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Effects of Prenatal Bisphenol A Exposure on Adrenal Gland Development and Steroidogenic Function

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Abstract

Developmental exposure to bisphenol A (BPA), a ubiquitous endocrine disrupting chemical, is associated with organ dysfunction and diseases in adulthood. However, little is known about its effects on the adrenal glands. Therefore, this thesis addresses this important question using both *in vivo* and *in vitro* approaches. BPA at environmentally relevant doses was administrated via diet to pregnant mice from embryonic day 7.5 to birth, following which mice were switched to a standard chow. At two months postnatally, adrenal glands and blood samples were collected from adult mouse offspring for structural and functional analysis. I found that: ***(a)*** BPA increased adrenal gland weight as well as plasma corticosterone levels; ***(b)*** BPA did not alter plasma levels of ACTH; and **(*c*)** BPA stimulated expression of the two key steroidogenic factors, steroidogenic acute regulatory protein (StAR) and cyp11A1 in female but not male offspring. To determine the molecular mechanisms underlying the BPA-induced StAR expression, I used human fetal adrenal cortical H295A cells as an *in vitro* model system, and showed that BPA increased StAR protein expression likely through an estrogen receptor (ER)-mediated mechanism independent of StAR gene transcription, translation and protein half-life. I then investigated the molecular mechanisms underlying the BPA-induced increase in adrenal gland weight using the same *in vitro* model system. I demonstrated that ***(a)*** BPA increased cell number and protein levels of the three universal markers of proliferation (proliferating cell nuclear antigen (PCNA), cyclin D1 and D2, as well as sonic hedgehog (shh) and its key transcriptional regulator Gli1; ***(b)*** cyclopamine, a shh pathway inhibitor, blocked these stimulatory effects of BPA on cell proliferation; ***(c)*** BPA increased the nuclear translocation of ERβ; and ***(d)*** the ERβ-specific agonist DPN mimicked while the ERβ antagonist PHTPP abrogated the stimulatory effects of BPA on cell proliferation, and prevented BPA-induced activation of the shh signaling. Taken together, these findings demonstrate that developmental exposure to BPA adversely affects adrenal gland development and steroidogenic function in adult mouse offspring. Furthermore, they reveal novel molecular signaling mechanisms of BPA actions in regulating adrenal steroidogenic function and adrenal cortical cell proliferation.

Keywords

Bisphenol A, endocrine disruptor, adrenal gland, steroidogenesis, steroidogenic acute regulatory protein, fetal development, estrogen receptor, sonic hedgehog

Co-Authorship Statement

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SM, GH, and KY designed the experiments. SM was responsible for animal care, blood and tissue collection and conducted the experiments. SM, GH and KY analyzed and interpreted the data. SM and KY wrote the manuscript. All authors approved the final version of the manuscript.

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List of Abbreviations

ACA	Adrenal adenomas
ACAT	Acyl-coenzyme-A-cholesterol-acyl-transferase
ACC	Adrenal cortical carcinomas
ACT	Adrenal cortical tumors
ACTH	Adrenocorticotrophic hormone
AKAP	A-kinase anchoring protein
AKT	Protein kinase B
AR	Androgen receptor
BPA	Bisphenol A
BPA-G	Bisphenol A glucuronide
BPA-S	Bisphenol A sulfate
BPAF	Bisphenol AF
BPF	Bisphenol F
BPS	Bisphenol S
Bw	Body weight
C/EBP	CCAAT-enhancer binding protein
CBG	Corticosteroid binding globulin
CD	Cushing's disease
CDK	Cyclin dependent kinase
CHX	Cycloheximide
CRH	Corticotrophin releasing hormone
CRHR1	Corticotrophin releasing hormone receptor 1
CS	Cushing's syndrome
Cyc	Cyclopamine
Cyp	Cytochrome P450
DAX-1	dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome factor 1
DHEA	Dehydroepiandrosterone
Dhh	Desert hedgehog
DOHaD	Developmental origins of health and disease

DPN	2,3-bis(4-Hydroxyphenyl)-propionitrile
E	Embryonic day
EDC	Endocrine disrupting chemical
ELISA	enzyme-linked immunosorbent assays
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular regulated kinase
ERR	Estrogen related receptor
EFSA	European Food and Safety Authority
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
Foxl2	Forkhead box L2
GPR30	G-protein coupled estrogen receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GS-MS	Gas chromatography mass spectrometry
HDL	High density lipase
Hh	Hedgehog
Hhip	Hedgehog interacting protein
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC-MS	High performance liquid chromatography–mass spectrometry
HSD	Hydroxysteroid dehydrogenase
HSL	Hormone sensitive lipase
IC	Inhibitory concentration
ICI	ICI 182, 780
IGF	Insulin growth factor
Ihh	Indian hedgehog
IMM	Inner mitochondria membrane
IMS	Intra-mitochondrial space

iNOS	Inducible nitric oxidative synthase
LAL	Lysosomal acid lipase
LDL	Low density lipoprotein
LOAEL	Lowest observable adverse effect level
MAPK	Mitogen-activated protein kinase
MC2R	Melanocortin 2 receptor
MEK	Mitochondrial kinases
miRNA	micro RNA
MLN64	Metastatic lymph node 64
MRP	Multidrug resistance associated protein
NMDRC	Non-monotonic dose response curves
NOAEL	No observed adverse effect level
OMM	Otter mitochondria membrane
PBR	Peripheral benzodiazepine receptor
PCNA	Proliferating cell nuclear antigen
PHTPP	4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol
PI3K	Phosphatidyl-inositol-3-kinase
POMC	Proopiomelanocortin
Ptch	Patched
PND	Postnatal day
qRT-PCR	Real time quantitative polymerase chain reaction
SF-1	Steroidogenic factor 1
Shh	Sonic hedgehog
SMO	Smoothened
SOR	StAR overload response
Sp1	Specificity protein 1
SRB1	Scavenger receptor B type 1
SREBP	Sterol regulatory element binding protein
StAR	Steroidogenic acute regulatory protein
Sufu	Suppressor-of-fused protein
SULT	Sulfotransferase

TDI	Tolerable daily intake
Tis11b	TPA-induced sequence 11b
TSPO	mitochondrial transport protein
UGT	UDP-glucuronosyltransferases
WNT	wingless-related mouse mammary tumor virus integration site
WT-1	Wilm's tumor 1
YY1	Ying yang 1
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticularis
zU	Undifferentiated cell zone

1 INTRODUCTION

1.1 Bisphenol A

Bisphenol A (BPA) is a widely used endocrine disrupting chemical (EDC) that has become a source of major health concerns [1-3]. With an estimated production of six billion tons per year, BPA ranks as one of the most highly produced chemicals [4]. Initially, it was synthesized by Dr. A.P. Dianin in 1891 for use as a synthetic estrogen, however, the discovery of more potent estrogenic compounds resulted in its discontinuation [2]. During the 1940s and 50s, BPA was identified as a potentially important component of plastics and began to be utilized in the manufacture of polymers, polyvinyl chloride plastics, and flame retardant tetrabromobisphenol-A [1-4]. Currently, it is commonly found in polycarbonate plastics, such as plastic containers, baby bottles, plastic water bottles, etc.; and in epoxy resins, which are used as an internal coating on food and beverage cans [5]. Additionally, BPA is present in medical and dental equipment, thermal paper, and cardboard and has been detected in soil, water and air samples [2-4,6-8]. The BPA used in packaging contains an ester bond linking BPA monomers onto polymers, making it susceptible to hydrolysis, thus allowing migration of BPA from the packaging into the contents [8]. BPA has been demonstrated to leach out of plastic containers and liners of cans into the food or beverage products, a process that is enhanced in acidic or high temperature environments [2-4,6-8]. The most common route of exposure to BPA is through ingestion, but exposure through dermal routes and inhalation is also possible [5]. BPA is ubiquitous in the environment and is detectable in the urine of 91% of Canadians [9].

Several human epidemiological studies have demonstrated an association between exposure to BPA and adverse health outcomes that include infertility, reproductive complications, childhood obesity, childhood asthma, and altered neurological development in children and adults [10-12]. Furthermore, animal studies have shown that developmental exposure to BPA results in a wide range of adverse health effects including reproductive, cardiovascular, immunological, metabolic, behavioral, and neurological disorders, as well as certain cancers in adult offspring, and that many of these effects are sex specific [2,6,13].

1.1.1 Mechanism of Action

BPA is considered an environmental estrogen and an EDC [2-4,6,7] with the ability to bind to both α and β estrogen receptor subtypes (ER), androgen receptors (AR), G-protein coupled estrogen receptors (GPER), estrogen related receptors γ (ERRγ) and glucocorticoid receptors (GR) [5,7]. Recent evidence also indicates that BPA exposure results in adverse effects through pregnane X receptors [14], peroxisome proliferator-activated receptors [15], thyroid hormone signaling [5,7,15], NF-κB signaling [16], ion signaling [17], and induces pro-inflammatory cytokines and chemokines [18,19]. Additionally, prenatal BPA exposure, even at low doses, has been shown to cause epigenetic alterations, including DNA methylation and histone modifications [20]. BPA's effects on these receptors and pathways are based on (1) the presence of the receptors, (2) the level of expression of these receptors, (3) the dose of BPA, and (4) the level of endogenous hormones that compete with BPA for binding to these receptors.

1.1.1.1 Estrogen Receptor

Of particular interest are the actions of BPA through the ERs, due to the structural similarities of BPA to estrogen and its previous use as a synthetic estrogen (**Figure 1.1**) [15]. There are two distinct ER subtypes, ERα and ERβ, which are specific to cell type, with ERα primarily expressed in reproductive and insulin-sensitive tissues [15]. Upon estrogen binding to the ER, a conformational change occurs and ER translocates to the nucleus of the cell. In the nucleus, ER can either bind ***(1)*** directly to estrogen response elements (ERE) on promoters of ER target genes to induce gene expression or ***(2)*** to transcriptional coactivators, such as Sp1 and Ap1 to induce gene expression [15,21].

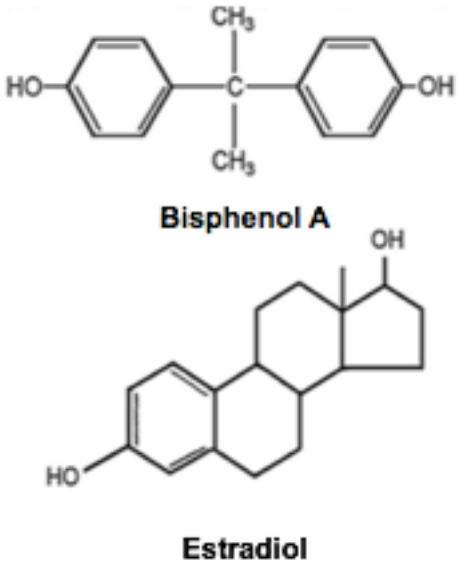

Figure 1.1: Chemical structure of BPA and estradiol.[2]

BPA acts as an agonist for ERβ and has dual agonist or antagonist actions for ERα and has a higher binding affinity to ERβ than to ERα [22-24]. Thus, the effects of BPA on ER are largely dependent on the cell type and ER subtype present [2]. However, BPA has been classified as a weak estrogen based on its low binding affinity for ER compared to naturally-occurring 17β-estradiol (~10,000-fold lower) [2]. This leads to the claim that BPA will not have a large impact on ER in the presence of endogenous estrogens. However, additive effects of BPA and estradiol together have been demonstrated [25]. Additionally, the effects of BPA may be explained by the "spare receptor" hypothesis, which states how a maximal response may be achieved by low concentrations of a hormone (or EDC), before occupancy of receptors are saturated [22]. In addition, BPA has been shown to have non-genomic actions that are distinct from its actions on classical ERs [22,26]. ER localized to the plasma membrane is known to activate a variety of pathways depending on receptor and cell type, including the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK), p38/MAPK, and phosphatidyl-inositol-3-kinase/protein kinase B (PI3K/AKT) pathways [15]. Moreover, BPA has been demonstrated to activate these non-genomic pathways by acting through membrane ER [15].

1.1.1.2 GPER

GPER (also known as GPR30) is a G-protein coupled intracellular membrane receptor that is activated by estrogen and responsible for rapid estrogen signaling [27]. BPA has been shown to be a strong agonist of the GPER, having non-genomic effects similar to estrogen [5,28]. BPA has a half maximal inhibitory concentration (IC50) of 630 nM for the GPER, which is 8-50× higher than for classic ERs [28]. Additionally, BPA can displace over 50% of the [^{3}H] E_2 binding to the plasma membrane of cells transfected with GPER, demonstrating BPA's ability to interfere with estrogen signaling [28].

1.1.1.3 Androgen Receptor

Since BPA also affects the male reproductive system, the results of BPA binding to the androgen receptor have been investigated. BPA acts as a moderate antagonist of the AR in numerous cell types [5,7,29,30]. BPA has an inhibitory concentration (IC50) value of 3.9×10^4 nM for the AR in MA-10 cells [29]. Additonally, BPA has been shown to

competitively bind to the AR and reduce AR nuclear translocation, and therefore its activity [30].

1.1.1.4 Estrogen Related Receptor γ

ERRγ are a subfamily of orphan receptors similar to ERs that can bind to estrogen related response elements as well as EREs [15]. BPA has been shown to be a potent ERRγ agonist [5], with an IC50 value of 13.1 nM, similar to that of the well-known strong ERRγ agonist 4-hydroxytamoxifen [31]. Thus, BPA may adversely affect signaling pathways through ERRγ binding [15]. Moreover, there is potential for interaction or interference between ERRs and ERs during both the developmental period and adulthood [15].

1.1.1.5 Glucocorticoid Receptor

Since BPA has been demonstrated to act as both a GR agonist and antagonist [5,29,32,33], the actions of BPA on the GR may be tissue and dose specific. The affinity of BPA to the GR is relatively low, with an IC50 value of 6.7×10^4 nM in MA-10 cells and has antagonistic activity toward the GR [29]. In the adipose cell line 3T3-L1, BPA displayed agonistic activity through the GR, which was also demonstrated in an *in silico* study [32,33]. Additional evidence from our lab has demonstrated that BPA interferes with the glucocorticoid signaling pathway, resulting in inhibition of glucocorticoid target genes in A549 lung cells [16].

1.1.1.6 Epigentic Modifications

Recent evidence has emerged, supporting a potential for environmental chemicals to alter the epigenome during the period of embryogenesis. BPA has been demonstrated to induce numerous epigenetic alterations in rodent studies, including DNA methylation and histone modifications [5,34,35]. For example, DNA methylation alterations have been observed to result in a shift in coat color after prenatal BPA exposure in viable yellow agouti mice [34]. Moreover, oocyte maturation in porcine subjects was disrupted by BPA as a result of DNA methylation and histone modifications [35].

1.1.2 Dose Response and Low Dose Effects

Regulatory agencies typically establish the lowest observable adverse effect level (LOAEL) and/or no observed adverse effect level (NOAEL) for chemicals such as BPA

[36]. This enables the calculation of a reference dose that is considered safe for human exposure, based on the theory that "the dose makes the poison," which stipulates that high doses of a chemical result in a larger physiological effect or in an increase in potential adverse effects [36]. This theory is challenged by the testing of EDCs [36]. In contrast to traditional monotonic response curves, many EDCs including BPA, have non-monotonic dose response curves (NMDRCs). Traditional monotonic response curves can be either linear or non-linear, but the slope of the curve always remains in the same direction [36]. NMDRCs are U-shaped or inverted U-shaped, such that the direction or slope of the curve changes over the range of doses being examined [36]. Thus, outcomes of low-dose exposure to BPA cannot be inferred from results of higher dose exposures [37], making the traditional development method of LOAEL and NOAEL for BPA problematic. Since all doses of BPA tested had adverse effects, negating the possibility of determining a NOAEL value, the reference dose for human exposure of BPA was calculated using the LOAEL [36]. Thus, the widespread use of BPA and consequent ubiquitous exposure to BPA even at very low doses is of concern.

