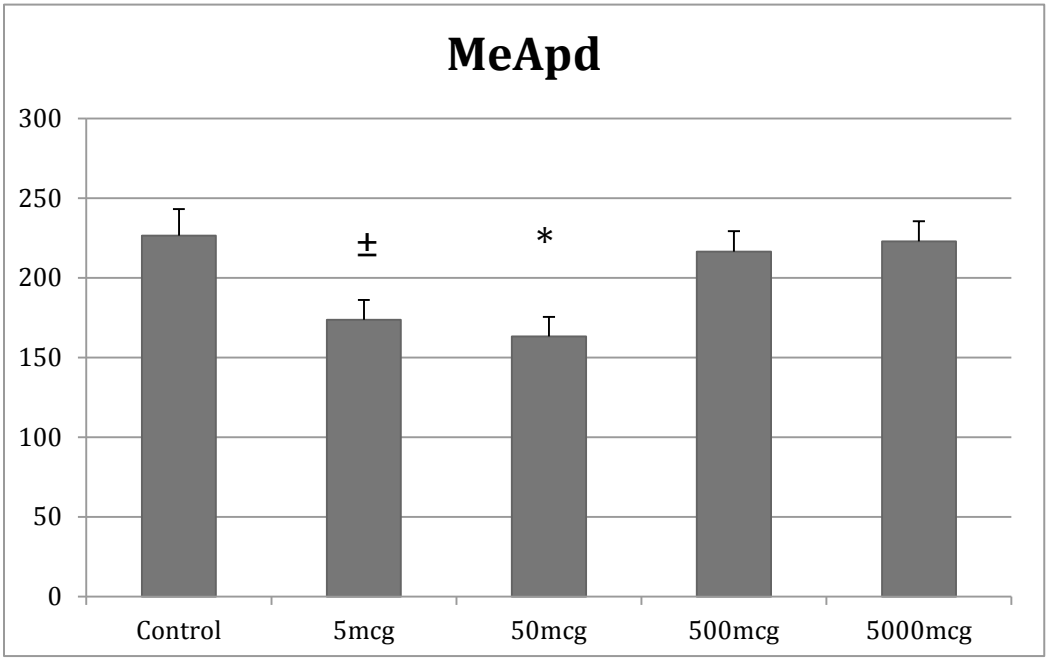
Statistical Analysis

One-way analysis of variance (ANOVA) with post-hoc Tukey's analyses was done using SPSS v.19 to determine any differences in the expression of ERα in these three regions. A one-tailed Pearson's correlation between the number of ERα-expressing cells in each of the MeApd, BNST and MPN and sexual performance composite scores calculated following behavioural analysis (Jones et al., 2011) was also conducted. These *a priori*, directional analyses were determined from previous work showing that a reduction in ERα expression led to an inhibition in sexual behaviour (Ogawa et al., 1998). For GR analysis, a 2X5 ANOVA (sex X dose) was used to determine any group differences in GR optical density.

Results

The ANOVA revealed that there were no differences between the groups in both the MPN ($F_{4,48} = 1.816$, $p = 0.141$) and the BNST ($F_{4,43} = 0.895$, $p = 0.475$), however, there was a significant difference in MeApd ($F_{4,45} = 4.971$, $p = 0.002$). A post-hoc Tukey's analysis showed that both the control and the 5000 μg/kg groups expressed significantly more ERα than the 50μg/kg ($p = 0.018$ & 0.019, respectively; Figure 12; Appendix B, photomicrographs 1 - 5). No other differences were detected, though Tukey's test showed a difference of $p = 0.058$ and $p = 0.065$ between the 5 μg/kg and the control and the 5000 μg/kg. The Pearson correlation showed that there was a modest but significant correlation between the composite score from the day of sexual naïveté and the level of ERα expression in both the MPN ($r = 0.255$, $p = 0.033$) and the MeApd ($r = 0.234$, $p = 0.051$). A significant relationship between sexual performance following multiple exposures and ERα expression in the MeApd ($r = 0.293$, $p = 0.019$) was also noted. No differences in GR expression was found, however (sex: $F_{1,92} = 0.000$, $p = 0.994$; dose: $F_{4,92} = 1.734$, $p = 0.303$; sex X dose: $F_{4,92} = 0.906$, $p = 0.464$)

Figure 12. ERα Expression in the MeApd



* $p < 0.05$ vs. Control and 5000 µg/kg groups

± $p = 0.058$ & 0.065 vs. control & 5000 µg/kg group, respectively

Discussion

The study here provides further evidence that adult levels of ERα can be altered by exposure to low doses of BPA perinatally. Specifically, the number of neurons expressing ERα is reduced in the MeApd, but not the BNST or MPN. It is intriguing that this pattern of expression is exceptionally similar to the pattern of sexual performance expressed by these animals both when naïve and after multiple sexual experiences (Jones et al., 2011). Furthermore, we have found a significant correlation between the expression of ERα in both the MPN and the MeApd and sexual performance.

The MeApd is involved in the processing of pheromonal cues from conspecifics (Bressler & Baum, 1996) and members of other species (Dielenberg et al., 2001) and many of its efferents innervate both the BNST and the mPOA, including the MPN (Canteras et al., 1995). Bilateral lesions of the MeApd have been found to eliminate reproductive behaviours in sexually inexperienced male rats (Kondo, 1992) showing that the function of this structure is critical in the expression of reproductive behaviours. However, the functional connections between the MeA and the mPOA are necessary for expression of male-typical reproductive behaviours, as contralateral, but not ipsilateral, lesions of these structures can virtually eliminate these behaviours (Kondo & Arai, 1995; Been &

Petrulis, 2011). Furthermore, as the expression of ERα is necessary for male-typical sexual behaviour in adults (Ogawa et al., 1998) and the inhibition of estrogen synthesis in adulthood prevents this behaviour (Vagell & McGinnis, 1997), it would seem likely that the activity of ERα in the MeApd is at least partially responsible for male-typical sexual behaviour. Our data converge with these previous findings and also indicate that the maximum recommended daily intake dose of BPA decreases ERα in the medial amygdala and inhibits copulatory behaviours.

The BNST appears to play less of a critical role in the expression of male-typical copulatory behaviours, but is important in other aspects. For example, the functional dissociation of the MeA from the BNST does not suppress sexual activity, but instead reverses the typical difference in the investigation of sex-specific odours and causes a preference for male-derived odours (Been & Petrulis, 2011). Lesions of the BNST have also been found to eliminate non-contact erections, though larger lesions, particularly those extending further along the caudal extent of this structure, do reduce male-typical performance, though not to the extent of mPOA lesions (Liu et al., 1997).

BPA has previously been shown to have varying effects on the expression of ERα in a region- and development-specific manner. For example, postnatal administration of a high dose (50 mg/kg) of BPA can increase the expression of ERα mRNA in the mPOA on P4, but decrease expression on P10 in female rats,

(Cao et al., 2012). However, that same dose, while increasing ERα mRNA in the P4 male brain had no effect on P10, but ERα mRNA expression in the mPOA of P10 males exposed to 50 μg/kg was decreased (Cao et al., 2012). But Ramos and colleagues, administering 25 and 250 μg/kg BPA during gestation only, found that the expression of ERα mRNA in the adult male mPOA was unaffected. Nor does a high dose (~100 mg/kg) of postnatally administered BPA alter ERα expression in the AVPV (Patisaul et al., 2006). We did not notice an effect of any dose of BPA administered both pre- and postnatally on the adult MPN, however, though while this differs from the juvenile pattern of expression, our data are similar to that seen by Ramos and colleagues (2003), and similar to that seen in the mPOA following exposure to other endocrine disrupting phenolic compounds (Dickerson et al., 2011).