1.1.3 Pharmacokinetics

The metabolism of BPA in humans, rodents, and primates is similar and is thought to occur through comparable mechanisms [38]. Pharmacokinetic studies of BPA indicate that large amounts of BPA are subject to first pass metabolism in the liver, where it is conjugated into either the main metabolite, BPA-glucuronide (BPA-G), by uridine 5′-diphospho-glucuronosyltransferase (UGT) or BPA-sulfate (BPA-S) by sulfotransferase (SULT). Among UGTs, UGT2B15 shows the highest activity for conjugating BPA into BPA-G in human liver microsomes [39]. In both rodents and humans, glucuronidation and sulfation produce inactive hydrophilic BPA metabolites that is excreted in the feces [38,40]. However, unconjugated BPA is cleared by the kidneys and eliminated in the urine of primates, but is excreted primarily in the feces of rodents [38]. This difference in route of elimination raises the question of potential differences in metabolism and circulating levels of BPA. Taylor, et al. [38] demonstrated that oral administration of BPA results in similar levels of unconjugated BPA in the circulation and identical rates of clearance in non-human primates and rodents. However, in pregnant rhesus monkeys, the systemic bioavailability or the percent of unconjugated BPA that reaches the systemic circulation

of BPA is considerably lower; only 0.48% [41]. Rapid conjugation of BPA was demonstrated when pregnant rhesus monkeys were injected with 100 μg/kg bodyweight (bw) of unconjugated BPA, and at the five-minute initial sampling point, 87% of the BPA detected was in the conjugated form. The presence of unconjugated BPA in the blood demonstrates either ***(1)*** incomplete first pass metabolism, ***(2)*** a bypass of first pass metabolism or ***(3)*** the potential for deconjugation of BPA metabolites into unconjugated BPA [4]. Indeed, evidence suggests that in the intestine and colon, conjugated BPA can be deconjugated back into active BPA in rodents [4]. Clearly, additional investigation is required to determine the pharmacokinetics of BPA and the potential differences in experimental animal models versus human exposure to comprehend the risks of BPA exposure.

During pregnancy, the placenta plays a critical role in controlling potential toxins that can reach the fetal circulation (**Figure 1.2**). Detectable levels of BPA were present in the placentae of rhesus monkeys given a single injection of 100 μg/kg bw BPA [41] and of that, 29% was unconjugated BPA [41]. Using a placental transfer model, 27% of unconjugated BPA was shown to cross from the maternal circulation of the placenta to the fetal circulation, indicating that unconjugated BPA can freely cross the placenta [42]. Moreover, this model demonstrated that the placenta plays no role in conjugating active BPA into BPA-G, since BPA-G was detected in neither the maternal nor the fetal placental circulation [42]. In addition to passive diffusion of BPA, there are active placenta transport proteins such as organic anion transporting polypeptide (Oatp) and multidrug resistance associated protein (Mrp) transporter family members that aid in the transport of conjugated BPA [40,43,44]. BPA-G has been shown to cross into the placenta via the Oatp4a1 transport and then into fetal circulation by Mrp1 transporter [40,44]. Thus, BPA has the potential to reach fetal circulation in both conjugated and active forms.

The fetal liver is reported to have a 36-fold lower level of UGT2B15 than the adult liver, suggesting that the fetus has a decreased potential to metabolize BPA [40,45,46]. While BPA-G is considered to be inactive, β-glucuronidase in the fetal liver can deconjugate BPA into the active form [40,47]. Thus, the risk of BPA to the fetus is compounded by its ability to de-conjugate BPA-G, as well as by its undeveloped drug detoxifying system [40,46]. Additionally, since BPA-G is water soluble and cannot cross back through the placenta

into maternal circulation after excretion in the fetal urine, it may become trapped in the amniotic fluid where it has the potential to continually re-enter fetal circulation [47]. Thus, there is growing concern about the risk of the developing fetus to prenatal exposure to BPA.

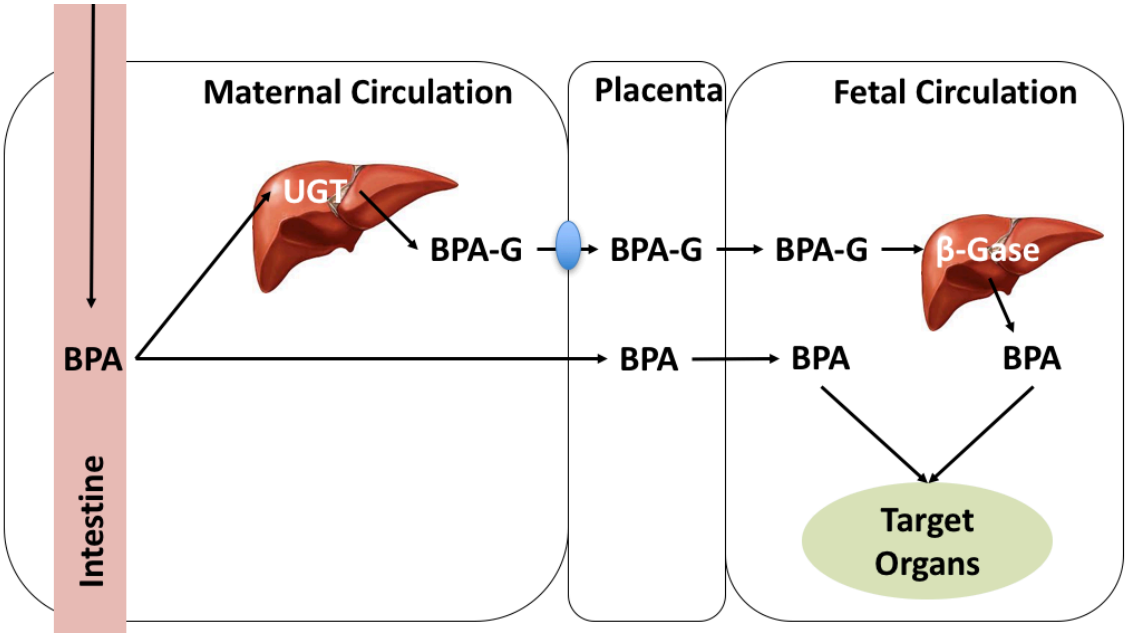

Figure 1.2: Prenatal pharmacokinetics of BPA

Absorption of BPA occurs in the maternal intestines, BPA can either (1) be metabolized by the maternal liver to BPA-G, by UGTs, and then transported across the placenta by transport proteins or (2) freely diffuse across the placenta unconjugated and reach the fetal circulation. Conjugated BPA in the fetus can be unconjugated by β-Gase by the fetal liver to unconjugated BPA. Unconjugated BPA can then reach target fetal organs and accumulate in the fetus. Abbreviations: BPA, Bisphenol A; UGT, uridine 5'-diphospho-glucuronosyltransferase; BPA-G, BPA-glucuronide; β-Gase, β-glucuronidase.

1.1.4 Human Exposure to BPA

It is estimated that 90-95% of people have detectable levels of BPA in their urine, demonstrating the universality of BPA exposure [9,15]. Ninety percent of BPA exposure is thought to result through food and drink exposure, with the remaining exposure through dust, dental products and surgery, and dermal contact [15]. Health Canada has designated the level of tolerable daily intake (TDI) of BPA to be 25 μg/kg bw per day for the average Canadian adult, and in 2010 banned the use of BPA in baby bottles and food containers [48,49]. Even with such regulations in place, estimated BPA daily intake levels in Canadian infants are reported as 0.22-0.33 μg/kg bw/day and levels in adults have been estimated at 0.052-0.081 μg/kg bw/day [50]. In the United States, the Food and Drug Administration (FDA), eliminated the use of BPA in epoxy resins of baby food containers due to marketplace demands in 2013 [49]. However, the FDA continues to state that BPA is safe for consumers at their current levels [49]. Similarly, the European Food and Safety Authority (EFSA) reported there was no health concern of dietary BPA for any age group [49], but as a precautionary measure, reduced the levels of safe BPA exposure from 50 μg/kg bw/day to 4 μg/kg bw/day, and banned the sale of baby bottles containing BPA [49]. Nonetheless, government agency regulations aimed at preventing exposure to BPA in infants and young children do not address the issue of fetal exposure to BPA during pregnancy via maternal sources, which has been shown to have permanent, long lasting effects on human health [11,12].

1.1.4.1 Developmental Origins of Health and Disease

Over thirty years ago, David Barker first considered the possibility that poor maternal malnutrition resulting in low birth weight of the fetus led to premature death due to metabolic and cardiovascular complications later in life for the offspring [51]. This concept was expanded to include early life exposure to environmental toxins that can lead to subtle changes during development that lead to dysfunction and/or diseases later in life and is now referred to as the Developmental Origins of Health and Disease (DOHaD) hypothesis [51]. Application of this hypothesis to BPA implies that prenatal exposure to BPA can have long lasting effects that span a lifetime due to alterations in gene and protein expression that occur during the critical period of organ development [51,52]. Epigenetic alterations in the fetus are also thought to play an important role in the

susceptibility to dysfunction and/or disease later in life [52]. Furthermore, many of these developmental effects can be sex-specific and are often irreversible [51]. As such, developmental exposure to environmental stressors (altered nutritional status, environmental chemical, stress, etc.) can have lasting effects on the offspring, which is not yet fully understood.

1.1.4.2 BPA during Pregnancy

Dynamic changes occur in drug metabolism and transport during pregnancy, and thus BPA metabolism may vary in pregnant versus non-pregnant populations [40,53]. During pregnancy, women have increased plasma volume and clearance as well as altered metabolism depending on the specific enzymes involved [40,53]. Consequently, there is a possibility of an increased half-life of BPA in the maternal circulation thereby increasing potential fetal exposure [47].

While the placenta is considered a protective barrier between the mother and fetus, many environmental chemicals pass through the placenta due to their high lipophilic properties [54]. Furthermore, the presence of both ERα and ERβ on the placenta make it vulnerable to estrogenic environmental contaminants such as BPA [54]. The ban of BPA from baby products (e.g. baby bottles and toys) does not reduce the risk of BPA exposure to the developing fetus [11,12]. Indeed, pregnant German women were found to have plasma levels of BPA as high as 9.2 ng/mL, and their fetuses had plasma levels of BPA averaging 12.7 ng/mL, proving that BPA crosses the placenta [55]. This finding was supported by North American and Canadian studies that determined BPA levels in maternal serum to be between 0.5-22.3 ng/mL [56], and BPA levels in urine from pregnant women to be between 0.16-43.20 ng/mL, respectively [57]. BPA has also been detected in cord blood, amniotic fluid, and breast milk [55,58-60]. Taken together, these studies show cause for concern about *in utero* exposure to BPA during fetal development [13].

1.1.4.3 Fetal Exposure to BPA

One of the major functions of the placenta is to act as a barrier preventing xenobiotics in the maternal circulation from reaching the fetus [42]. However, the placenta appears to be ineffective at preventing the transfer of BPA, since unconjugated BPA readily crosses the placenta by passive diffusion in both directions [42,61]. While members of the BPA

metabolizing enzyme UGT and SULT families are expressed in the placenta, they have negligible efficacy in conjugating BPA in this milieu [42]. In addition to its detection in cord blood, fetal blood, placental tissues, and amniotic fluid [3,42,55], BPA was found in fetal tissues, including fetal liver samples (9.02 ng/g unconjugated BPA, 25.8 ng/g total BPA) collected from the greater Montreal area between 1998 and 2008 [62]. Thus, BPA is readily crossing the placental barrier and reaching the fetus.

Various animal models employed to examine the effects of fetal exposure to BPA have demonstrated that fetal BPA exposure has a wide range of adverse effects including reproductive, cardiovascular, immunological, metabolic, behavioral, and neurological disorders; contributes to the development of certain cancers in adult offspring; and that many of these effects are sex-specific [2,6,13]. In mouse models, pre- and post-natal exposure to low doses of BPA affected the organization of the central nervous system and neurotransmitter receptor systems, resulting in reduced and/or reversed sexual differences in the emotional behavior of offspring [63,64]. Moreover, in this model, low doses, but not high doses of BPA, resulted in metabolic disruption (increased body weight, adipocyte number, abdominal fat, insulin levels, and impaired glucose tolerance) in male offspring [63,65]. Our laboratory has demonstrated that prenatal exposure to BPA impairs the development of the fetal liver [66], pancreas [67] and lungs [68]. Therefore, due to the ubiquitous nature of BPA, there is increasing concern for the potential long-term consequences of developmental exposure to BPA.

1.2 The Adrenal Gland

The adrenal glands are an important endocrine organ that synthesizes hormones in its cortex and medulla [69,70]. The adrenal cortex produces steroid hormones during fetal and adult life, including glucocorticoids, aldosterone, progesterone, and precursors of testosterone and estradiol [69,70]. These hormones are produced via the steroidogenic pathway, which starts with cholesterol and involves a number of cytochrome P450 enzymes and hydroxysteroid dehydrogenases [69,70].

The adult adrenal cortex is divided into three zones: the zona glomerulosa (ZG), zona fasciculata (ZF) and the zona reticularis (ZR) [70] (**Table 1.1**). The outermost adrenal zone, the ZG, secretes aldosterone and is a key component of the renin-angiotensin-aldosterone

axis, which is responsible for the regulation of water balance [71,72]. Aldosterone transcriptionally regulates a number of proteins and enzymes involved in maintaining water and sodium balance, and potassium excretion in the kidney [71,72]. Cells of the ZG contain numerous mitochondria, and some cytoplasmic lipid droplets [71]. The middle adrenal cortex zone, the ZF, secretes glucocorticoids, a key hormone in the hypothalamic-pituitary-adrenal (HPA) axis [71]. Cells in the ZF are arranged in bundles, called fascicles that are surrounded by numerous capillaries [71]. These cells contain large numbers of mitochondria, as well as prominent smooth endoplasmic reticulum and large lipid droplets used for steroidogenesis [71]. The ZR, the innermost adrenal cortex zone, secretes dehydroepiandrosterone (DHEA), a precursor of testosterone and estrogens [71]. Cells of the ZR are similar in shape and size to the ZF but have more lysosomes and fewer lipid droplets [71]. The adrenal medulla is composed of chromaffin cells that synthesize epinephrine and norepinephrine [73]. All adrenal gland hormones play various critical roles in maintaining homeostasis. However, this thesis specifically focuses on the regulation of adrenal glucocorticoid synthesis.

Table 1.1: Hormone production in the adrenal cortex.

	Zona Glomerulosa	Zona Fasciculata	Zona Reticularis
Stimulus	Angiotensin II	ACTH	ACTH
Primary Receptor	Angiotensin II receptor	MC2R	MC2R
Hormone Product	Mineralocorticoids (Aldosterone)	Glucocorticoid (cortisol/corticosterone)	Androgens (DHEA)
Function	Regulation of intravascular volume	Glucose homeostasis Stress response Immune response	Adrenarche

Abbreviations: ACTH, Adrenocorticotropic hormone; MC2R, melanocortin 2 receptor; DHEA, dehydroepiandrosterone.