Given that the mPOA seems to be critical for copulatory behaviour (Hansen et al., 1984; Powers et al., 1987; Arendash & Gorski, 1983; Hurtazo et al., 2008), it is not surprising that we have noted a relationship between ERα expression in the mPOA and sexual performance. Taken together, this suggests that estrogen, acting via ERα in the mPOA and the MeApd is important for reproduction and that perinatal exposure to BPA can act to disrupt the development of this system, thus decreasing reproductive competency in adult males. To our knowledge, this is the first report indicating that BPA can alter ERα expression in the medial amygdala.

BPA did not exert any effect on the expression of GR in the CA1 of hippocampus. It is unclear why this may have occurred, as prenatal manipulations of corticosteroid exposure has been seen to alter GR expression in the adult hippocampus. For example, Wilcoxon & Redei (2007) found an increase in GR expression in the HPC following maternal adrenalectomy during gestation, while Brabham et al. (2000) noted that, at least in the dHPC, DEX treatment did not alter GR mRNA expression in adulthood. However, this may be due to the timing of DEX administration. Welberg and colleagues (2001) found that administering DEX during the final week of gestation, but not throughout the entire gestational period, decreased GR mRNA in the CA1 of male rats suggesting some specific critical period; we dosed our animals during both the 2nd and 3rd week *in utero*, thus it is possible that we may have reduced our ability to detect potential changes to alterations of the HPA axis as per Welberg et al. (2001). Regardless, as this administration regime is likely to mimic the typical ecological pattern of BPA exposure, we suggest that there is likely little effect on this parameter. Future studies should examine other regions, however, such as the dorsal hippocampus, which appears to play a role in specific parameters on the FST (Korte et al., 1996) and the amygdala, where GR activation increases anxiety in the EPM (Weiser et al., 2010).

Limitations

Our analysis of ERα expression was done in animals that had four different exposures to proceptive/receptive females in the span of a week; thus, it is possible that the sexual experiences enjoyed by our animals could alter the expression of ERα. One report has suggested that a single ejaculation in rats can lead to increased ERα expression in the MeApd 24 hours later (Phillips-Farfan et al., 2007), though this was not replicated in mice (Swaney et al., 2012). However, in the former study, all animals (including the no-sex controls) were sexually experienced. In the latter study, experienced mice were killed after a one-week delay during which they were housed with no-receptive females. In our study, the animals were exposed to other tests, with a time of approximately 60 days between the final reproductive test and the removal of tissue; it is unknown what changes may occur to the expression of ERα in animals given that length of time and no further opportunity for the expression of sexual behaviour, though given the results of Swaney et al. (2012), we would expect that any temporary, experience-dependent changes would have resolved by the time that we examined the expression of this protein. Also, given that non-copulating rats were seen to express greater number of ERα-expressing cells in the MeApd than rats given copulatory experience (Portillo et al., 2006), it is likely that the results noted here are due to BPA exposure, specifically.

Similarly, our testing protocol may have altered the pattern of expression of GR-ir thus preventing us from finding an effect. Acute stress has been seen to alter GR expression, though this has typically been examined in the dHPC (Tritos et al., 1999; Paskitti et al., 2000), however, data from Romeo et al. (2008) have suggested that acute stress in adulthood does not alter expression of GR in the ventral CA1, though there is a decrease in the dorsal CA1. As such, our data here are likely to accurately represent the effects of BPA on GR expression in the ventral CA1.

Conclusion

Despite the differences in methodologies, including the differences in timing of administration, dosage and timing of the histological analysis, the weight of evidence suggests that BPA exposure can, particularly during hormone-sensitive critical periods in development, alter the expression of ERα in tissues responsible for the expression of male typical reproductive behaviours. Our previous work has shown that BPA exposure does not significantly alter the number of anogenital investigations despite the drastic decrement in copulatory behaviour. It may be the case that BPA, through alterations in the normal pattern of expression of ERα in the MeApd, is preventing the normal level of processing of pheremonal cues, thus inhibiting the typical expression of sexual behaviour. Given that BPA can alter the organisation of other systems that are involved in this behaviour, such as dopamine (Patisaul et al., 2006; Tando et al., 2007), there may be a number of neurophysiological changes that could potentially explain the poor copulatory behaviour of males exposed to this compound.

The weight of evidence, then, suggests that the organisation of neural systems critical in social behaviours are altered by early life exposure to BPA, and as a result, the behaviours of these organisms in adulthood is thus altered. The Canadian government has recognised this fact and now regards BPA as a

toxic compound and forbids the sale of baby bottles containing BPA. Unfortunately, BPA can be detected in baby formula (Schechter et al., 2010; Cao et al., 2011) and as shown here, the mother can also serve as a vehicle for BPA administration to her developing children. While a number of European nations and American states have also passed legislation banning the sale of BPA-containing baby bottles, no other country has gone so far as to declare this substance toxic.

Chapter 5: The effects of BPA on an androgen-sensitive neuromuscular system.

Introduction

BPA is an endocrine-disrupting compound originally known to have weak estrogenic activity (Dodds & Lawson, 1936). However, this ubiquitous compound has also been found to alter a number of other systems. For example, BPA can block thyroid receptors (Moriyama et al., 2002) and aryl-hydrocarbon receptors (Bonefeld-Jorgenson et al., 2007), activate glucocorticoid receptors (Sargis et al., 2010) and the membrane-bound estrogen receptor GPR30 (Thomas & Dong, 2006) and bind with high affinity at the estrogen-related receptor gamma (ERRγ; Takayanagi et al., 2006). A number of *in vitro* studies have also noted that BPA can act as an antagonist at the androgen receptor (AR) in a number of models (Sohoni & Sumpter, 1998; Lee et al., 2003; Sun et al., 2006; Jolly et al., 2009). The evidence for AR-antagonism *in vivo* has been less convincing, however. Leranth et al. (2008) have found that BPA administration to adults can inhibit synaptogenesis in intact male rats and prevent the testosterone (T)-induced increase of synaptogenesis in gonadectomised males. Furthermore, Xu et al. found that perinatal BPA can inhibit performance in an androgen-sensitive spatial-learning task in both adults (2007) and pre-pubescent male rats (2010a), though this effect in adults was not found by either Nakamura et al. (2012) or

Jones and Watson (2012). As such, the National Toxicology Program (2008) has stated that the weight of evidence does not support the claim that BPA is an AR-antagonist.