1.2.1 The HPA Axis

The production of glucocorticoids is regulated by the HPA axis (**Figure 1.3**) [74,75]. Upon stimulation, corticotrophin releasing hormone (CRH) is synthesized and secreted from the paraventricular nucleus in the hypothalamus into the hypophyseal portal vessels to be transported to the anterior pituitary gland [75]. Binding of CRH to the CRH receptor 1 (CRHR1) in the anterior pituitary gland induces synthesis of adrenocorticotrophic hormone (ACTH) from the prohormone proopiomelanocortin (POMC) [75]. ACTH is released into the systemic circulation where it binds melanocortin 2 receptor (MC2R), a G-protein coupled receptor expressed on the adrenal cortex, to stimulate steroidogenesis [76]. The stimulatory effect of ACTH can increase steroidogenesis in a number of ways including ***(1)*** promoting adrenal cortex growth over the long term [77]; ***(2)*** promoting the up- regulation of its receptor MC2R [78]; ***(3)*** increasing the presence of the scavenger receptor class B member 1 (SRB1) and low-density lipoprotein (LDL) receptors, thereby enabling enhanced uptake of cholesterol [78]; and ***(4)*** up-regulating key steroidogenic enzymes such as cyp11A1 [77,78] and steroidogenic acute regulatory protein (StAR) [78].

The HPA axis is tightly regulated by a glucocorticoid negative feedback mechanism [75]. Glucocorticoids produced from the adrenal gland provide a negative feedback signal to the hypothalamus and pituitary gland to inhibit further glucocorticoid production through altering transcription of HPA components upon binding to glucocorticoid responsive elements (GREs) or interaction with various transcription factors [75]. Disruptions in the development and formation of the HPA axis pathways during the critical window of fetal development has long lasting health consequences that extend into adulthood, including metabolic syndrome [79] and anxiety/mood disorders [80,81].

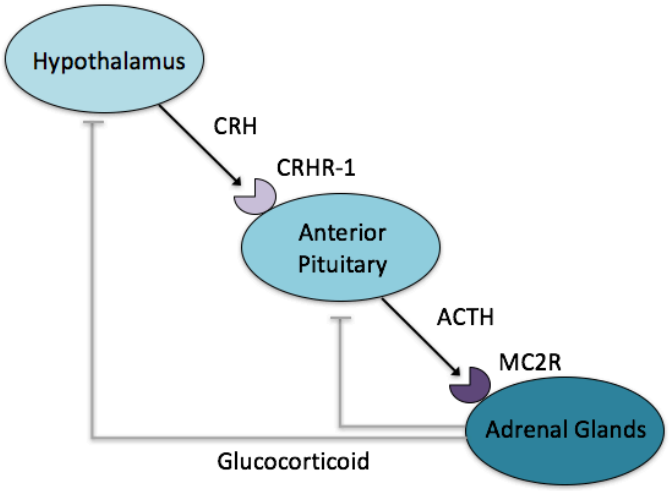

Figure 1.3: Hypothalamic-pituitary-adrenal (HPA) axis.

Hypothalamus releases CRH binding to CRHR-1 receptors on the anterior pituitary gland. Binding of CRH stimulates the release of ACTH which binds to MC2Rs on the adrenal cortex, causing the release of glucocorticoids; cortisol in humans and corticosterone in mice. Glucocorticoids will negatively feedback to both the hypothalamus and anterior pituitary gland to regulate its expression. Abbreviations: CRH, corticotrophin releasing hormone; CRHR-1, corticotrophin releasing hormone receptor-1; ACTH, adrenocorticotrophic hormone; MC2R, melanocortin 2 receptor.

1.2.2 Physiological Function

Glucocorticoids are essential in maintaining whole body homeostasis through their various actions in numerous tissues. Glucocorticoids play a major role in glucose metabolism in stressful environments through increasing serum glucose and amino acids by ***(1)*** increasing catabolism of muscle to increase circulating amino acids; ***(2)*** increasing amino acid uptake in the liver to increase gluconeogenesis and glycogenesis; and ***(3)*** decreasing peripheral glucose uptake in muscle and adipose tissue [78,79,82,83]. Additionally, glucocorticoids increase general catabolism by increasing lipid hydrolysis and increasing fatty acids and by increasing bone and connective tissue catabolism, which may result in osteopenia, and thinning of skin and support structures [78,79,82-84]. Glucocorticoids also play an important role in the immune system by suppressing the inflammatory response while promoting anti-inflammatory actions [78,85].

1.2.2.1 Cushing's Disease

Cushing's disease (CD) is an endocrine disorder characterized by the overproduction of ACTH due to a pituitary tumor, commonly an adenoma, which results in overstimulation of the adrenal gland [86-89]. While CD is the most common form of Cushing's syndrome, other causes include excessive use of glucocorticoids [86-89]. Cushing's syndrome is defined by increased cortisol in both the serum and urine, with a disruption of the HPA axis and cortisol circadian rhythm [86]. The prevalence of CD is reported as 40 cases per million, and has highest prevalence in women aged 40-60 years old [86]. Symptoms of excessive glucocorticoids include increased weight gain, fatigue, insulin resistance, skin thinning, and bruising [86,87,89]. The wide range of comorbidities associated with CD includes hypertension, diabetes mellitus, dyslipidemia, osteoporosis, depression, impaired sexual function in men, menstrual disorders in women, and infertility in both men and women [86,88]. Additionally, patients with persistent or recurring CD or excessive glucocorticoid production have increased risk of mortality [86]. Untreated CD has a five-year survival rate of less than 50% [89]; with the most common causes of mortality being cardiovascular disease and infection [86,87]. Patients with CD report a significant decrease in their quality of life, physically, mentally, and emotionally [86-88]. Treatment for CD varies, depending on the source of the condition, but the first line of treatment is surgery (pituitary and/or adrenal gland) [86,87]. Pharmacological therapies, including steroidogenesis inhibitors (e.g.

ketoconazole, metyrapone, etomidate) and glucocorticoid receptor antagonists (e.g. mifepristone) are used preoperatively or for reoccurrences [87]. Treatment of CD may reduce symptoms of the disease, but comorbidities may be irreversible, therefore potential risk persists throughout life [88].

1.2.2.2 Addison's disease

Opposite to adrenal over-activity is adrenal insufficiency, often known as Addison's disease, which is a rare chronic endocrine disease that results in loss of adrenal function, with a subsequent decrease in adrenal production of glucocorticoids, as well as mineralocorticoids in certain cases [90-92]. Adrenal insufficiency is classified as primary or secondary adrenal insufficiency [90,91]. Primary insufficiency, which affects 0.01% of the population, results from direct inhibition of glucocorticoid production from the adrenal gland. Primary adrenal insufficiency can be caused by autoimmune adrenalitis, infectious adrenalitis (e.g. AIDS, tuberculous adrenalitis), bilateral adrenal hemorrhage, adrenal infiltration, adrenalectomy, drug-induced adrenal insufficiency, or various genetic disorders (mutations in any of the adrenal steroidogenic enzymes, regulatory transcription factors, or receptors) [90,91]. Secondary adrenal insufficiency has a prevalence of 1 in 5,000 and is a result of decreased stimulation of adrenal gland steroidogenesis due to decreased ACTH levels [90,91]. This can be a result of tumors of the pituitary gland or hypothalamus, chronic glucocorticoid use, head trauma, isolated congenital ACTH deficiency, proopiomelanocortin-deficiency, or combined pituitary-hormone deficiency [90,91]. Adrenal insufficiency presents with a range of symptoms depending on whether the adrenal insufficiency involves decreased glucocorticoids, mineralocorticoids, and/or androgens [92]. Common clinical manifestations of both primary and secondary adrenal insufficiency include fatigue, anorexia, muscle weakness, weight loss, light-headedness, nausea, vomiting, headache, sweating, salt craving, and, in women, dry itchy skin and loss of libido [90-92]. In addition, adrenal insufficiency can result in an adrenal crisis [90,92], which is a severe lack of glucocorticoids during extreme stress, infection, or trauma, and can be life threatening [90]. Monitoring of glucocorticoid levels and adjustment during times of stress can prevent adrenal crisis, but mortality rates are still 1.5-2 fold higher in patients suffering from adrenal insufficiency [90]. Current treatment of adrenal insufficiency is to compensate for the glucocorticoid and mineralocorticoids deficiency (only in primary

adrenal insufficiency), with multiple daily tablets of hydrocortisone or prednisone and fludrocortisone in doses that mimic normal hormone secretion patterns [91,92]. Primary adrenal insufficiency results in a decrease in lifespan of 3.2 years in women and 11.2 years in men, compared to the general population, mainly due to increased acute adrenal failure, infections, and sudden death [92]. People suffering from adrenal insufficiency report a decreased perception of health status and quality of life [92].

1.2.2.3 Adrenocortical Cancer

Adrenal cortical tumors (ACTs) are classified as either malignant adrenal cortical carcinomas (ACCs) or benign adrenal adenomas (ACA) [93,94]. Small ACA's are relatively common, affecting about 3-10% of the population [94]. In contrast, ACCs are a rare form of cancer with an annual incidence rate of between 1 to 2 million [93,94]. These tumors present with an aggressive phenotype and the patients have a poor prognosis [94,95]. They are characterized by altered production of steroid hormones, uncontrolled tumor growth and metastases to other tissues [95]. Between 50-80% of patients present with hypercortisolism and 40-60% of patients present with excess adrenal androgen production [94].

Numerous genetic mutations and alterations have been linked to the development of ACTs [95]. Mutations in *TP53* predisposes children to pediatric ACTs (as well as other conditions). This is particularly relevant in southern Brazil, which has a 10-fold higher rate of pediatric ACTs due to mutations in *TP53* [96]. Levels of insulin growth factor II (*IGFII)* is commonly used as an ACC marker due to its overexpression in 90% of ACCs [95,97]. Alone, mutations that result in increased *IGFII* levels are not a significant factor for ACC development, but these mutations may contribute to ACC progression in combination with other factors [95,98]. Activating mutations of β-catenin, leading to increased activation of Wnt signaling, has been detected in ACC patients [95,99]. Additionally, mutation in genes shown to regulate or be involved in Wnt signaling potentially lead to an increase in ACTs [95]. Mutations in other genes including multiple endocrine neoplasia 1 (*MEN1),* mutL homolog 1 *(MLH1),* mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6)*, post meiotic segregation increased 2 (*PMS2)* and post meiotic segregation increased 2 (*PRKAR1A*) have all been observed in patients with ACCs [95].

Alterations in gene expression in ACTs are commonly divided between ACC and ACA [95]. Changes in gene expression frequently seen in ACC are the overexpression of cell proliferation and cell cycle genes, such as cyclin E1, cyclin E2, and cyclin dependent kinase 2 and 4 (CDK2 and 4) [95,100]. Additionally, ACCs have alterations of steroidogenic enzymes, including cyp11A1, StAR, cyp17A1, while these enzymes are generally upregulated in ACAs [95]. Other pathways known to be affected in ACTs include, IGFII [101], sonic hedgehog (Shh) [102,103], Wnt [95,99], fibroblast growth factor receptor (FGFR) [101], and retinoic acid signaling pathways [101].

1.2.3 Adrenal Development

The adrenal gland is developed from two different cells types: the adrenal medulla arises from neural crest cells, while the adrenal cortex arises from coelomic epithelium (urogenital ridge) (**Figure 1.4)** [104]. The adrenogonadal primordium develops from the coelomic epithelium, with the presence of developmental regulatory factors, Wilm's tumor 1 (WT-1), and wingless-related mouse mammary tumor virus integration site 4 (WNT4) [104]. In mice, at embryonic day 9 (E9), the key developmental factors steroidogenic factor-1 (SF-1) and dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome factor 1 (DAX-1) can be detected in the adrenogonadal primordium [104,105]. Migration of adrenal progenitor cells from the adrenocortical primordium, happens in parallel to an upregulation of SF-1 [71,95]. The importance of SF-1 in adrenal development is demonstrated in studies utilizing knockout mice, where SF-$1^{-/-}$ mice lacked adrenal glands and died at birth due to adrenal insufficiency [106]. The expression of steroidogenic enzymes begins at E11 in mice, indicating the possibility of steroidogenesis [105]. At approximately E12-14, neural crest cells migrate and disperse into the developing adrenal gland, preceding their development into the chromaffin cells of the adrenal medulla [107]. Encapsulation of the adrenal cortex is completed by E14.5 [104,105], and adrenal cortex zonation is completed between postnatal days (PND) 1-7 in mice [105]. The formation of the X-zone surrounding the adrenal medulla develops between PND10-14 in mice, and continues to proliferate until PND21 [105]. The function of the X-zone and its presence postnatally is still not fully understood [105]. In male mice, the X-zone will disappear at sexual maturity, whereas in females, it

remains until the first pregnancy [105]. Encapsulation of the medulla by a fibrous tissue layer is completed only after complete regression of the X-zone [105].

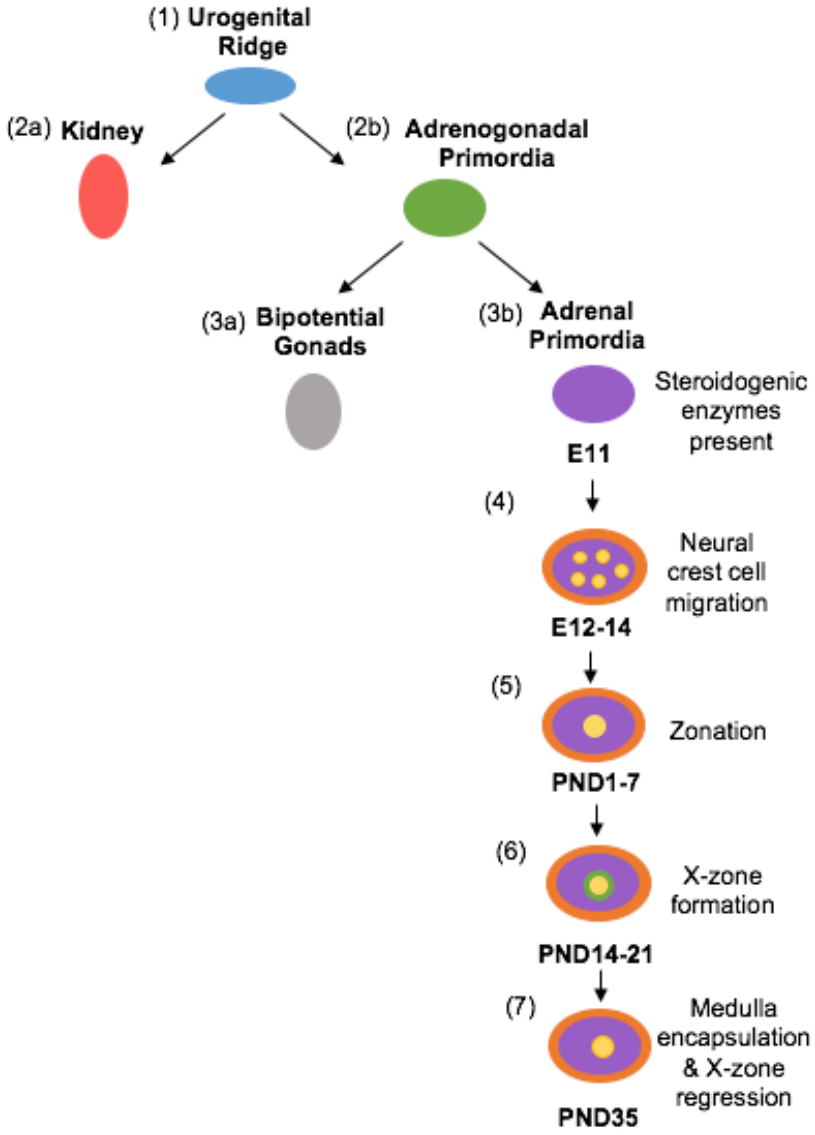

Figure 1.4: Adrenal gland development in mice.