To further examine the putative AR-blocking effects of BPA, we examined how this compound alters the physiology of a highly androgen sensitive pool of motor neurons within the lower lumbar spinal cord (L5 & L6). This group of neurons, the spinal nucleus of the bulbocavernosus (SNB) innervates the bulbocavernosus/levator ani (BC/LA) muscles of the penis (Breedlove & Arnold, 1980). During gestation, both males and females initially contain about 300 of these neurons but as the organism develops, the process of apoptosis – or pre-programmed cell death – pares this number down to approximately 200 in males and 60 in females (Nordeen et al., 1985). It is unclear exactly how this occurs, but the activity of the androgen receptor at some site outside of the SNB is required for male-typical survival of these neurons, likely at the BC/LA muscle (Kurz et al., 1992). In the same region of the spinal cord are two other pools of motor neurons. One, the dorsolateral nucleus (DLN), is also sexually dimorphic as it innervates the ischiocavernosus muscle in the penis and is dependent on the action of the AR for survival, while the other, the retrodorsolateral nucleus (RDLN) innervates the monomorphic flexor digitorum brevis muscle and does not require androgens for survival (Leslie et al., 1991). In adulthood, androgens are known to be involved in “housekeeping” (Matsumoto et al., 1994) and deprivation of androgenic activity following castration reduces the soma size in the SNB but

not RDLN (Hamson et al., 2009). I took advantage of these known developmental parameters to examine the effects of BPA on these structures following either perinatal exposure to BPA or chronic exposure in adulthood. Specifically, I looked to see if motor neuron survival is altered following perinatal exposure to BPA or if soma size is decreased following chronic (28 days) exposure to BPA in adulthood.

Methods

Experimental Design 1: Perinatal Exposure

Fifteen 60-day old Long-Evans (LE) females from Charles River Laboratories (Quebec) served as dams for the BPA treatment manipulation. These females were maintained in a colony room in the Animal Resource Center at Simon Fraser University, and had access to standard rat chow (Purina) and water *ad libitum*. All procedures conformed to the guidelines of the Canadian Council on Animal Care, and were subject to prior approval by the University Animal Care Committee.

The females were trained to drink corn oil from a syringe, to ensure proper administration of the vehicle and also to reduce stress associated with handling and exposure to a new stimulus. They were then paired with LE stud males and, when vaginal plugs were found in the cages (denoting copulation), they were moved to single housing in polysulfone cages with BPA-free water sacks. The pregnant females were randomly assigned to one of 5 BPA dose groups: oil vehicle, 5 µg/kg bw/day, 50 µg/kg bw/day, 500 µg/kg bw/day, or 5000 µg/kg bw/day, such that there were 3 gestating females in each group. Although copulatory plugs were found for each female, one female assigned to the 500 µg/kg bw/day group did not become pregnant. As such, there were only two

females in that group. The day that the plugs were found was considered gestational day 1 (GD1), and BPA dosing began on GD7. Dosing continued through post-natal day 14 (P14), with the day of birth being considered P0. Resultant litters were culled to 4 males and 4 females each, except in the 500 µg/kg bw/day group, in which litters were culled to 6 males & 6 females each; thus, each dose group had a total of 12 males and 12 females. Upon weaning (P21), pups were group housed in polysulfone cages with same-sex littermates, and access to tap water and standard Purina rat chow *ad libitum*. A number of behavioural tests were conducted between the ages of 90 & 150 days, as described previously (Jones et al., 2011; Jones & Watson, 2012).

Following behavioural testing, animals were killed with carbon dioxide and then perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PARA). Brains and spinal cords were removed and placed in PARA for 2 hours and then transferred to 30% sucrose in PBS as a cryoprotectant. The tissue was then frozen with a sliding microtome and sliced in serial sections of 3 at a thickness of 50µm and then transferred into de Olmos solution for storage at -20°C. One series was used for analysis. Specifically, spinal cords were mounted on slides and then stained with thionin, and put through a series of alcohols and xylene, then coverslipped immediately. Some tissue was unusable, due to technical difficulties. However, group size was 9 or above in every case.

Experimental Design 2: Adult Exposure

Fifty adult males were maintained in a colony room in the Animal Resource Center at Simon Fraser University, and had access to standard rat chow (Purina) and water *ad libitum*. All procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the University Animal Care Committee prior to the study.

BPA was dissolved in water and each male was randomly assigned to one of 5 BPA (Sigma, Oakville, ON; >99% pure) dose groups: water vehicle, 5 µg/kg bw/day, 50 µg/kg bw/day, 500 µg/kg bw/day, or 5000 µg/kg bw/day and then group housed in polysulfone cages with access to tap water and rat chow *ad libitum*. Animals were administered BPA-containing water for 28 days following which they were killed and had their spinal cords removed. BPA concentrations in water for each group were determined following a previous pilot study in which daily BPA-containing water consumption was tracked in age-matched male LE rats in our facility. Water consumption throughout the study was continuously monitored to ensure that animals were exposed to the doses described above. Tissue was sliced at 50µm thick and then stained with thionin – as described above – to reveal motor neurons.

Statistical Analyses

One-way ANOVAs using SPSS 17 with post-hoc tukeys tests were used to determine group differences in motor neuron survival, for those animals in experiment 1, and in soma size, for animals in both experiments.

Results

Experiment 1

There were no differences in adult motor neuron survival from any of the three pools examined (SNB, $F_{4,50} = 0.713$, $p = 0.587$; DLN, $F_{4,50} = 0.243$, $p = 0.912$; RDLN, $F_{4,50} = 0.529$, $p = 0.715$) nor did we see any differences in adult soma size (SNB, $F_{4,50} = 0.292$, $p = 0.882$; DLN, $F_{4,50} = 0.505$, $p = 0.732$; RDLN, $F_{4,50} = 0.18$, $p = 0.948$) following perinatal exposure to BPA.

Experiment 2

Chronic exposure to BPA in adulthood had significant effect on the size of motor neurons in each of the three pools quantified here (SNB, $F_{4,50} = 13.9$, $p < 0.001$; DLN, $F_{4,50} = 7.476$, $p < 0.001$; RDLN, $F_{4,50} = 9.802$, $p < 0.001$; Figures 1 – 3, respectively). The post-hoc Tukey's analysis revealed that in the SNB, the control group had a significantly larger soma size than each of the other 4 BPA dose groups ($p < 0.001$ for each group), while no differences emerged across the four dose groups (data not shown). This pattern of results was seen in the DLN as well where controls were different than all other dose groups ($p = 0.006$ vs. 5 µg/kg; $p = 0.002$ vs. 50 µg/kg; $p = 0.006$ vs. 500 µg/kg; $p < 0.001$ vs. 5000 µg/kg); again, no differences emerged amongst the four dose groups (data not

shown). For the RDLN, controls were also larger than all of the BPA groups ($p = 0.055$ vs. 5 µg/kg; $p = 0.005$ vs. 50 µg/kg; $p < 0.001$ vs. 500 & 5000 µg/kg); furthermore, the 5000 µg/kg group was significantly smaller than the 5 µg/kg group ($p = 0.029$).

Figure 13. SNB soma size following chronic BPA exposure in adulthood.

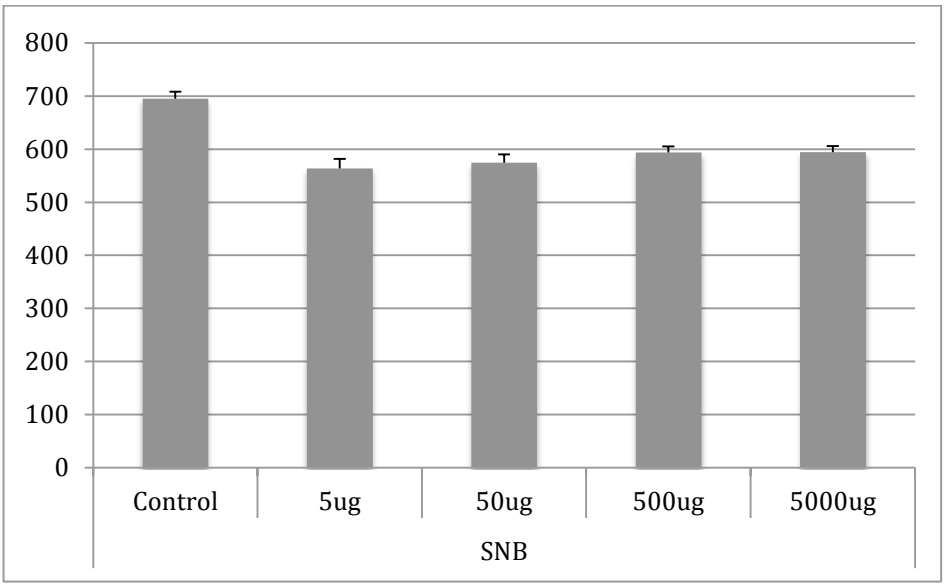


Figure 14. DLN soma size following chronic BPA exposure in adulthood.

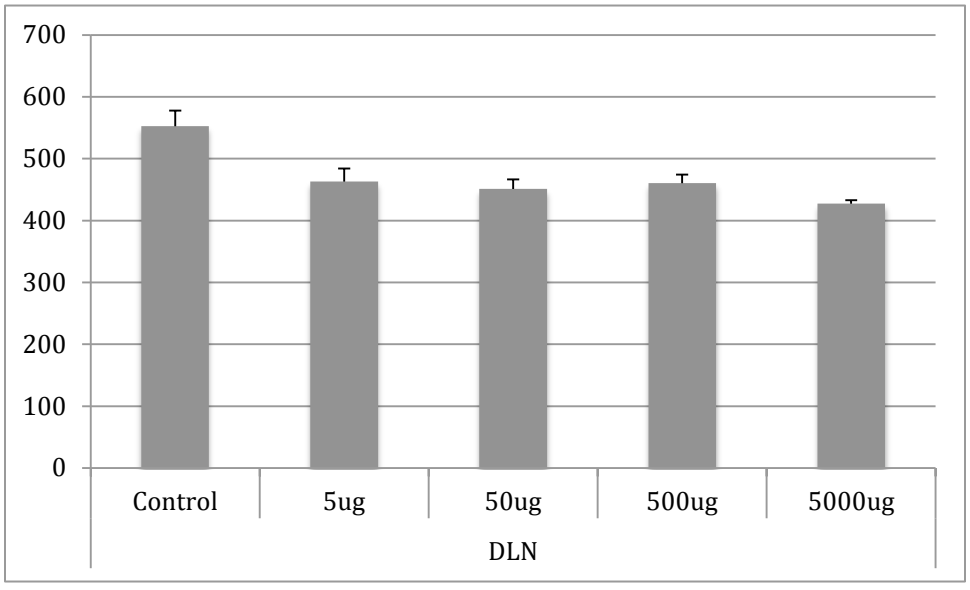
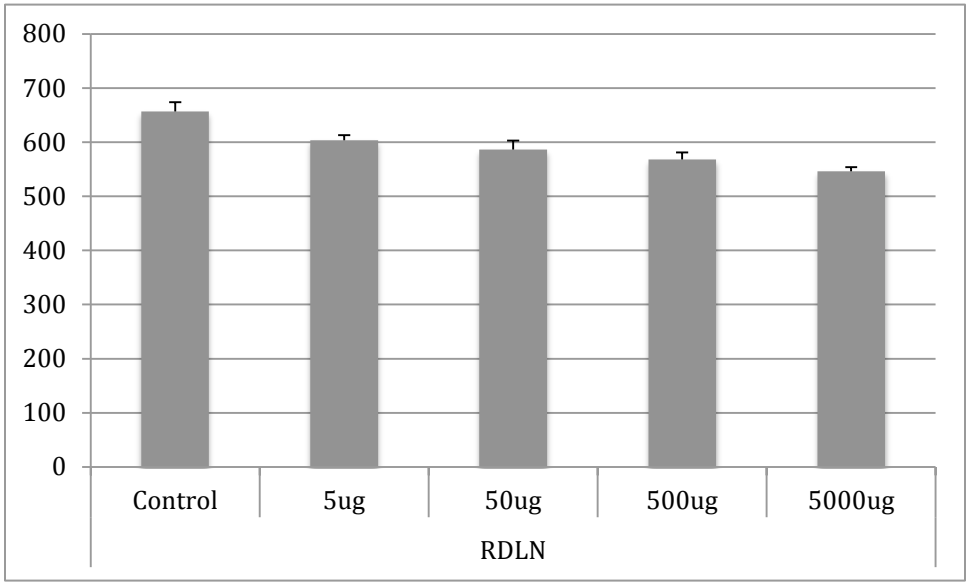


Figure 15. RDLN soma size following chronic BPA exposure in adulthood.



Discussion

The administration of BPA during development had no effect on known AR-dependent parameters in the SNB or the DLN. Furthermore, the BPA-mediated effects on soma size following chronic exposure in adulthood are not consistent with a purely anti-androgenic mechanism of action.

A growing literature has suggested that BPA is capable of acting to prevent normal AR-mediated activity. These studies, however, have typically examined human AR (hAR) transfected into either yeast (Sohoni & Sumptor, 1998) or monkey kidney cells (Sun et al., 2006; Xu et al., 2005), or the AR found in LNCaP cells, including T877A, the mutant AR found in human prostate cancer which is capable of exerting genomic effects following binding to AR antagonists inlcuding flutamide and BPA (Wetherill et al., 2005). With the exception of the N-terminal domain, AR is one of the most highly conserved proteins studied to date (Thornton & Kelley, 1998). There is one AR variant, AR45, which is found in many species, including humans, and expressed in skeletal muscle, but is not found in *rattus norvegicus* or *mus musculus*, and is differentiated from other variants by its N-terminus (Weiss et al., 2007). It is possible that this variant, which can either promote or inhibit AR-mediated genomic activity and is also

found in prostate (Ahrens-Fath et al., 2005) could explain differences between the *in vitro* and *in vivo* studies.

The evidence to date, with the exception of one study using high doses (Jolly et al., 2009), suggests that BPA does not exert strong androgenic effects *in vivo* in the models studied thus far; in fact, the pattern of effects seen are more consistent with its estrogenic actions. For example, this has been found in the Hershberger assay – a test of a number of androgen-sensitive physiological parameters used to assess compounds, including environmental endocrine disruptors (EDCs), for decades (Gray et al., 2005). Specifically, vinclozolin, the anti-androgenic fungicide, showed comparable efficacy to flutamide in preventing the typical development of external genitalia and the urogenital tract in males (Kang et al., 2004), while BPA did not (Kim et al., 2002). Other research has shown that BPA exposure during the prenatal period leads to an enlarged adult prostate as well as gene expression changes similar to that seen with DES or estradiol treatment (Taylor et al., 2011). Conversely, flutamide treatment during this period prevents prostate formation, leading instead to a vaginal pouch (Welsh et al., 2007) and, when paired with castration, leads to a dramatic reduction in the number of SNB motor neurons in adulthood (Breedlove & Arnold, 1983).