Urogenital ridge separates into either the *(2a)* fetal kidney or *(2b)* the adrenogonadal primordia, which derives into the *(3a)* bipotenial gonads or the *(3b)* adrenal primordia, where steroidogenic enzymes are first detected at E11. Followed by *(4)* neural crest cells migration at E12-14. *(5)* Zonation occurs at PND1-7, and *(6)* X-zone development at PND14-21. *(7)* Medulla encapsulation and X-zone regression occurs at sexual maturity in males and at the first pregnancy in females. Abbreviation: E, embryonic day; PND, postnatal day.

While development of the adrenal gland in humans differs from that of the mouse in terms of timing of developmental processes, the factors responsible for adrenal gland development are thought to be similar between the two species (**Table 1.2**) [105]. In humans, migration of coelomic epithelial cells starts during week 5-7 of pregnancy, and the formation of the fetal zone, which produces DHEA, begins at week 7 [105,107]. Despite considerable differences in timing of development, the fetal zone in humans is thought to be equivalent to the X-zone in mice [105]. The adrenal primordia develops at around 8 weeks [105,107]. At 9 weeks, migration of mesenchymal capsular cells to encapsulate the adrenal cortex and neural crest cells to form the adrenal medulla [95,105]. Regression of the fetal zone takes place shortly after birth (postnatal weeks 1-6), followed by the encapsulation of the medulla (postnatal months 12-18) [105,107]. Complete adrenal cortex zonation follows later, around the time of puberty in both males and females (10-20 years) [105].

Estrogen and ERs are thought to play critical roles in the development of the adrenal gland [108]. The discovery of both ERα and ERβ in the fetal adrenal cortex suggests a role for estrogens in regulating adrenal development and function [108]. Upon binding to Ers, estrogens induce direct and indirect effects in the fetal adrenal gland, affecting sensitivity and responsiveness to ACTH, and altering the synthesis of DHEA [108,109]. However, the role of estrogens or Ers in programming glucocorticoid synthesis in the fetal adrenal gland remains elusive.

Table 1.2: Human and mouse adrenal development.

	Human	Mouse
Migration of coelomic epithelial cells	5-7 weeks	E9
Development of the adrenal primordia	8 weeks	E11
Migration of neural crest cells	8 weeks	E12-14
Regression of fetal zone/X-zone	Postnatal week 1-6	PND35 (males), after first pregnancy (females)
Medulla Encapsulation	Postnatal month 12-18	PND35
Adrenal Cortex Zonation	10-20 years	PND1-7

Abbreviations: E, embryonic day; PND, postnatal day.

1.2.4 Adrenal Remodeling and Growth

Differentiation and renewal of the adrenal cortex zones is not fully understood. However, a few theories have been proposed for adrenal zonation: ***(1)*** Migration Theory that hypothesizes the centripetal proliferation of cells from the outermost zone, ZG, towards the ZF, then the ZR and finally undergoing apoptosis at the edge of the adrenal medulla; ***(2)*** Transformation field theory that postulates the presence of two transformation fields between the ZG and ZF and between the ZF and ZR, where proliferation and differentiation occurs; ***(3)*** Zonal theory that proposes that all proliferation in each zone comes from cells located in the same zone [110,111]. The most probable theory is migration theory [95,107,111,112], which posits that proliferation begins with specialized cells located peripherally in the cortex and that these cells will transit inwards through the cortex layers, and finally to the medulla border where they undergo apoptosis, a process referred to as centripetal displacement [111,112]. This process and the location of specific stem cells varies in rats, where these cells are likely located in an undifferentiated cell zone (zU), between the ZG and ZF [111]. Continued remodeling and growth is essential in the adrenal gland after birth and throughout life [112-114]. These progenitor cells are not only important for the development of the adrenal cortex, but also are involved in adrenal cortex remodeling in adults [112-114].

1.2.4.1 Hedgehog Signaling

The Hedgehog (Hh) signaling pathway is essential in embryogenesis, adult remodeling homeostasis, and carcinogenesis in a variety of tissues [115]. The Hh signaling pathway regulates genes involved in proliferation, the stem-cell signaling network, stem-cell markers, survival, and epithelial-to-mesenchymal transition [115].

The Hh secretory proteins were first discovered in *Drosophila* for their role in specific embryonic segmentation [116]. The three main mammalian HH genes are Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). All are critical for development, as demonstrated in loss of function studies that result in structural abnormalities and malformations [116,117]. Dhh is localized mainly to the gonads, including Sertoli cells and granulosa cells. While $Dhh^{-/-}$ mice are viable and do not have a notable phenotype, males are infertile [117]. Ihh expression is limited to the primitive endoderm and prehypertrophic chondrocytes, resulting in 50% lethality in knockout mice, with surviving $Ihh^{-/-}$ mice having bone abnormalities, including cortical bone defects and aberrant chondrocyte development [117]. Shh is more broadly expressed both during embryogenesis and later life [117]. Mutations in Shh have been shown to cause cyclopia, as well as defects in the foregut and ventral neural tube patterning [117]. Additionally, defects present later in life as malformations of the limbs, ribs, and lungs [117].

All Hh secretory proteins bind to the Hh receptors, Patched (Ptch) 1 and 2 to activate the signaling pathway (**Figure 1.5**). Patched is a 12-pass transmembrane receptor located on the primary cilium of target cells. Co-receptors of the Hh signaling pathway include CDON, BOC, and GAS1 [115]. When not bound, Ptch inhibits another transmembrane protein, smoothened (SMO), by keeping it sequestered in the plasma membrane [117-119]. SMO is a 7-pass G-protein coupled receptor that is also located in the plasma membrane of the primary cilium. In the plasma membrane SMO is tethered to a complex containing the key Shh transcription factors Gli [117-119]. When the Shh pathway is activated, Hh prevents Ptch from inhibiting SMO and enables the translocation of SMO into the cytoplasm [117-119]. Upon SMO translocation, the complex containing the Gli transcription factors are released. In mammals, there are three Gli transcription factors (Gli1-3), with various activities [117-119]. Gli1 and Gli2 are primarily activators of the Shh signaling pathway and have similar roles [117-119]. However, Gli1 is also responsible for positive

feedback of the Shh signaling pathway, and is a direct transcriptional target of Shh activation, to extend cellular response [117-119]. The activation of SMO blocks proteolysis of Gli1 and 2, which leads to accumulation of the full-length activator forms of Gli1 and Gli2, ultimately leading to transcription of target genes [117-119]. Gli3 is primarily a repressor; in the absence of Shh, Gli3 is cleaved into an active repressor form, to inhibit transcription of target genes, but the presence of Shh prevents Gli3 cleavage and thus inhibits Gli3 activity [117-119]. Additional factors known to be involved in the regulation of the Shh pathway include suppressor-of-fused protein (Su(fu)) and hedgehog interacting protein (Hhip), both of which attenuate Shh signaling [115,118].

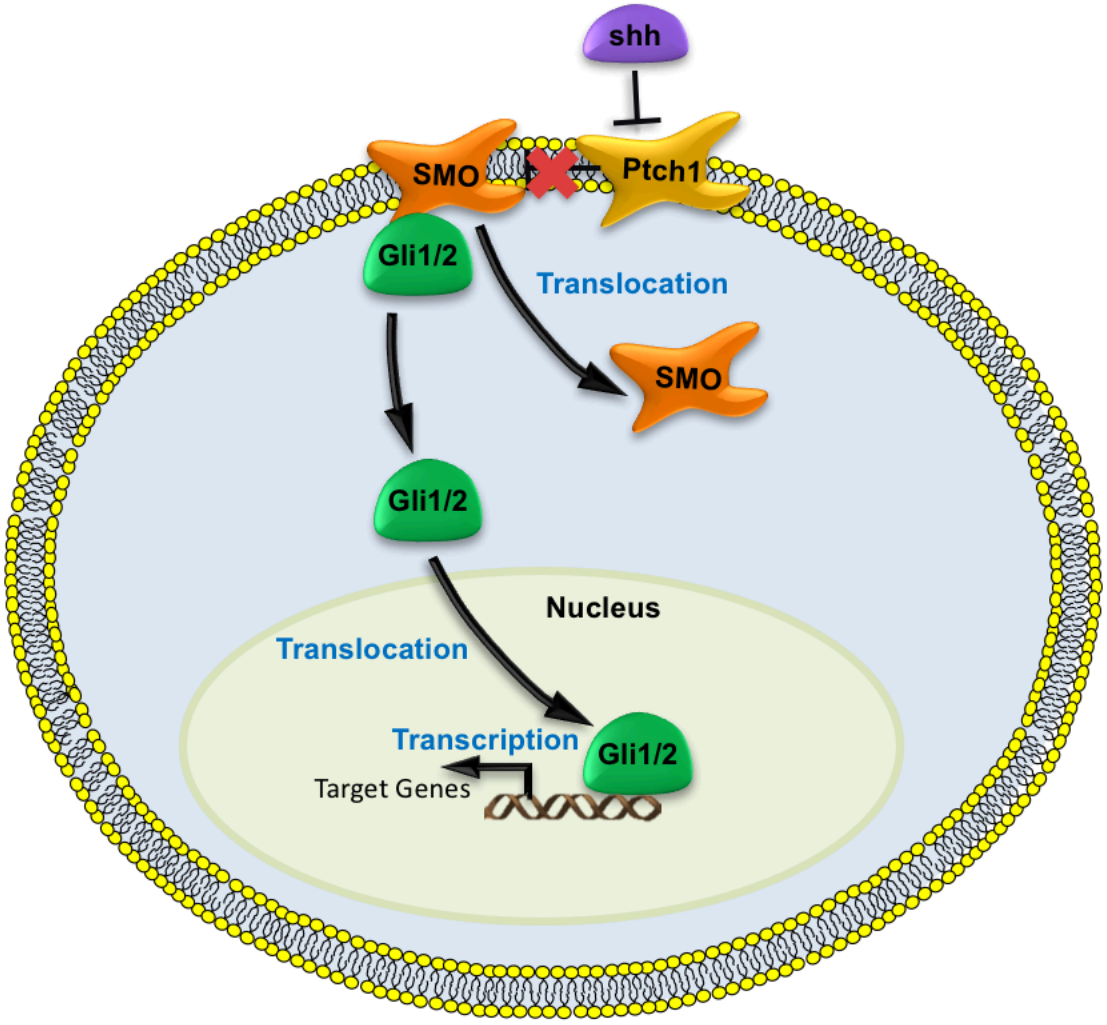

Figure 1.5: Simplified schematic of the Shh signaling pathway activation.

The secretory protein Shh, acts in an autocrine/paracrine fashion and binds to Ptch1 receptor, preventing Ptch1 from inhibiting SMO. SMO is then released from the plasma membrane into the cytoplasm, leading to the release of key Shh transcription activators Gli1 and Gli2. Gli1/2 translocate to the nucleus where they bind to the promoters of target genes to regulate gene expression. Abbreviations: Shh, sonic hedgehog; Ptch1, Patched 1; SMO, smoothened.

Due to the importance of Shh signaling in development and organ remodeling later in life, the Shh signaling pathway has been investigated in both rodent and human adrenal glands. Expression of mRNA for Shh, Ptch1, and Gli1 begins at E12.5 in the mouse adrenal cortex, and is localized in clusters at the periphery of the adrenal cortex, which continues throughout development [112,114,120]. Shh, Gli1, 2, and 3 proteins are detected throughout development and postnatally in the fetal human adrenal gland, with higher expression than is found in adult human adrenal glands [102]. In contrast, Werminghaus et al. demonstrated undetectable levels of Shh protein in both normal adult adrenal glands and adrenocortical carcinomas and adenomas [103]. However, protein levels of Gli1 were detectable in all adrenal cortex zones, but concentrated in the subcapsular area of the ZG of human adult adrenal glands, and was not detectable in adrenocortical carcinomas or adenomas [103]. While additional studies have also detected mRNA for Shh, Gli1, 2 and 3, Ptch1 and SMO in human adrenal glands, adrenocortical carcinomas and adenomas [102,103]. Additionally, there was an upregulation of *SHH* in both cortisol producing and non-cortisol producing adrenal adenomas compared to normal adrenal tissues, suggesting that Shh activation is involved in adrenal tumorigenesis [102,103]. Moreover, Shh, Gli1, Ptch1 and SMO mRNA has been found in human adrenal cortical carcinoma cell lines, H295R and H295A[102,103]. The presence of Dhh or Ihh in the adrenal glands has yet to be determined [113].

Shh and Shh pathway components (Ptch1, SMO, Gli1) mRNA and protein are localized in the outer cortex cells, which do not express the steroidogenic enzymes cyp11b1 or cyp11b2, during early organogenesis and throughout adulthood in mice [112]. This indicates the presence of a specialized population of cells in the adrenal cortex that lacks the ability to produce steroid hormones [112]. This demonstrates the essential role of Shh signaling in the development and expansion of the adrenal cortex [114], and supports the theory of adrenal growth and remodeling through a centripetal displacement process, where Shh containing cells are progenitor cells that differentiate into steroidogenic cells (**Figure 1.6)** [112,113]. Indeed, previous studies using genetic lineage analyses performed using a constitutive Cre model, demonstrated that Shh-positive cells give rise to cortex cells in all zones except the medulla [112]. Moreover, lineage analysis in adults, shows that cells

transition from the outer ZG to the ZF, demonstrating that Shh marks progenitor cells in the adrenal cortex during development and remodeling in adults [112,113].

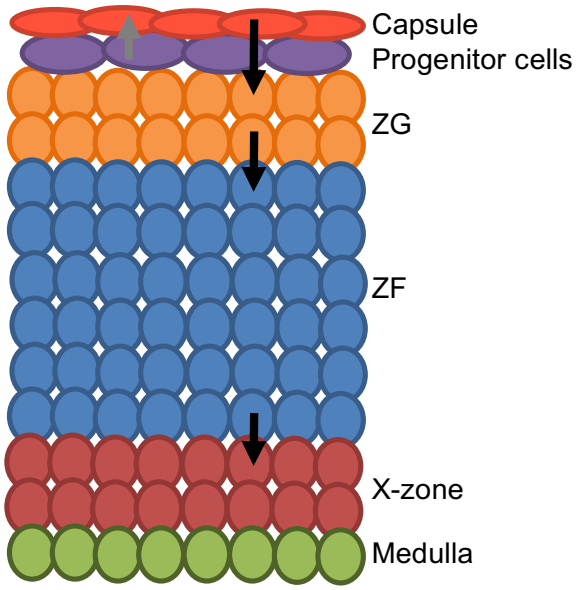

Figure 1.6: Migration theory of adrenal gland remodeling in mice.

Progenitor cells (purple), which are $Shh^{+}/Sf\text{-}1^{+}/cyp11B2^{-}$, signal to $Gli1^{+}/cyp11B2^{-}$ capsule cells (red), to differentiate into functional ZG steroidogenic cells (orange). The migration theory suggests a centripetal displacement process where ZG cells move inward and differentiate into ZF cells (blue) and on to X-zone cells (dark red), before undergoing apoptosis at the medulla border. Abbreviations: Shh, sonic hedgehog; Sf-1, steroidogenic factor-1; ZG, zona glomerulosa, ZF, zona fasciculata.

Gli3 mutation was found to be lethal in embryonic mice and to have an adrenal aplasia phenotype [121]. However, this phenotype was not observed by Laufer, et al. [113]. Adrenal Shh conditional knockout mice, created with a Sf-1-cre driver, exhibit severe hypoplasia and underdevelopment of the adrenal gland, but have no changes in zonation or differentiation of the adrenal cortex [112,114,120]. There was a significant decrease in both the thickness of the adrenal cortex and the capsule in these mice [112,114]. These effects were visible as early as E13.5, with no visual changes to the adrenal medulla [112,114,120]. However, despite the reduction in cortex size, the expression of steroidogenic enzymes was unaltered in $Shh^{-/-}$ mice [112,114,120]. Corticosterone levels were normal until approximately one year of age, when they became reduced along with an increase in ACTH plasma levels [114]. Moreover, $Shh^{-/-}$ mice had reduced levels of proliferating cells in their adrenal cortex, with no change in apoptosis levels [114]. In H295R cells, blocking Shh signaling with the antagonist cyclopamine resulted in decreased proliferation, and decreased production of aldosterone and DHEA [103].