Neurologic and behavioural data suggesting a putative anti-androgenic mechanism of BPA activity are also inconclusive. For example, administration of 300 µg/kg BPA has been found to prevent the T-induced synaptogenic effect in

the hippocampus and prefrontal cortex of castrated rats while reducing spine density in intact animals (Leranth et al., 2008), however, the authors acknowledge that this may be mediated by estrogenic activity. Furthermore, low doses of BPA can cause rapid spinogenesis in hippocampal slices from adult rat, an effect thought to be due to the activity of ERRγ, as this was prevented by hydroxytamoxifen, but not ICI (Tanabe et al., 2012). Furthermore, tests of an androgen-sensitive spatial learning task, the Morris water maze (MWM), have yielded inconsistent results. The oft-found male advantage was eliminated in juveniles given 100, but not 250, μg/kg postnatally, while juvenile males exposed perinatally to high (5 & 50 mg/kg), but not low (50 & 500 μg/kg) doses showed poor performance in this task (Xu et al., 2010a). This same study found that doses of 500μg/kg and higher inhibited performance of young adult males (P56). That same group also found that males given 100 μg/kg perinatally took longer to solve the MWM (Xu et al., 2007), however, while the former study suggests that these effects are not due to locomotion (as determined by swim speed) the latter study has no swim velocity data. However, the study I have shown here (Jones & Watson, 2012) agrees with Nakamura et al. (2012) that there are no BPA-mediated changes in performance in the MWM. Regardless, complete masculinization of this behaviour is not dependent solely on the AR, as estrogens have been shown to play a role as well (Jones & Watson, 2005; Isgor & Sengelaub, 1998). Indeed, Xu et al. (2010a) found that adult mice exposed perinatally to BPA exhibited decreased ERβ expression in whole HPC, an important finding given that the activity of this receptor has a mnemonic effect in

male rats (Osborne et al., 2009). Xu et al. (2010b) further noted that this estrogenic effect might be mediated through altered expression of the NMDA receptor, though this mediation is more likely to be dependent on the activity of GPR30 (Liu et al., 2012) or ERα as opposed to ERβ (Morissette et al., 2008).

The data here and in the literature are not consistent with a strictly androgenic mechanism of action following BPA exposure in adulthood either. Hamson et al. (2009) found that 28 days following castration, motor neurons in the SNB & DLN had decreased in size, but those in the RDLN were unaffected. In agreement with this, Osborne and colleagues (2007) found that the monomorphic motor neurons innervating the quadriceps muscle did not exhibit changes in soma size following castration or with or without T replacement as compared to controls. This suggests that the effects of BPA on somatic morphology are likely due to some other mechanism. For example, thyroidectomy decreases the soma size of motor neurons innervating the female soleus (lower calf) muscles (Bakels et al., 1998) while decreased maternal thyroid hormones in the uterine environment leads to smaller facial (masseter) motor neuron sizes prior to puberty (Ganji & Behzadi, 2007). Dexamethasone, the corticosteroid agonist, has also been found to reduce soma sizes in motor neurons (presumably the RDLN) in L4 & L5 following transection of the tibial nerve (Prodanov et al., 1998); and while this study was done in the neonatal rat, other research has shown that GR is expressed in the intact adult rat motor neuron both before and after spinal cord damage (Yan et al., 1999). However,

basal levels of corticosteroid in adult rats have not been found to alter SNB somatic morphology (Niel et al., 2011). Finally, ERRγ expression has been found in the motor neurons of the cranial nerves of mouse (Lorke et al., 2000), though in the spinal cord, restriction appears to be limited to gamma motor neurons, with little, if any, expression in alpha motor neurons (Friese et al., 2009) and are presumably absent in the Onuf's nucleus – the primate SNB homologue – of the macaque monkey (Yamamoto et al., 2011). Finally, ample evidence has accumulated to suggest that the reduction in motor neuron soma size is not likely to be due to estrogen receptors (Breedlove & Arnold, 1990; Verhovshek et al., 2010). These lines of evidence suggest that non-androgenic pathways possibly mediate the neuromuscular effects of BPA following adult exposure.

Taken together, the evidence suggests that BPA does not act as an anti-androgen *in vivo*, in stark contrast to the effects seen *in vitro*. The exact reasons for this are unknown and will require further experimentation to elucidate, but may well be due to a number of different factors. For example, the development of the SNB requires androgens, and AR are not heavily expressed in that system until P10 (Jordan et al., 1997), and removal of the BC/LA muscle itself prevents androgen-dependent survival (Kurz et al., 1992), indicating that the muscle is the site of androgenic activity in the survival of the SNB, and ERRγ expression is seen in skeletal muscle both postnatally and in adulthood (Heard et al., 2000). It is possible that the interaction of these systems may explain the different effects seen during development exposure patterns here and even offer some hint as to

the lack of *in vivo* androgenic activity seen in the literature thus far. Any putative suggestion for a mechanism of action is hindered, however, by the gaps in our knowledge with respect to the endogenous ligand for ERRγ and how BPA may affect its activity.

As such, the evidence to date suggests that BPA cannot be considered an anti-androgen *in vivo* in mammalian species. However, its ability to alter the neuromuscular physiology of reproductive systems in rats at environmentally relevant doses as well as the documented association with decreased sperm motility and HPG activity in humans is cause for concern.

Chapter 6. Conclusion

This research clearly indicates that bisphenol A alters certain neuroendocrine and behavioural parameters following exposure both perinatally and in adulthood. Combined with the rapidly growing literature as reviewed here, it seems entirely plausible that BPA poses a risk to human health and may alter the organization of behaviours along typical masculine and feminine lines.

I have found that perinatal BPA exposure is capable of reducing sexual performance in males, and that continued exposure and access to proceptive, receptive females does not remove this inhibition. I have also noted that BPA is capable of demasculinizing emotionality, particularly anxiety and depressive-like behaviours. Furthermore, neuroendocrine development is also affected, as the expression of ERα is reduced in the medial amygdala, which may partially explain the deficits in sexual performance seen in males; I also saw that ERα expression in the MPN is associated with sexual behaviour. Finally, I did not notice any anti-androgenic effects of BPA in the SNB or DLN of males following perinatal exposure, but the size of the motor neurons in these groups as well as in the monomorphic RDLN were reduced at virtually all doses following chronic exposure in adulthood. This may be due to effects at AR, but may also be consistent with effects on other steroid systems as well, such as thyroid and

glucocorticoid receptors or even possibly ERRγ. Given these findings and those in the literature to date, there seems to be little room for misgivings about the safety of this product, even if the effects of this compound are indeed subtle.