1.2.4.2 Wnt-1 Signaling

In both fetal and adult adrenal glands, the Wnt/β-catenin signaling pathway is critical for adrenocortical homeostasis [122]. β-catenin is present in the fetal adrenal cortex, and is localized to the ZG subcapsule [122]. Mice null for β-catenin in SF-1 expressing adrenal cortex cells, show abnormal adrenal development starting at E12.5, resulting in adrenal failure [123]. β-catenin *Sf-1*-cre mice, which expressed β-catenin in approximately half of their adrenal cortex cells, developed normally [123]. However, a thinning of the adrenal cortex and decreased steroidogenic function was observed starting at 30 weeks of age [123]. Additional evidence for the role of Wnt-1/β-catenin signaling in adrenal development and function is demonstrated when the signaling pathway is over-activated [123,124]. Constitutive over-activation of β-catenin results in increased proliferation of undifferentiated progenitor cells, with the eventual development of ACTs [123,124]. Activation of the Wnt signaling pathway is commonly seen in adrenocortical neoplasms [125,126]. The exact mechanism of Wnt/β-catenin signaling in adrenal cortex function and development remains unknown [107]. However, potential mechanisms include direct activation of Dax1 by β-catenin [127] and β-catenin induced inhibition of ZF differentiation, supporting the undifferentiated phenotype of progenitor cells [126,128].

1.2.5 Sex Specificity in Adrenal Glands

Female adrenal glands are significantly heavier than those of male mice from weeks 3-11, relative to body weight [129]. Additionally, female mice have a significantly larger ZF size and cell number compared to male mice after 3 weeks of age [129]. Both sexes have an X-zone until approximately week 5 postnatally, when the X-zone starts to recede in male mice [129]. In female mice, the X-zone persists until after the birth of their first litter, when it will start to recess. However, the role of X-zone and the sex-specificity of this zone is not fully understood yet [105]. Studies investigating corticosterone levels between sexes in a variety of species including humans have reached different conclusions with some reporting sex differences in corticosterone levels [78,130], while others found no differences in corticosterone levels between sexes [129,131-133]. Species investigated, time of collection, method of collection, and age of animal may all be confounding factors, contributing to the disagreement between the studies. Females are commonly shown to have a higher corticosterone response to stress, which is also sustained longer than it is in males [78]. Additionally, female mice have a greater number of lipid droplets stored in the adrenal glands than their male counterparts [129], which indicates a potential for different steroidogenic activity and capabilities. Additionally, levels of plasma corticosteroid binding globulin (CBG) vary between sexes, due to role of estrogen in promoting synthesis of CBG [78]. After puberty females are reported to have 2 to 5-fold higher CBG levels than males [78]. Thus, there are numerous sex differences in the growth and development in the adrenal gland, but the exact mechanism behind these differences remains largely unknown.

1.3 Adrenal Steroidogenesis

1.3.1 Steroidogenic Pathway

Steroidogenesis is the synthesis of all steroid hormones by a variety of P450 enzymes and hydroxysteroid dehydrogenases, generally located in the adrenal glands, placenta, and reproductive organs [69]. However, low levels of steroidogenesis have been reported in other tissues[134], such as the nervous system [135], skin[136], heart [137], and lungs [138]. The steroid hormones produced in each organ is dependent on the specific steroidogenic enzymes expressed in that organ [69].

The initial step of adrenal steroidogenesis begins in the adrenal cortex, where cholesterol is necessary to produce steroid hormones (**Figure 1.7)**. Most cholesterol for adrenal steroidogenesis originates from either high-density lipoprotein (HDL) or LDL in the blood and transport of cholesterol into the cell is mediated by SRB1 receptors for HDL or LDL receptors for LDL [77]. Humans preferentially utilize cholesterol from LDL endocytosis, while rodents use cholesterol transported by SRB1 receptors [77]. Additional free cholesterol can be produced from *de novo* synthesis, primarily from the endoplasmic reticulum [77]. Cholesterol in endosomes can be converted into free cholesterol by lysosomal acid lipase (LAL) [77,139]. Free cholesterol can be released from cholesterol esters stored in lipid droplets by hormone sensitive lipase (HSL) [77,139]. Re-esterified excess free cholesterol by acyl-coenzyme-A-cholesterol-acyl-transferase (ACAT) can be stored in lipid droplets for future use [77].

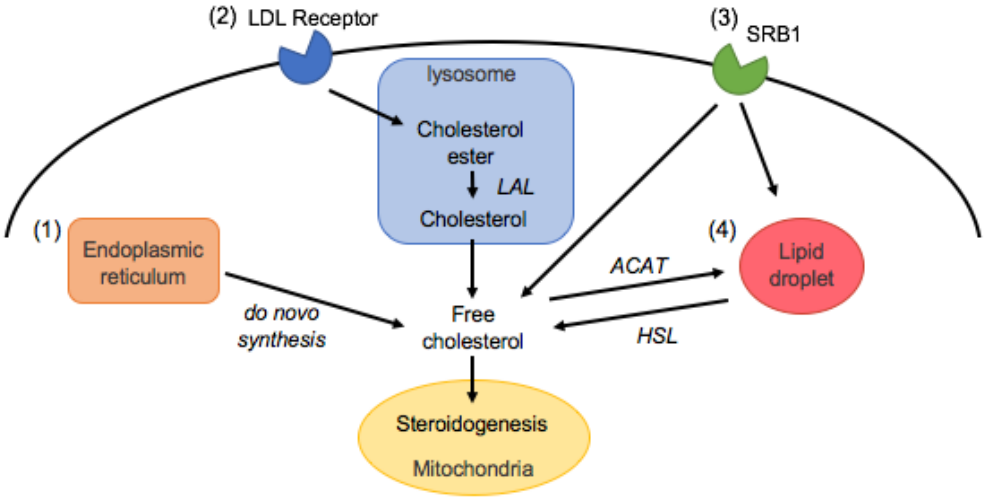

Figure 1.7: Cholesterol transport for adrenal steroidogenesis.

Free cholesterol for adrenal steroidogenesis is generated by 4 sources. *(1)* de novo synthesis from the endoplasmic reticulum; *(2)* LDL binding to the LDL receptor, which is taken up by endocytosis into lysosomes, where it will be synthesized from cholesterol esters to free cholesterol by LAL; *(3)* HDL cholesterol will bind to SRB1, which can be immediately used for steroidogenesis or stored in lipid droplets; or *(4)* HSL provides free cholesterol from lipid droplets. Excess cholesterol can be stored in lipid droplets after esterification by ACAT. Abbreviations: LDL, low-density lipoprotein; LAL, lysosomal acid lipase; HDL, high-density lipoprotein; SRB1, Scavenger receptor B type 1; HSL, hormone sensitive lipase; ACAT, Acyl-coenzyme-A-cholesterol-acyl-transferase.

Within the adrenal cortex cells, steroidogenesis begins within the mitochondria. The rate-limiting step of adrenal steroidogenesis is the transport of free cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondria membrane (IMM), which is facilitated by the protein StAR (**Figure 1.8**) [140]. Once cholesterol is in the mitochondria, it can be converted to pregnenolone by the P450 enzyme, cyp11A1 (formally referred to as P450 side chain cleavage; P450scc). Cyp11A1 conversion is a process of 3 reactions ***(1)*** 22-hydroxylation of cholesterol; ***(2)*** 20-hydroxylation of 22(R)-hydroxycholesterol; and ***(3)*** oxidative scission of the C20-22 bond [77,141]. Conversion of cholesterol to pregnenolone is critical for the production of all steroid hormones, so knockout of *cyp11A1* or mutations in this gene result in loss of steroidogenic activity [141]. For the synthesis of glucocorticoids, pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). In primates that synthesize mainly cortisol but also low levels of corticosterone, Cyp17 converts pregnenolone to 17α-hydroxylase pregnenolone and converts progesterone to 17α-hydroxylase progesterone. Progesterone or 17α-hydroxylase progesterone will then be further converted to 11-deoxycorticosterone or 11-deoxycortisol by Cyp21 in rodents and humans, respectively [69]. Finally, corticosterone/cortisol will be synthesized from 11-deoxycorticosterone/11-deoxycortisol by an adrenal specific P450 enzyme, Cyp11B1. Corticosterone/cortisol can either exit the adrenal gland in the plasma, bound to CBG and transported to most target organs throughout the body [78] or can be further converted by Cyp11B2 to aldosterone in the ZG of the adrenal cortex [142].

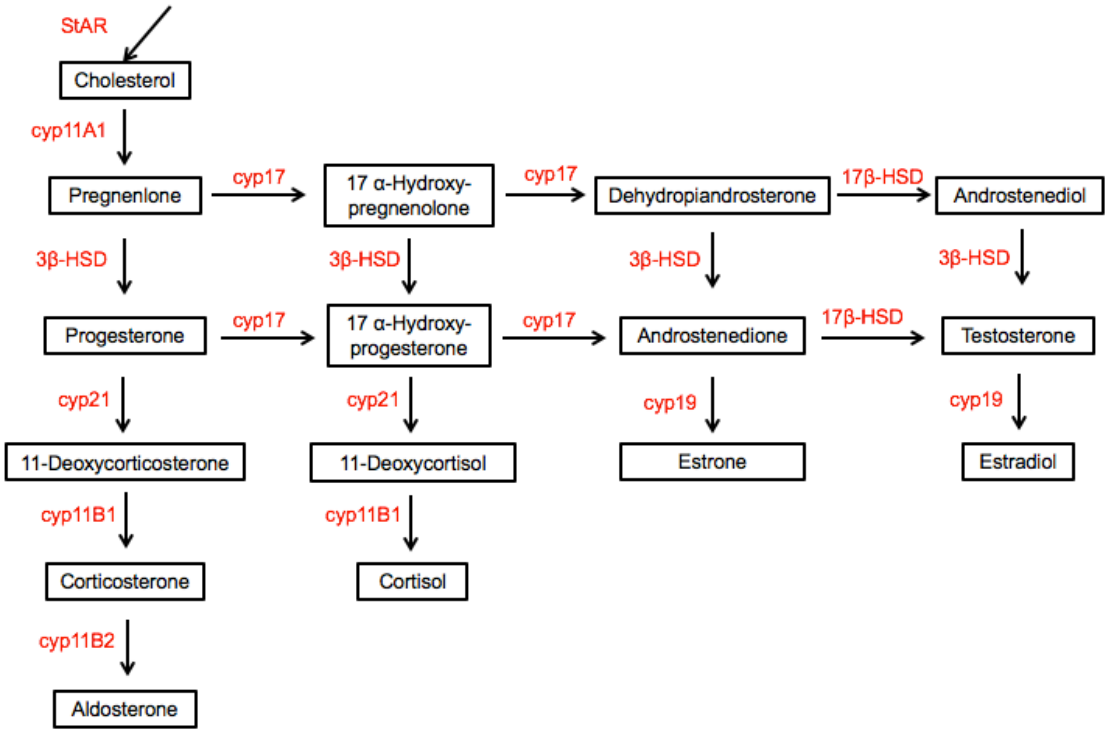

Figure 1.8: Steroidogenic pathway involved in glucocorticoid synthesis.

Steroidogenesis starts with the transport of free cholesterol from the outer mitochondria membrane to the inner mitochondria membrane by StAR, which can then be further converted to all the major steroid hormones. Abbreviations: StAR, steroidogenic acute regulatory protein; cholesterol side chain cleavage enzyme, cyp11A1, 3β-HSD, 3β-hydroxysteroid dehydrogenase; cyp21, 21-hydroxylase; cyp11b1, steroid 11β-hydroxylase; cyp11b2, aldosterone synthase; cyp17, cytochrome P450 17A1; cyp19, aromatase; 17β-HSD, 17β-hydroxysteroid dehydrogenases.

1.3.2 Steroidogenic Acute Regulatory Protein

StAR is the rate-limiting step in steroidogenesis, due to its essential role of transporting cholesterol from the OMM to the IMM for steroidogenesis. Mutations of StAR in humans produce congenital lipoid adrenal hyperplasia, resulting in a lack of steroid hormone production that requires life-long hormone therapy [143]. Moreover, StAR knockout mice lack the ability to synthesize steroid hormones, and accumulate cholesterol in both the adrenal glands and gonads [144-147]. *StAR*$^{-/-}$ mice have external female genitalia and fail to grow after birth [147]. A proportion of StAR$^{-/-}$ mice die shortly after birth due to respiratory distress, and the remainder die a week after birth from an imbalance in fluid and electrolytes, a result of secondary adrenal insufficiency [147]. While StAR$^{-/-}$ mice can be rescued by treatment with steroid hormones (corticosterone and aldosterone), they retain notable abnormalities in adrenal and gonad structure and function [146]. Steroidogenesis is not completely abrogated in StAR$^{-/-}$ mice until the accumulation of lipid droplets in adrenal and gonads builds up enough to destroy steroidogenic cells [146].

StAR originates from a 37-kDa StAR pre-protein with an N-terminal mitochondrial targeting sequence that directs it to the mitochondria [77,141]. The 37-kDa StAR cytoplasmic precursor has a short half-life, and is rapidly degraded if not imported into the mitochondria (**Figure 1.9**) [141]. Cleavage of the 37-kDa StAR pre-precursor into a 30-kDa "mature" molecule by removal of the N-terminus occurs at the OMM [148]. Although the 30-kDa protein (referred to in this thesis only as StAR) is considered "mature", the removal of the N-terminus is not necessary for activation [77]. The cleavage, however, seems to contribute to the localization of StAR on the OMM, which does determine its activity [77,149]. Thus, the time of StAR residency on the OMM is directly proportional to its activity [77,141]. StAR contains a sterol binding pocket allowing it to transport a single cholesterol molecule from the OMM to the IMM. [150]. However, each StAR molecule will transport hundreds of cholesterol molecules before it undergoes cleavage and removal from the OMM, terminating its activity [141]. There are currently four proposed models to account for StAR's ability to transport cholesterol [151]. ***(1)*** *Contact sites*: the 37-kDA StAR forms contact sites with the OMM and IMM that permit cholesterol to flow down a concentration gradient into the mitochondrial matrix [151,152]. ***(2)*** *Desorption*: StAR "desorbs" cholesterol at the OMM, permitting its entry into the intra-mitochondrial space

(IMS), potentially as micro droplets [151,153]. There is little evidence to support this theory, since micro droplets of cholesterol have not been observed [151]. ***(3)*** *IMS Shuttle*: StAR acts in the IMS to shuttle cholesterol from the OMM to the IMM [151,154]. This theory is no longer accepted, due to the observation of StAR activity on the OMM [151]. ***(4)*** *Molten Globule*: StAR undergoes a conformational change at the OMM caused by protonated phospholipids [149,151]. This is confirmed by the dependence of StAR on a proton pump on the mitochondria for its activity [151,155]. Additionally, at the OMM StAR interacts with a multi-protein complex containing translocation protein (TSPO, previously the peripheral benzodiazepine receptor; PBR), voltage-dependent anion channel 1, and phosphate carrier protein, which may all be involved in StAR-mediated cholesterol transport [148,149,156,157]. The activity of steroidogenesis is partially controlled by the phosphorylation of StAR at Ser194/5, which doubles its rate of cholesterol transport [77,158]. In the absence of StAR, steroidogenesis is possible when cholesterol transport occurs with the help of metastatic lymph node 64 protein (MLN64) using a yet to be determined mechanism [159]. This MLN64 mediated process of cholesterol transport occurs mostly in the human placenta, which lacks StAR protein, and has about 50-60% of the cholesterol transport ability of StAR [159].