Unfortunately, it should be noted that the issue of physiological and/or psychological perturbations from BPA exposure is not simply a matter of public health and safety, but also has considerable economic ramifications thus making it a political issue as well. As this compound is cheap to synthesize and is used in the production of a large number of items, the potential regulation or elimination of this compound could prove costly to those industries in which BPA is used. Companies, as corporate citizens, are also allowed to lobby governments and make contributions to political parties and individual candidates within the regulations pertaining to specific jurisdictions. Given the low cost of BPA and the costs associated with the research and development that would be required to discover and produce a suitable alternative and retrofit production to accommodate such a change, it is in the interest of industry to maintain the status quo in this regard, if possible. I do not wish to assert that industries would deliberately allow humans to be exposed to a known injurious substance for the sake of a healthier quarterly financial report – though little time would be required for even the most uninformed of individuals to unearth documented evidence of such corporate malfeasance – but we should be aware that such conflict does in fact exist. Indeed, vom Saal (2008) – in his comments to the NTP report from that same year – has already noted that none of the 13 studies funded by chemical

corporations had found any effect of BPA, in contrast with the 152 government-funded studies examined there, of which 138 noted a change in some parameter. Some of these discrepancies, and even some of the negative findings from the publicly funded studies, may be reducible to the strain of rat used. As previously noted (vom Saal & Hughes, 2005), one particular Sprague-Dawley (SD) strain from Charles River Laboratories (CD-SD) is relatively insensitive to exogenous estrogens, and in the vom Saal analysis, none of the studies using this strain have noted any differences, including 10 conducted by government-funded laboratories, though this strain is over-represented in studies from industrial laboratories. Regardless, there are a number of issues that plague not just the research, but also interpretations of research and thus, any policy decision that may or may not be made with regard to this chemical.

However, the financial – not to mention the personal – burden that we may incur as a result of continued widespread exposure to BPA is potentially staggering. This has been a particularly pressing issue for a number of reasons particularly due to the fact that both prostate and breast cancer cases have been rising consistently since the 1970s to over 25,000 cases of prostate cancer and over 23,000 cases of breast cancer in Canada in 2011 (Canadian Cancer Society, 2011) compared to 223,307 and 202,964 cases of prostate and breast cancer, respectively, in the US in 1997 (Centers for Disease Control, 2012a & b). It was estimated that in 2011, 29.6% of all deaths in Canada were from cancer, 487 Canadians were diagnosed with some form of cancer daily, and 205 people

died of cancer every day (Canadian Cancer Society, 2011) while 1 in 3 Americans will eventually be diagnosed with some form of cancer, costing $226.8 billion annually in direct and indirect costs (American Cancer Society, 2012).

One of the newest concerns regarding our widespread exposure to BPA has to do with the epidemic of obesity that exists in North America, a problem that is becoming increasingly common around the globe. Between 2007-2009, according to a joint report by the Canadian Health Measures Survey (CHMS) and the National Health and Nutrition Examination Survey (NHANES), 24.1 of Canadians and 34.4% of Americans were obese (Shields et al., 2011). According to Statistics Canada (Shields et al., 2011), 15% of obese Canadians had diabetes, compared to only 5% of non-obese citizens, while obesity is also a known risk factor for strokes, heart disease, reproductive problems including, infertility, erectile dysfunction, depression, osteoarthritis and cancer (Haslam & James, 2005) and intrauterine fetal death (Arendas et al., 2008), amongst other problems. The economic cost of obesity alone is $4.3 billion dollars in Canada (Public Health Agency of Canada, 2011) and $147 billion dollars in the U.S. (CDC, 2012c), values which most certainly underestimate the true cost of this epidemic, particularly as these estimates are from those individuals considered obese, not just overweight.

While BPA may play a small, but epidemiologically significant, role in these issues, we must also consider that this compound, while the most ubiquitous in modern society, is but one of several thousand chemicals still not

tested for safety. In fact, there may be more than 80000 chemicals currently in use that have never been tested for safety (NTP, 2005). Many of these compounds are endocrine disruptors, including heavy metals such as cadmium (Stoica et al., 2000), the phthalates and phytoestrogens, such as genistein and and daidzein. Of particular concern is that there may be additive or even synergistic effects of combinations of these compounds. Clearly, given the number of chemicals in regular use, a full examination of these interactions is simply not possible, but testing the effects of the most common disruptors in combination should be done. To date, there is sparse research in this area, however, one such study found that BPA and genistein could dramatically alter rat embryonic CNS development more than either compound alone (Xing et al., 2010).

Human bio-monitoring studies have determined that the average person in the U.S. and Canada has detectable levels of BPA in their bodies, estimated to be anywhere from 0.5ng/ml serum to 4 ng/ml serum (Calafat et al., 2008; Volkel et al., 2002; Bushnik et al., 2010; Vandenberg et al., 2007 & 2010); as such, it has been thought that human exposure was in the range of <1 – 7 μg/kg. Recent work has debunked these estimates (Vandenberg et al., 2010) and may be anywhere from 400 μg/kg or higher (Taylor et al., 2011). The studies here have shown that perinatal BPA exposure at doses of 5, 50 and 500 μg/kg are able to perturb behaviour in adulthood and disrupt hormone-sensitive physiological processes in adulthood. As such, the recent decision by the Food and Drug

Administration in the U.S. to not regulate the use of BPA in food tins is concerning. Furthermore, government should require industry to fully test compounds for safety before allowing their use for industrial purposes or making it commercially available.

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Appendices

Appendix A. Tables

Table 1. Sex Differences in the EPM

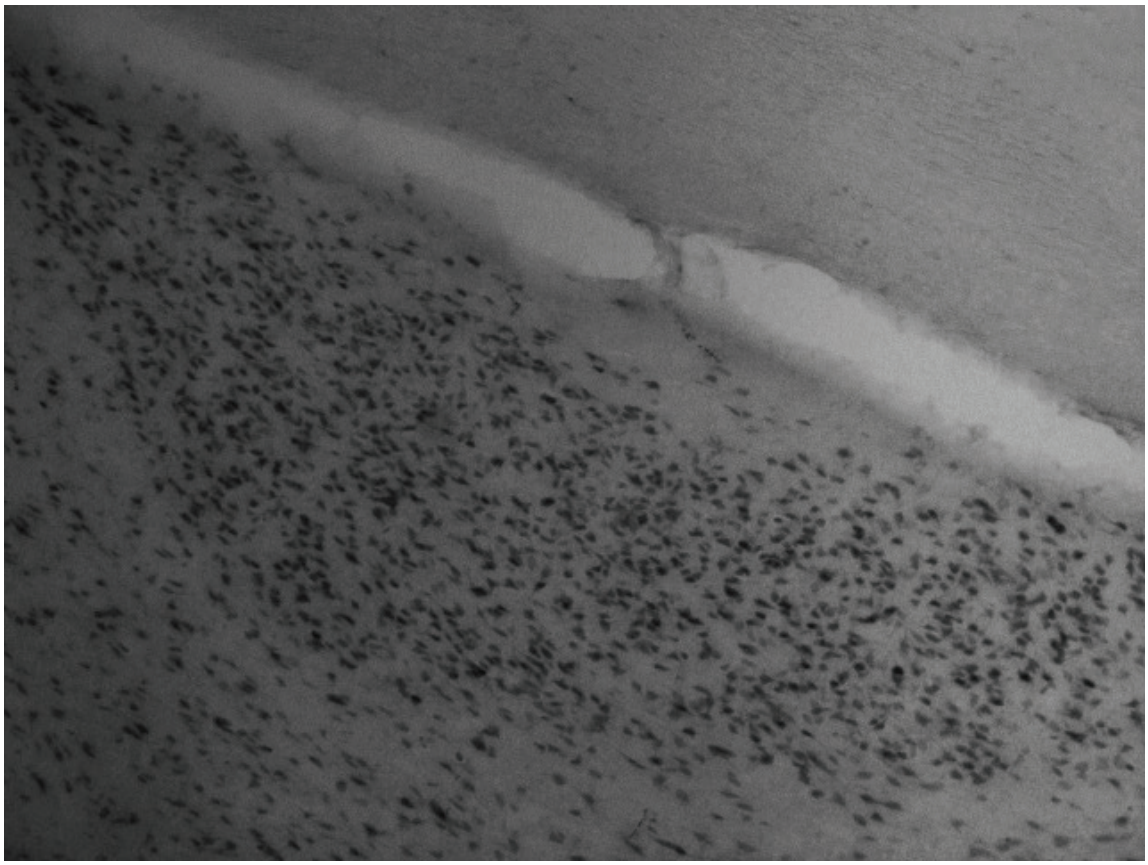
		Control		5ug		50ug		500ug		5000ug	
		XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Distance	Mean ±SEM	1.05 ±0.11	1.41 ±0.11	1.45 ±0.1	1.39 ±0.09	1.17 ±0.09	1.5 ±0.08	1.12 ±0.07	1.46 ±0.14	1.26 ±0.12	1.48 ±0.08
	p	p=0.27		N/S		p=0.012		p=0.028		N/S	
Time Mobile	Mean ±SEM	113.06 ±13.9	160.37 ±12.57	149.76 ±14.6	164.04 ±7.74	128.73 ±12.35	184.8 ±10.8	115.28 ±9.2	161.69 ±13.7	112.41 ±11.31	167.5 ±8.8
	p	p=0.19		N/S		p=0.002		p=0.01		p=0.001	
#OAE	Mean ±SEM	3.58 ±0.69	5.92 ±0.82	5.08 ±0.89	7.08 ±0.48	4.58 ±1.1	7.5 ±0.78	4.67 ±0.93	6.14 ±0.99	5.00 ±0.71	6.92 ±0.75
	p	p=0.041		N/S		p=0.042		N/S		N/S	
%CAE	Mean ±SEM	51.47 ±2.61	42.48 ±1.62	47.64 ±4.68	39.69 ±2.55	48.09 ±4.82	38.08 ±2.13	46.79 ±4.05	42.06 ±2.39	45.04 ±3.2	43.43 ±0.91
	p	p=0.008		N/S		N/S		N/S		N/S	
#Hub	Mean ±SEM	3.5 ±0.68	6.5 ±0.63	5.00 ±0.79	5.00 ±0.51	4.25 ±0.8	8.33 0.77	2.67 ±0.36	5.57 ±0.92	3.67 ±0.92	5.17 ±0.6
	p	p=0.004		N/S		p=0.001		0.003		N/S	
Boli	Mean ±SEM	3.417 ±0.84	0.25 ±0.18	3.455 ±0.87	0.333 ±0.22	2.75 ±0.54	0.58 ±0.34	5.25 ±0.84	2.57 ±1.04	3.5 ±0.7	0.67 ±0.17
	p	p=0.001		p=0.002		p=0.002		N/S		p=0.001	

Table 2. Sex Differences in the Forced Swim Test

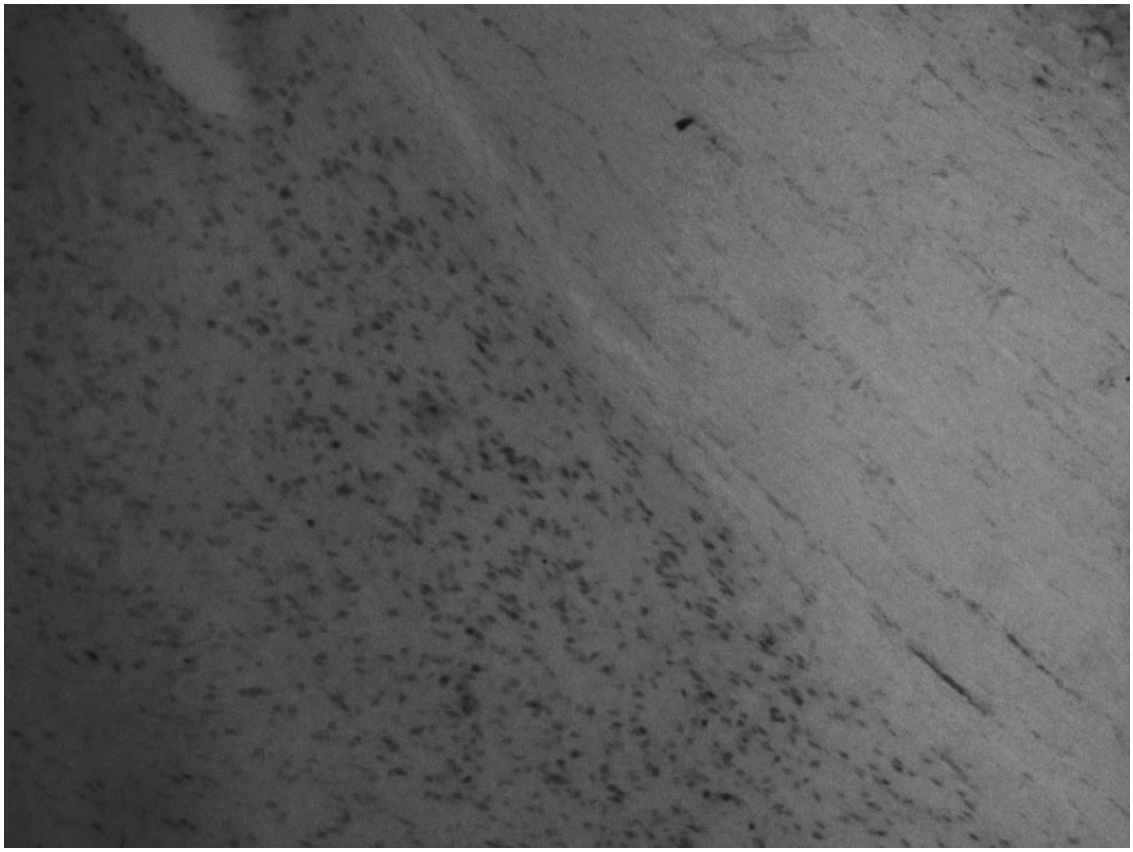
		Control		5		50		500		5000	
		XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Time Immobile	Mean ±SEM	173.37 ±22.53	66.79 ±9.33	137.93 ±18.2	44.3 ±5.91	146.84 ±16.58	70.97 ±13.13	147.7 ±17.58	67.44 ±15.81	109.73 ±12.72	42.2009.15
	p-value	0.001		0.001		0.002		0.007		0.001	
Rotations	Mean ±SEM	8 ±1.34	12.75 ±0.98	11.3 ±1.78	15.33 ±1.32	9.5 ±1.08	12.92 ±1.75	8.92 ±1.74	11.71 ±2.25	9.08 ±0.85	17.25 ±1.15
	p-value	0.009		N/S		0.001		0.008		0.001	
Latency Immobile	Mean ±SEM	8.87 ±3.59	35.4 ±10.29	7.47 ±2.81	28.33 ±9.91	4.68 ±1.77	34 ±7.81	10.41 ±5.19	41.49 10.32	8.68 ±2.55	49.58 ±17.03
	p-value	0.023		N/S		N/S		N/S		0.027	
Immobile Episodes	Mean ±SEM	19.92 ±2.35	14.67 ±1.6	19.42 ±1.9	12.5 ±1.58	22.67 ±2.07	17.17 ±2.32	18.67 ±1.49	15.86 ±2.6	18.08 ±1.47	10.17 ±1.71
	p-value	N/S		0.01		N/S		N/S		0.002	
Mobile Episodes	Mean ±SEM	20 ±2.39	15.5 ±1.66	19.92 ±1.87	13.42 ±1.58	23.08 ±2.1	18 ±2.32	19 ±1.49	16.71 ±2.67	18.58 ±1.44	11.08 ±1.69
	p-value	N/S		0.014		N/S		N/S		0.003	