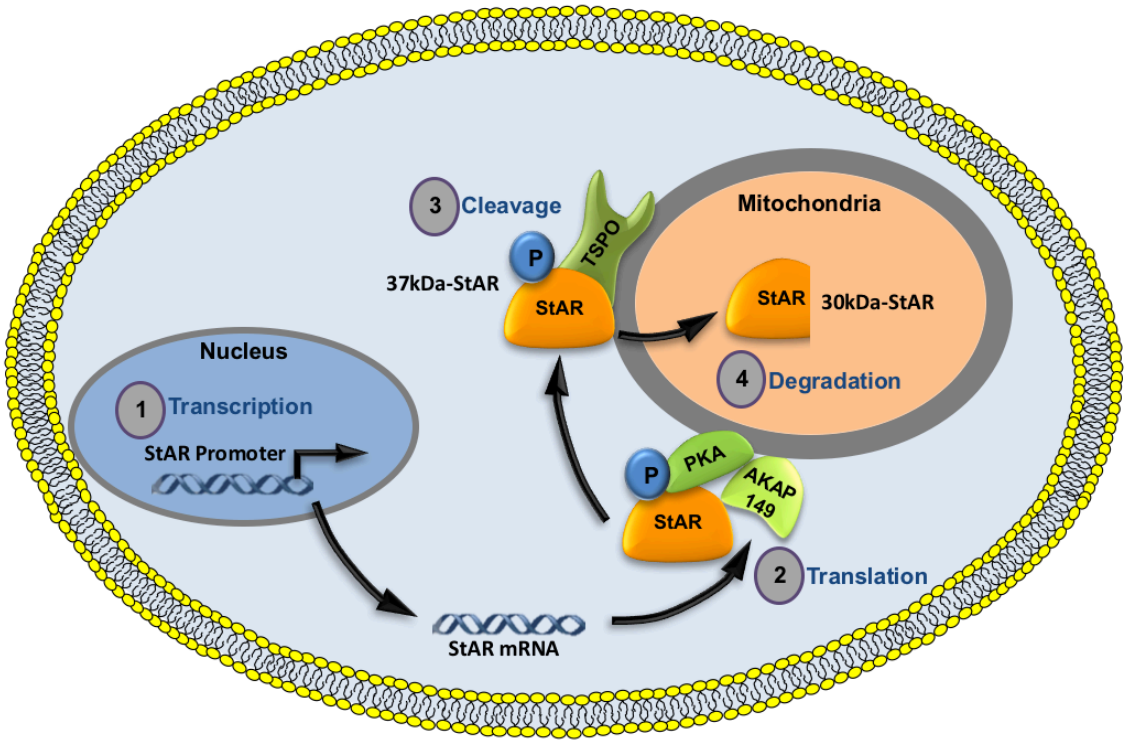

Figure 1.9: Synthesis of steroidogenic acute regulatory protein (StAR).

(1) Transcription of StAR occurs in the nucleus of steroidogenic cells. ***(2)*** StAR mRNA is then transported to the mitochondria where it binds to AKAP149, which is involved in its translation into a 37-kDa pre-protein and phosphorylated by PKA at Ser194/5. ***(3)*** 37-kDa StAR will then interact with the multi-protein complex, TSPO on the outer mitochondria membrane, where the n-terminus of StAR is cleaved. StAR then facilitates the transport of cholesterol from the outer mitochondria membrane to the inner mitochondria membrane. ***(4)*** After cholesterol transport, 30-kDa StAR will enter the mitochondria where it will be degraded. Abbreviations: StAR, steroidogenic acute regulatory protein; AKAP149, A-kinase anchor proteins; PKA, protein kinase A; TSPO, Translocator protein.

1.3.2.1 Regulation of StAR

Due to the essential role of StAR in facilitating cholesterol transport from the OMM to the IMM for steroidogenesis, the regulation of this protein is of great importance. The regulation of StAR is not fully understood but has been shown to be quite complex, involving numerous hormones, transcription factors, and receptors [160,161].

1.3.2.1.1 Epigenetic Regulation of StAR

StAR expression is affected directly and indirectly by epigenetic modifications, such as histone modifications, and micro-RNA (miRNA) [162-166]. For example, induction of StAR was associated with acetylation of histone 3 but not histone 4 on the proximal StAR promoter in MA-10 cells and in primate granulosa cells after stimulation [162,163]. Additionally, epigenetic factors can have indirect effects on StAR expression by altering transcription factors known to bind the StAR promoter, such as miRNA-133b inhibition of the negative transcription factor forkhead box L2 (Foxl2), which results in increased StAR transcription [164].

1.3.2.1.2 Transcriptional Regulation of StAR

The first 150 bases of the proximal StAR promoter are highly regulated by numerous transcription factors. Positive regulators of StAR transcription include SF-1, CCAAT-enhancer-binding protein β (C/EBPβ), GATA-4, specificity protein 1 (Sp1), sterol regulatory element binding protein (SREBP), CREB/CREM, and AP-1 [140,162,167], all of which have numerous putative binding sites on the StAR promoter [140,167]. Additionally, DAX-1, Foxl2 and Yin Yang 1 (YY1) negatively regulate transcription of StAR mRNA. Transcription factor expression and regulation of StAR have been shown to be cell/tissue-specific as well as time dependent [162].

SF-1 not only plays a critical role in adrenal development, it is also known as a master regulator of steroidogenesis [168]. There are six binding sites for SF-1 on the StAR promoter, where it can regulate both basal and stimulated StAR transcription [140,167]. The SF-1 binding sites -43/-37, -102/-96 and -105/-99 have all been shown to be essential for both basal and stimulated StAR regulation in reproductive cells [167]. Mutations in any SF-1 binding sites, result in a significant decrease, but not an elimination in cAMP induced

StAR activity, demonstrating the involvement of other transcription factors or regulatory responses in StAR expression.

These transcription factors are able to act alone or in combination by binding to the StAR promoter [167]. It is suggested that C/EBPβ can form a complex with SF-1 on the StAR promoter, as well as interacting with Sp1 to promote StAR transcription [140,167]. Additionally, SF-1 can interact with Sp1, and C/EBPβ cooperates with GATA-4 to regulate StAR expression, [167]. Therefore, the regulation of StAR transcription is thought to involve the interaction of numerous transcription factors at the StAR promoter region.

1.3.2.1.3 Post-transcriptional Regulation of StAR

Initially, StAR was thought to be mainly transcriptionally regulated, however recent evidence points to a role of post-transcriptional regulation of StAR [160]. The stability of StAR mRNA and post-translational regulation of StAR have been investigated [160]. Currently, no known proteins have been shown to bind to StAR mRNA to regulate its stability, however, possible candidates include the mRNA stabilizing proteins, TPA-induced sequence 11b (Tis11b) and HuR, which are expressed in steroidogenic cells [160].

Additionally, StAR mRNA may be targeted by proteins such as mevalonate kinase, DAX-1 and A-kinase anchoring protein 121/149 (AKAP121/149) that alter its rate of translation [160]. AKAP121/149 contains an N-terminal KH domain that targets and recruits StAR mRNA to its location at the OMM where it can be translated [169]. StAR protein expression at the OMM has been shown to be enhanced by AKAP121/149 in MA-10 cells [170]. In addition, AKAP121/149 has been demonstrated to recruit PKA, which phosphorylates StAR to increase its activity [170].

The N-terminal of the 37-kDa StAR directs it to the mitochondria, but it may also destabilize the protein, promoting its degradation and contributing to its short half-life [152,160]. StAR degradation in the cytoplasm has been shown to be extremely rapid in many tissues [171]. Proteasome-mediated degradation of the 37-kDa StAR protein has been demonstrated, and it is suggested that this may occur without ubiquitinylation of StAR [171].

StAR physically and/or functionally interacts with numerous proteins at or around the OMM, including cyclin dependent kinase-5 (CDK5), mitochondrial kinases (MEK) 1/2,

PAP7, TSPO, HSL [160]. While these proteins and others could potentially contribute to StAR's expression and activity, more evidence is needed to understand StAR–protein interactions.

1.4 Effects of BPA on Steroidogenesis

1.4.1 Male Reproductive Steroidogenesis

Due to its estrogenic nature, the effect of BPA on the male reproductive system has been the subject of considerable research effort. BPA exposure, both during the prenatal period and during adulthood results in increased prostate size [6,172]. Rodent studies have demonstrated that exposure to BPA during the developmental period alters spermatogenesis and reduces sperm quality in adult offspring [173]. Additionally, BPA adversely affects androgen production, which is essential for functional spermatogenesis [173]. Decreases in testosterone levels and/or steroidogenic enzymes was observed in rodents exposed to BPA prenatally, as well as during the postnatal period [173-176].

Investigation of the effects of BPA on the rate limiting step of steroidogenesis, StAR, in the male reproductive system yielded inconsistent results that vary between model systems (**Table 1.3 and 1.4**). In male rats, Qui et al. 2003 [177] observed an increase in StAR and cyp11A1 gene expression in the testis after acute BPA exposure. In contrast, D'Cruz, et al. [175] demonstrated that BPA exposure resulted in decreased protein expression of StAR in male rats, a finding in agreement with two other studies showing that acute BPA exposure decreased StAR levels in testes [174,178]. Chouhan, et al. [178] concluded that the decrease in StAR protein after BPA exposure could be attributed to a BPA-induced increase in oxidative stress, resulting in increased inducible nitric oxidative synthase (iNOS). Moreover, both perinatal and acute exposure to BPA significantly inhibited StAR in the testis of fetal and offspring rodents [179-181]. However, BPA treatment for 17 h did not significantly alter levels of StAR in primary mouse Leydig cells [182]. Therefore, more research is needed to understand the variability and mechanism behind the effects of BPA on StAR in the male reproductive system.

1.4.2 Female Reproductive Steroidogenesis

Due to BPA activity as an estrogen mimicking chemical, the effects of BPA on the female reproductive system and fertility has also been investigated. Of interest is a study

by Ikezuki, et al. [59] that used enzyme-linked immunosorbent assays (ELISA) to determine BPA levels of 1-2 ng/mL in 36 human follicular fluid samples. In contrast, when measuring BPA with high-performance liquid chromatography and mass spectrometry (HPLC-MS), no BPA was detected in the five human follicular samples examined [183].

Ovarian steroidogenesis is fundamental for estrogen production, which is essential for ovarian function [184]. In ovaries, theca cells use cholesterol to produce testosterone via the steroidogenic pathway, which is then further converted to estrogen in granulosa cells by the enzyme aromatase (cyp19A) [184]. The effects of BPA on estrogen, androstenedione and DHEA have been widely reported in the literature [184]. *In vitro* studies have demonstrated increased estrogen synthesis, and as shown by Peretz, et al. [185], impairment of follicular growth. However, BPA exposure in rodent models result in inconsistent outcomes[184]. Gamez, et al. [186] reported increased estradiol and FSH levels in pre-pubertal female rats exposed to 3µg/kg/d BPA prenatally. In contrast, ovine female offspring prenatally exposed to three different BPA doses (0.05, 0.5, or 5mg/kg bw/day) had no changes in estradiol levels, but did have a shortened time of estradiol surge compared to the LH surge peak, indicating potential fertility problems [187]. Epidemiological studies have also found that higher BPA levels lead to higher serum estradiol levels in most cases [184]. However, few human studies employ healthy female subjects, tending to focus on women attending clinics for *in vitro* fertilization or other reproductive conditions [184]. Thus, the effects of BPA on ovarian steroidogenesis and function appear to be dose-, species- and time-dependent, with more investigation necessary to provide conclusive results.

Numerous investigators have looked at levels of the key steroidogenic protein StAR, since it is involved in all steroid hormone production as well as being the rate-limiting step in steroidogenesis (**Table 1.3 and 1.4**). BPA inhibits StAR in cultured mouse ovarian follicles in a variety of mouse strains [185,188,189]. Conversely, BPA increases cyp11A1 and StAR mRNA in rat ovarian theca-interstitial (T-I) cells and granulosa cells [190]. However, BPA had no effect on StAR mRNA in luteinized human granulosa cells [191]. *In vitro* studies showed that BPA inhibited StAR protein expression in T-I and granulosa cells [192]. In contrast, Xi, et al. [179] found no change in ovarian StAR expression with either prenatal or postnatal BPA exposure. Thus, the effects of BPA exposure on steroidogenic

enzymes are animal- and tissue-specific and vary depending on the type of BPA exposure. Nevertheless, it is important to note that regulation of steroidogenesis in reproductive tissues differs from that of the adrenal gland.

Table 1.3: Effects of BPA on testicular and ovarian StAR expression *in vitro*.

Cell type	Time of BPA	Dose	Results	Reference
Primary antral follicles from FVB, and C57BL/6	24-96 h	1-100 μg/ml	Decreases mRNA of StAR after 72-96 h after 10-100 μg/ml BPA.	[189]
Primary CD-1 mouse antral follicles	24-96 h	1-100 μg/ml	Decreases mRNA of StAR at 72-96 h with 10-100 μg/ml	[188]
Primary mouse follicles from FVB mice	120 h	4.4-440 μM	Decreases StAR after 440 μM	[185]
Primary rat theca-interstitial and granulosa cells	72 h	10^{-7}-10^{-4} M	Increases StAR mRNA after 10^{-5}-10^{-4} M in theca cell and 10^{-4} M in granulosa cells	[190]
Luteinized human granulosa cells	48 h	0.02, 0.2, 2, 20 μg/ml	No effect on StAR mRNA	[191]
Primary culture of mouse leydig cells	17 h	10 μM	No effect on StAR mRNA	[182]

Table 1.4: Effects of BPA on testicular and ovarian StAR expression *in vivo*.

Animal Model	Tissue	Time of exposure	Dose and method	Results	Reference
Swiss albino male mice	Testes	60 days	IP injection of 0.5, 50 and 100 µg/kg body weight/day	Decrease StAR protein at all doses	[178]
Wistar/ST male rats	Testes	42 days	SC injections of 20, 100, or 200 mg BPA/kg/day	StAR mRNA and protein decreased with 100 and 200 mg dose	[174]
Wistar male rats	Testes	45 days	Gavaged 0.005, 0.5, 50, and 500 µg/kg bw/day	Decreased StAR protein at all doses	[175]
Sprague Dawley male Rats	Testes	56 days	Gavaged 0.0005, 0.5, 5 mg/kg/bw	Increases StAR mRNA and protein at 5mg/kg/bw dose	[177]
Sprague Dawley female rats	Ovary	90 days	Gavaged with 0.001, or 0.1 BPA mg/kg bw	Decrease StAR protein at all doses	[192]
Sprague-Dawley rats	Fetal testes	E11-20	SC injections of 0.02, 0.5, or 400 mg/kg/day	Decreased StAR mRNA at 400 mg/kg/day	[180]
ICR Mice	Offspring adult testes	E1-5	20 µg/kg/day orally	StAR mRNA decreased at PND 35-50	[181]
CD-1 mice	Ovary and testes	Cohort A: E1-PND 49 Cohort B: PND 20-49	Gavaged Cohort A: 12-50 mg/kg/day Cohort B: 25-50 mg/kg/day	Cohort A: decreased StAR mRNA and protein at 50mg/kg/day in testes, no change in ovaries Cohort B: no change in StAR mRNA	[179]

1.4.3 Adrenal Steroidogenesis

Due to the essential role of glucocorticoid in maintaining whole body homeostasis, epidemiological studies have associated high levels of BPA with HPA dysfunction [57,193]. A recent study by Giesbrecht, et al. [57] demonstrated that pregnant women with high urinary BPA (1.66-43.20 ng/mL) had lower waking cortisol levels and flatter diurnal cortisol rhythms, providing evidence for the potential of BPA to alter the HPA axis and cortisol response in adults [57]. The offspring of the same women were examined after parturition, to determine the effects of high BPA exposure on infant cortisol levels and reactivity [193]. This study showed an association between high maternal BPA levels and increased basal salivary cortisol levels in female infants, but decreased cortisol levels in male infants compared to infants exposed to low BPA levels [193]. Additionally, cortisol reactivity was decreased in female infants and increased in male infants exposed to high prenatal BPA [193]. Taken together, these studies shown an association of chronic prenatal exposure to BPA with dysfunction of the HPA-axis in humans [57,193].