Appendix B. Photomicrographs

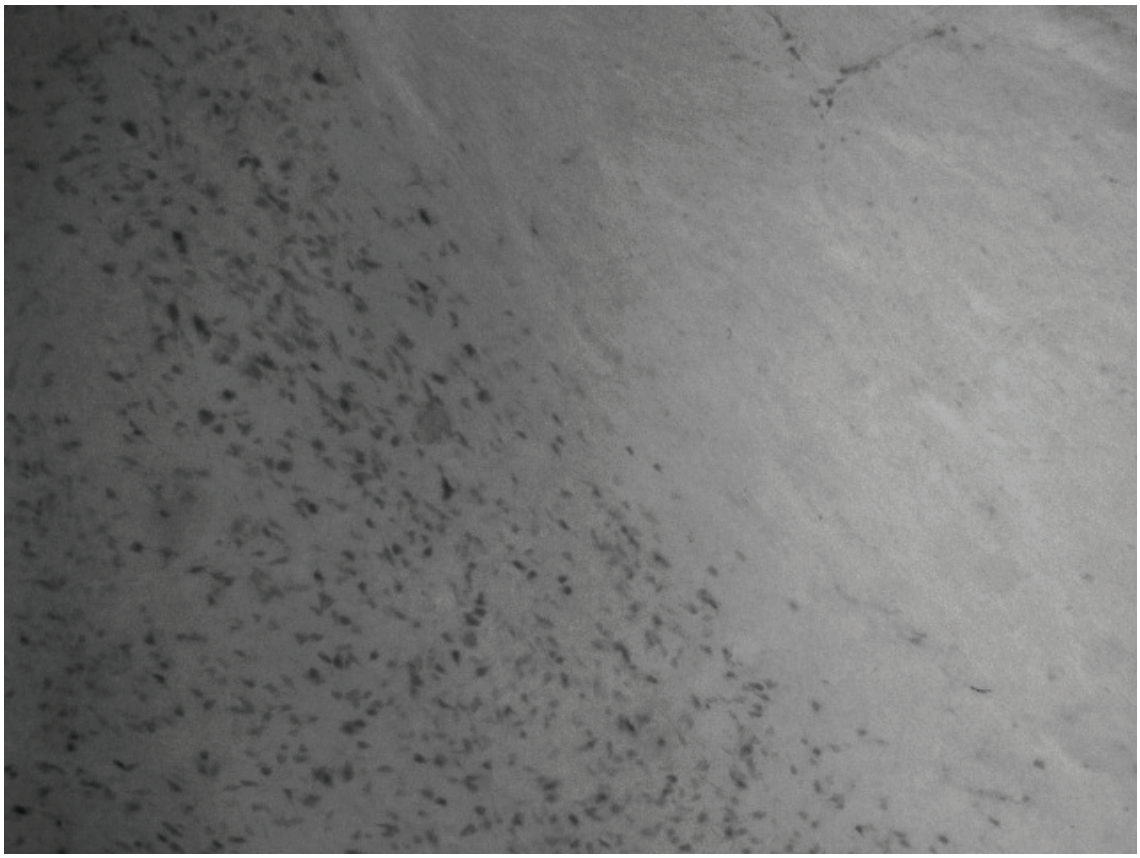
Picture 1: ERα in the MeApd of representative control animal



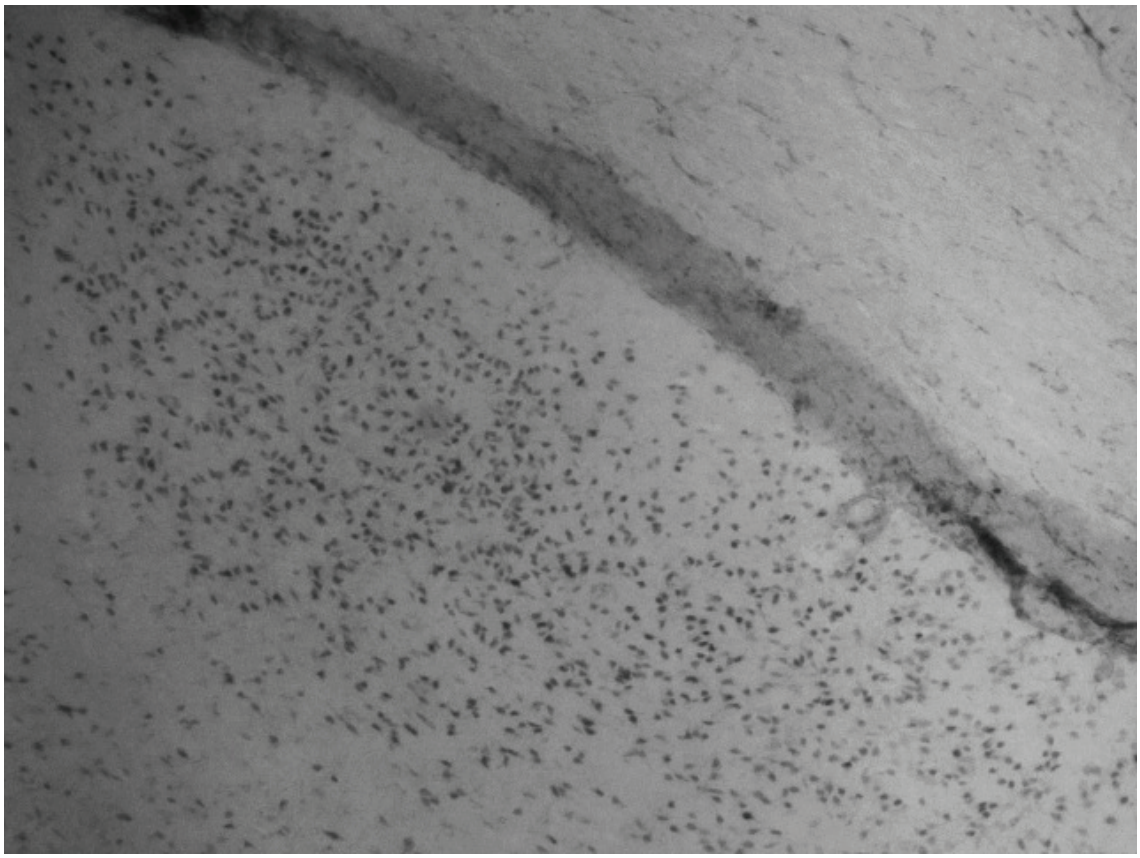
Picture 2: ERα in the MeApd of representative animal in 5 µg/kg group



Picture 3: ERα in the MeApd of representative animal in 50 µg/kg group



Picture 4: ERα in the MeApd of representative animal in 500 µg/kg group



Picture 5: ERα in the MeApd of representative animal in 5000 µg/kg group