The effects of BPA on plasma levels of corticosterone have been evaluated in numerous experimental animal studies, however the effects appear to be dependent on the animal used, dosage of BPA, timing and length of exposure, route of exposure, and time of corticosterone measurement (**Table 1.5**). The sex-specificity of BPA effects on corticosterone levels remain disputed. An increase in corticosterone levels in male but not female adult offspring was seen in rats pre- and post-natally exposed to 2 μg/kg subcutaneous injections of BPA from E10 to PND7 [80,81]. However, an increase in corticosterone levels was seen in female, but not male, mid-adolescence rat offspring when pre- and post-natally exposed to 40 μg/kg BPA in orally throughout pregnancy and lactation [194,195]. No changes in corticosterone were seen in either sex of rats at PND21 when gavaged with 2.5 or 25 μg/kg/day BPA from E6 to PND21 [196].

The effects of acute BPA exposure on adrenal steroidogenic enzymes have been demonstrated in the adrenal mouse cell line Y-1, as well as in rats acutely exposed to BPA [197]. Lan et al. [197] demonstrated that *in vitro* exposure to BPA levels from 50-10,000 nM was sufficient to elevate cyp11A1 protein levels in a dose-dependent manner, but did not affect SF-1 levels [197]. Additionally, this group showed that daily subcutaneous BPA injections of 0.5 μg/kg for three days resulted in increased plasma corticosterone and

adrenal cyp11A1 protein levels in male Sprague-Dawley rats [197]. These studies show that BPA alters adrenal steroidogenesis in cell and animal models; however, the effects of prenatal BPA exposure on adrenal steroidogenesis have yet to be investigated.

Table 1.5: Effects of BPA on basal corticosterone levels.

Animal Model	Time of exposure	Dose and Method	Age of evaluation	Basal Corticosterone levels	Reference
C57BL/6	E7.5-E18.5	25 mg BPA/kg in food	E18.5	No change	[68]
Sprague-Dawley rats	E10-PND7	orally administered 2 μg/(kg/day) of BPA	PND80	Increased in males	[81]
Sprague-Dawley rats	E6-E21 prenatally, PND1-PND21 directly to pup	Orally gavaged 2.5 and 25 μg/kg/day	PND21	No change in either sex	[196]
Sprague-Dawley rats	Throughout pregnancy and lactation	orally gavaged 40 μg/kg/day of BPA	PND40-50	Increased in females	[198]
Wistar rats	Throughout pregnancy and lactation	orally administered 40 μg/kg/day of BPA	PND46	Increased in females and not males	[195]
Wistar rats	Throughout pregnancy and lactation	orally administered 40 μg/kg/day of BPA	PND46	Increased in females but not males	[194]
Sprague-Dawley rats	E10-PND7	2μg/(kg/day) BPA SC injections	PND80	Increased in males and not females	[80]
Deer mice	2 weeks prior to mating and throughout pregnancy and lactation	50mg of BPA/kg feed weight	PND90	No changes in males	[199]
Sprague-Dawley rats	3 days	0.5μg/kg BW BPA SC injections	8 weeks	Increased in males	[197]

1.5 Rationale

BPA as an EDC in numerous tissues is well established [2,10,15]. Moreover, the potential adverse effects of *in utero* BPA exposure on fetal development and the long-term consequences of this exposure is of great concern [2,6,13]. Indeed, prenatal BPA exposure has a wide range of adverse health effects, including reproductive [179-181], cardiovascular [200,201], respiratory [68,202], immunological [203,204], metabolic [66,67,205], behavioral and neurological [11] disorders, in both the fetus and adult offspring. Thus, brief exposure to BPA during critical periods of development can have lifelong health consequences.

The adrenal gland plays a critical role in production of glucocorticoids which are necessary in maintaining whole body homeostasis. Furthermore, the adrenal gland is highly vulnerable to environmental toxin insult due in part to its potential for free radical generation during steroidogenesis, ability to take up lipophilic agents, high vascularity allowing delivery of toxins, and high levels of CYP enzymes available to activate toxins [206,207]. Given the above, my thesis focuses on the long-term effects of prenatal BPA exposure on adrenal gland development and steroidogenic function in adulthood.

Prenatal BPA exposure has been shown to increase plasma glucocorticoid levels in offspring, in a sex-specific manner. However, the precise nature of these sex-specific effects on plasma corticosterone levels remains obscure [80,194,195,198]. Moreover, whether the BPA-induced increases in plasma corticosterone levels are a result of enhanced adrenal steroidogenesis is not known. Importantly, whether prenatal BPA exposure alters adrenal development remains to be demonstrated. Therefore, this thesis addresses these important questions.

1.6 Hypothesis

I hypothesize that prenatal exposure to BPA disrupts adrenal gland development and steroidogenic function in adult mouse offspring.

1.7 Objectives

i. To determine the effects of prenatal BPA exposure on adrenal gland development, and adrenal steroidogenic function *in vivo*.

ii. To determine the molecular mechanisms that underlie the BPA-induced aberrant adrenal gene expression *in vitro*.

iii. To determine the molecular mechanisms underlying the BPA-induced aberrant adrenal gland development *in vitro*.

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2 PRENATAL EXPOSURE TO BISPHENOL A DISRUPTS STEROIDOGENESIS IN ADULT MOUSE OFFSPRING[1]

[1] Reproduced (adapted) from: Medwid S, Guan H, Yang K (2016) Prenatal exposure to bisphenol A disrupts steroidogenesis in adult mouse offspring. *Environ Toxicol Pharmacol.* **43**: 203-8

2.1 Introduction

Bisphenol A (BPA) is a ubiquitous endocrine disrupting chemical, being present in polycarbonate plastics, epoxy resins, paper receipts, and cardboards, as well as water and air samples [1-3]. Of foremost concern is exposure to BPA during the critical period of organ maturation [4]. Indeed, BPA has been detected in placental tissues and fetal blood, demonstrating BPA's ability to cross the placenta and reach the fetus [3,5]. Numerous human epidemiological studies have demonstrated an association between gestational exposure to BPA and pregnancy complications, male genital abnormalities, childhood obesity, childhood asthma and altered neurological development in children [6]. Furthermore, *in vivo* animal studies have shown that developmental BPA exposure results in a wide range of adverse effects, including reproductive, cardiovascular, immunological, metabolic, behavioural, and neurological disorders as well as certain cancers in adult offspring. In addition, many of these adverse effects are sex specific [3,4,7].

Due to the critical role of glucocorticoids (cortisol in humans, and corticosterone in rodents) in maintaining whole body homeostasis, the effects of BPA on the hypothalamic-pituitary-adrenal axis had been examined previously. It was found that maternal exposure to BPA during pregnancy and lactation resulted in increased basal corticosterone levels in juvenile female, but not male rats [8-10]. Furthermore, perinatal BPA exposure led to abnormal adrenal cortex structure, including increased adrenal gland weight in females, accompanied by a reduction in the zona reticularis and hyperplasia of the zona fasciculata in both sexes [8]. In contrast, Chen, et al. [11] reported that prenatal BPA exposure resulted in increased corticosterone levels in adult male rats only. Thus, the precise nature of these sex-specific effects of developmental exposure to BPA on circulating corticosterone levels remains obscure. Importantly, whether the BPA-induced increases in circulating corticosterone levels are a result of enhanced adrenal steroidogenesis is unknown. Therefore, the present study was undertaken to address these two important questions.

2.2 Materials and Methods

2.2.1 Animal Experiments

The use of animals in this study was approved by the Council on Animal Care at the University of Western Ontario, following the guidelines of the Canadian Council on Animal Care. Breeding pairs of adult C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). To minimize environmental BPA exposure, mice were housed in polypropylene cages with glass water bottles. Mice were allowed food and water *ad libitum*, and maintained in humidity and temperature-controlled rooms on a 12 h/12 h light-dark cycle. Female mice were placed overnight with males, and detection of vaginal plug the next morning indicated pregnancy, and marked as embryonic day 0.5 (E0.5). Pregnant dams were fed either a control diet (phytoestrogen-free food pellets supplemented with 7% corn oil; TD.120465, Harlan Teklad, Madison, WI) or the control diet supplemented with 25 mg BPA/kg feed weight (equivalent to 5 mg BPA/kg body weight; TD.120466, Harlan Teklad, Madison, WI) from E7.5 to postnatal day (PND) 0.5. The gestational age of E7.5 was chosen as the start of the feeding regime in order to avoid any confounding effects of BPA on embryo implantation. After birth, both control- and BPA-fed dams were switched to regular chow for remainder of the study. Pups were weaned on PND 21, separated by sex and fed regular chow. Five litters of control and BPA were used, with number of pups between 6 and 11 pups per litter. Litters were culled at 5 pups per sex per litter. They were group housed by litter, experimental treatment and sex. Offspring were sacrificed between 8 and 10 weeks of age using carbon dioxide asphyxiation. Blood samples were collected *via* cardiac puncture in heparinized capillary tubes (Fisher brand Cat. No. 22-260-950), and centrifuged at 2000*g* for 10 min at 4 °C. Plasma was then harvested were stored at −80 °C. Adrenal glands were dissected, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C. All sacrifices and sample collection were done between 9:00–11:00 am.

2.2.2 Western Blot Analysis

Due to the limited tissue quantity (*i.e.*, the tiny size of mouse adrenal glands), we made a strategic decision to determine changes in protein, rather than mRNA, abundance following exposure to BPA during critical periods of adrenal gland development. This is because information on alterations in protein levels is more biological meaningful.

Furthermore, the limited tissue availability also precluded the possibility of conducting enzyme activity assays, which would require greater amounts of tissues in comparison to western blot analysis.

Levels of various proteins were analyzed using standard western blot analysis, as previously described [12]. Briefly, sodium phosphate buffer, (pH 7.0), was used at a 10 times volume dilution to hand homogenize 2–3 adrenals gland from the same sex and the same litter before being mixed with equal amounts of SDS gel loading buffer (50 mM Tris-HCL, pH 6.8, 2% wt/vol SDS, 10% vol/vol glycerol, 100 mM DTT and 0.1% wt/vol bromophenol blue) to be loaded to a standard 10% SDS-PAGE gel. Protein was then transferred to a PVDF transfer membrane (Amersham Hybond-P, Cat. No. RPN303F, GE Healthcare Lifesciences, Baie D'Urfe, QC), and blocked overnight with 5% milk in TTBS (0.1% vol/vol Tween-20 in TBS) to decrease non-specific antibody binding. Membranes were then probed with primary antibodies (**Table 2.1**) for 1–2 h at room temperature. Washing was done with TTBS, 3 × 10 min before labeling with horseradish peroxidase-labeled rabbit secondary antibody (**Table 2.1**), for 1 h at room temperature. After 3 × 10 min TTBS washes, proteins were detected using ECL and visualized using a chemiliminescence (Cat. No. WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON) and captured on the VersaDoc Imaging System (BioRad, UK). Densitometry was performed using Image Lab Software, comparing levels of proteins expressed as percent of controls.

Table 2.1: Primary and secondary antibodies used for western blotting.

Antibody	Company	Catalog Number	Dilution Used
Perilipin	Cell Signaling	3470	1:1000
StAR	Santa Cruz	Sc-25806	1:4000
Cyp11A1	Bioss	Bs-3608R	1:200
SF-1	Abcam	Ab168380	1:1000
GAPDH	Imgenex	IMG-5567	1:10000
Anti-Rabbit	R&D systems	HAF008	1:3000

2.2.3 Hormone Assays

Levels of corticosterone and ACTH in plasma samples (plasma from one litter and the same sex were pooled, and used as one sample) were determined with an ELISA Kit following the manufacturer's instructions (corticosterone: Abcam, ab108821, 1:60 plasma dilution, Toronto, ON; ACTH: Phoenix Peptide EKE-001-21, 1:1 plasma dilution, Burlingame, CA). To eliminate inter-assay variations, all samples were analyzed in triplicate in one assay, and the intra-assay coefficient of variation was <5%.

2.2.4 Statistical Analysis

Results are presented as mean ± SEM of four to five different litters, as indicated in figure legends. Data were analyzed using two-way ANOVA followed by Tukey's post-hoc test, or Student's *t*-test as indicated. Significance was set at $p < 0.05$. Calculations were performed using Graphpad Software Prism version 6.

2.3 Results

2.3.1 Effects of prenatal BPA exposure on adrenal gland weight

To determine if prenatal BPA exposure affected body weight, mice were weighed at 8 weeks. No significant differences in body weight were observed in either sex of BPA-exposed and non-exposed control mice (**Figure 2.1A & B**). To investigate if prenatal BPA exposure resulted in altered adrenal gland weight, the weight of adrenal glands and the ratio of adrenal gland weight to body weight were determined. An increase in adrenal gland weight was observed in both male ($P < 0.05$) and female ($P < 0.01$) mice prenatally exposed to BPA when compared with controls (**Figure 2.1C & D**). Furthermore, the ratio of adrenal gland weight to body weight was significantly increased in both BPA-exposed male ($P < 0.05$) and female ($P < 0.01$) mice (**Figure 2.1E & F**).

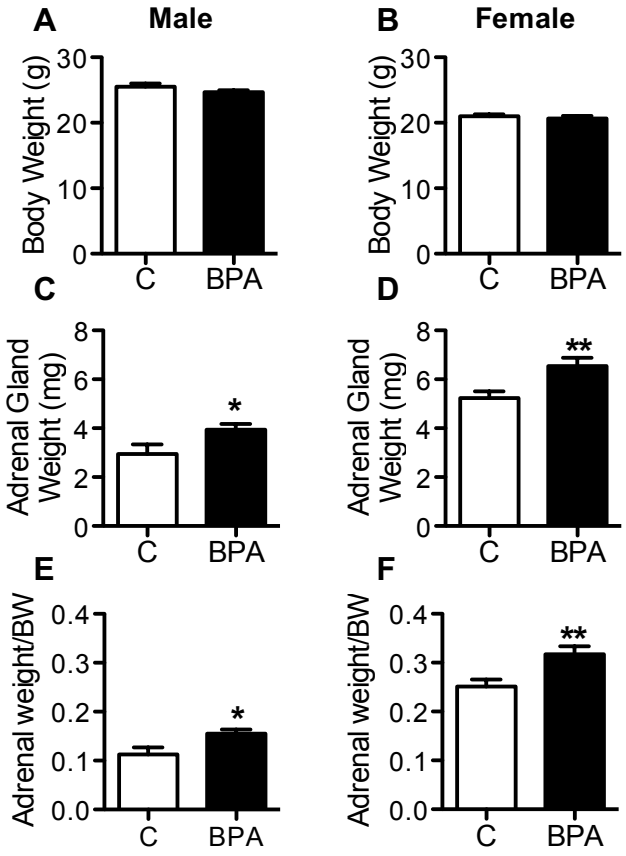

Figure 2.1: Effects of prenatal BPA exposure on adrenal gland weight.

Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from E7.5 to birth. At eight weeks of age, offspring were sacrificed, body weight (A & B) and adrenal gland weight (C & D) were recorded, and adrenal gland to body weight ratio (E & F) was then calculated. Data are presented as mean ± SEM (n = 16–22; *P < 0.05, **P < 0.01, *vs.* control).

2.3.2 Effects of prenatal BPA exposure on basal plasma corticosterone and ACTH levels

To determine if prenatal BPA exposure affected adrenal plasma corticosterone, plasma corticosterone levels were measured using ELISA. We found that corticosterone levels were significantly increased in both male ($P < 0.01$) and female ($P < 0.05$) mice prenatally exposed to BPA when compared to control mice (**Figure 2.2A**). To ascertain if elevated corticosterone levels were a result of hyper-pituitary activity, plasma ACTH levels were measured with ELISA. We observed no differences in plasma ACTH levels between control and prenatally BPA-exposed mice (**Figure 2.2B**).

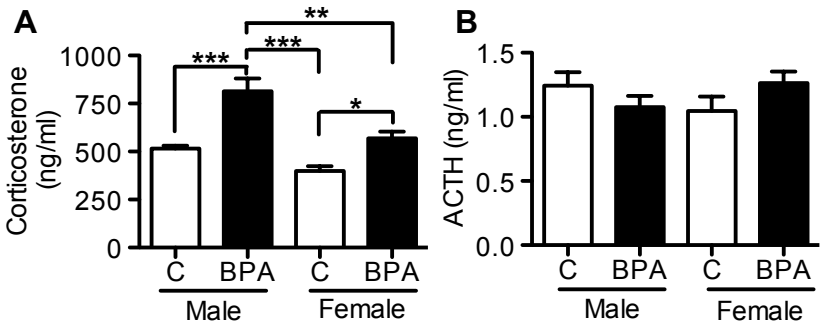

Figure 2.2: Effects of prenatal BPA exposure on plasma corticosterone and ACTH levels.

Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from E7.5 to birth. At eight weeks of age, offspring were sacrificed, and plasma samples were collected. Plasma levels of corticosterone (A) and ACTH (B) were measured by standard ELISA. Data are presented as mean ± SEM; statistical significance was determined using a 2-way ANOVA followed by Tukey's post-hoc test (n = 3–5; *P < 0.05, **P < 0.01, ***P < 0.001).

2.3.3 Effects of prenatal BPA exposure on perilipin protein levels

To determine if prenatal exposure to BPA altered substrate availability for adrenal steroidogenesis, adrenal levels of perilipin, a surrogate for cholesterol content [13], were measured using western blot analysis. The level of perilipin protein was similar between control and prenatally BPA-exposed mice in both sexes (**Figure 2.3**).

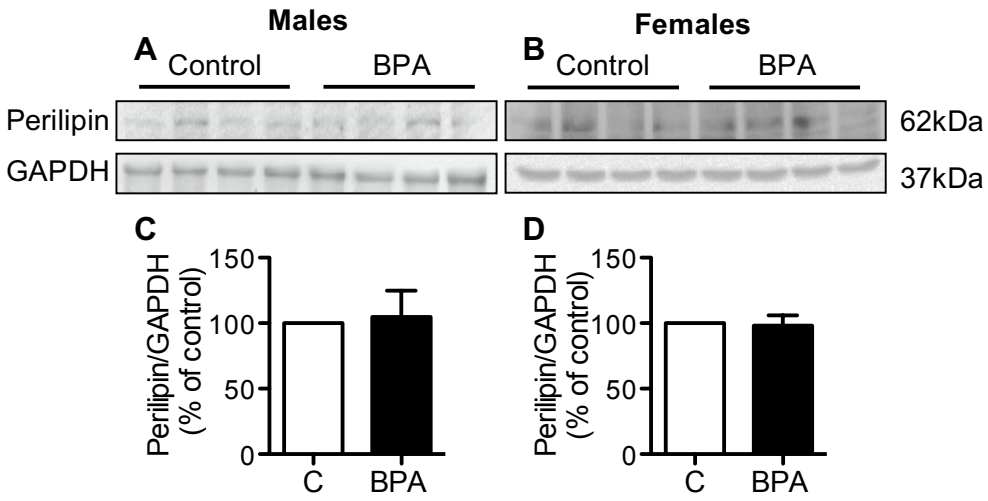

Figure 2.3: Effects of prenatal BPA exposure on perilipin protein levels in adrenal glands.

Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from E7.5 to birth. At eight weeks of age, offspring were sacrificed, and adrenal glands were collected. Levels of perilipin protein in adrenal gland tissue homogenates were determined separately in males (A & C) and females (B & D) by western blot analysis. Data are presented as mean ± SEM (n = 4).

2.3.4 Effects of prenatal BPA exposure on StAR and cyp11A1 protein levels

To study the effects of prenatal exposure to BPA on the rate-limiting steps of steroidogenesis, levels of StAR and cyp11A1 proteins were measured by western blotting. We found a significant increase in both StAR ($P < 0.01$) and cyp11A1 ($P < 0.05$) protein levels in prenatally BPA exposed female mice compared to controls (**Figure 2.4B, D & F**). By contrast, no changes in either StAR or cyp11A1 protein were observed in male mice prenatally exposed to BPA when compared to control males (**Figure 2.4A, C & E**).

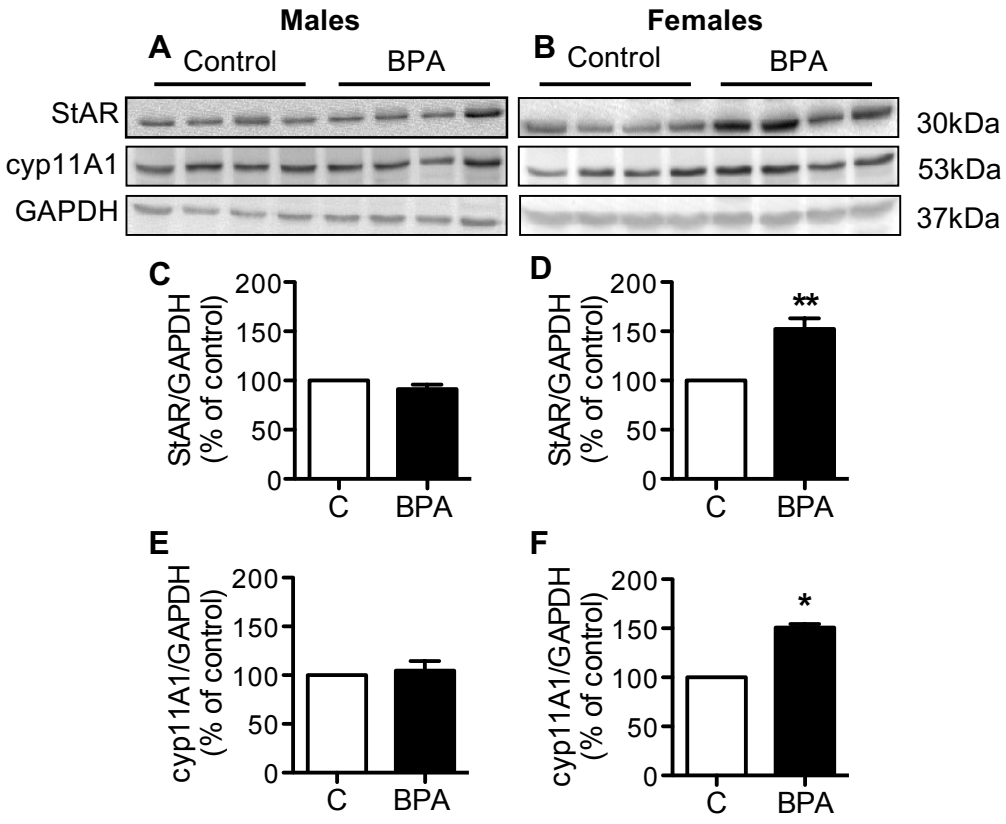

Figure 2.4: Effects of prenatal BPA exposure on StAR and Cyp11A1 protein levels. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from E7.5 to birth. At eight weeks of age, offspring were sacrificed, and adrenal glands were collected. Levels of StAR and cyp11A1 protein in adrenal gland tissue homogenates were determined separately in males (A, C & E) and females (B, D, & F) by western blot analysis. Data are presented as mean ± SEM (n = 4; *P < 0.05, **P < 0.01 *vs.* control).

2.3.5 Effects of prenatal BPA exposure on SF-1 protein levels

To investigate the effects of prenatal exposure to BPA on the regulatory mechanisms of adrenal steroidogenesis, we examined the expression of steroidogenic factor-1 (SF-1), a key transcription factor involved in the regulation of StAR and cyp11A1. We found no significant changes in the level of SF-1 protein in either female or male mice prenatally exposed to BPA when compared to control mice of the same sex (**Figure 2.5**).

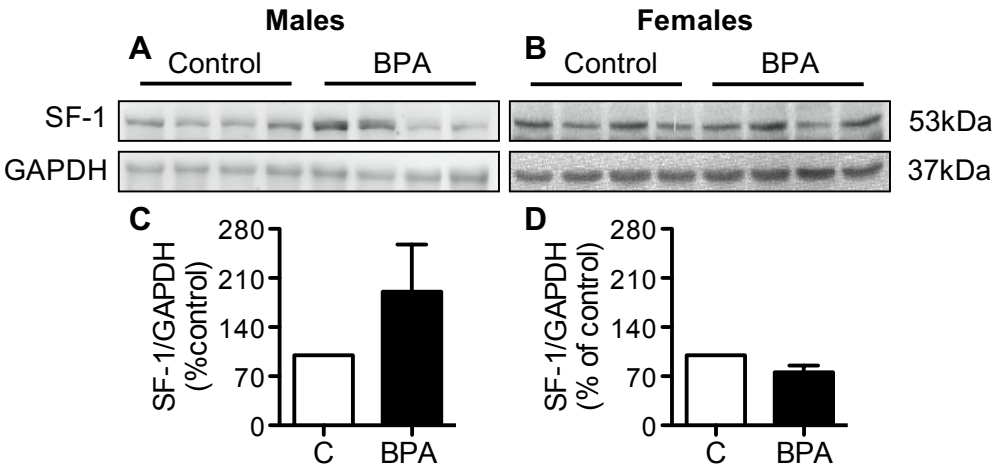

Figure 2.5: Effects of prenatal BPA exposure on SF-1 protein levels in adrenal glands.

Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from E7.5 to birth. At eight weeks of age, offspring were sacrificed, and adrenal glands were collected. Levels of SF-1 protein in adrenal gland tissue homogenates were determined separately in males (A & C) and females (B & D) by western blot analysis. Data are presented as mean ± SEM (n = 4).

2.4 Discussion

In the present study, we demonstrate that prenatal exposure to BPA results in increased basal corticosterone levels independent of circulating ACTH levels in both male and female adult mouse offspring. Furthermore, we provide evidence indicating that BPA-induced increases in basal corticosterone levels are likely a consequence of up-regulated adrenal steroidogenesis in female mice while the mechanisms behind the BPA-induced increase in corticosterone levels in males are unknown. Thus, our present findings provide novel insight into the long-term and sex-specific effects of developmental BPA exposure on adrenal steroidogenesis.

The dose of BPA used in this study (25 mg BPA/kg diet; equivalent to 5 mg BPA/kg body weight) was chosen based on our previous dose-response studies in which we found that prenatal exposure to this dose of BPA led to impaired fetal lung maturation without any effect on fetal body weight or litter size [14]. This dose is also one tenth of the no observed adverse effect level (NOAEL) for rodents (50 mg/kg/day), as determined by the U.S. Environmental Protection Agency (IRIS 2012). Importantly, maternal concentrations of BPA in our mouse model were determined to be 1.7 ng/ml measured using gas chromatography–mass spectrometry (GC–MS) [14], which is at the lower end of the range of those reported in the serum of pregnant women in the US [15].

As a first step in investigating the effects of prenatal BPA exposure on adrenal gland development and function, we sought to determine changes in adrenal gland weight. We found that the weight of adrenal glands was significantly increased in both male and female mice prenatally exposed to BPA when compared to offspring of control mice. Since BPA did not alter body weight, we observed a similar increase in the ratio of adrenal gland weight to body weight in prenatally BPA-exposed offspring in both sexes. This is in marked contrast to the findings of a previous study, which showed that maternal BPA exposure during pregnancy and lactation led to increases in both adrenal gland weight and the ratio of adrenal to body weight only in female but not male juvenile rats [8]. Although the precise reasons for the discrepancy between the two studies are not clear, it is possible that differences in the dosage of BPA and the length of its exposure as well as the animal species and the offspring age (at which the study was conducted) are

important contributing factors. Increased adrenal weight can result in increased adrenal steroid output, which can be a result of increased output per adrenal cell and/or an increase in the number of adrenal cells.

We then determined the functional significance of the BPA-induced increases in adrenal gland weight by examining changes in plasma levels of corticosterone, the principal glucocorticoid synthesized by the adrenal gland in rodents. We found that although prenatal exposure to BPA resulted in a significant increase in basal corticosterone levels in both male and female mice, this increase was greater in BPA exposed males compared to BPA females. This suggests that plasma corticosterone levels are more vulnerable to BPA exposure *in utero* in male than in female offspring. Previous studies reported sex-dependent changes in plasma corticosterone resulting from developmental exposure to BPA. For example, one study showed that prandial administration of 40 μg BPA/kg body weight per day throughout pregnancy and lactation led to elevated plasma corticosterone levels in juvenile female and but not male rats [8,9]. In another study, Zhou, et al. [10] also observed an increase in basal corticosterone in female juvenile rats exposed to 40 μg BPA/kg body weight per day throughout pregnancy and lactation; however male rats were not examined in that study. In contrast, Chen, et al. [11] reported an increase in corticosterone levels in adult male but not female rats as a result of daily subcutaneous administration of 2 μg BPA/kg body weight from gestation day 10 to lactation day 7. These discrepancies can be attributed to differences in the study design, including the dosage, the timing, the duration, and the mode of BPA administration as well as the age at which corticosterone levels were measured. Consistent with previous studies, there were no differences in basal corticosterone levels between control male and female adult mice [16-18]. It is interesting to note that basal corticosterone levels were slightly higher in both males and females in our study when compared to those published previously [19-21], which may be attributed to differences in the time of the day when blood samples were collected, because corticosterone is known to be released in a circadian fashion [22,23]. Furthermore, the presence of varying degrees of potential stressors in the animal housing environment, such as noise, human traffic and lighting conditions, may also be a contributing factor [24-26].

Given that elevated circulating corticosterone levels are commonly associated with enhanced ACTH release from the anterior pituitary [27], we measured plasma ACTH levels and found that they were not altered in either female or male offspring following prenatal BPA exposure. This suggested that the BPA-induced increases in basal plasma corticosterone levels are independent of pituitary ACTH, and likely the result of a direct effect of BPA on the adrenal gland. Our present findings are in marked contrast with those reported previously showing a concomitant increase in plasma levels of corticosterone and ACTH in adult male rats [11] and juvenile female rats following perinatal exposure to BPA [10]. As discussed above, differences in the timing, dosage and duration of BPA exposure likely accounted for the contrasting findings between these studies.

Our conclusion of BPA exerting a direct effect on the adrenal gland is supported by previously published *in vitro* evidence showing that BPA inhibited cortisol and corticosterone secretion in human adenocarcinoma H295R cells, by inhibiting cyp17A1 (17,20 lyase) [28]. Furthermore, BPA reduced the mRNA levels of StAR and cyp11A1, the two rate-limiting factors in the *de novo* steroidogenesis, in cultured mouse ovarian follicles [29,30], while another study reported increased mRNA levels in rat ovarian theca-interstitial (T-I) cells and granulosa cells following exposure to BPA [31]. Furthermore, BPA has been shown to alter mRNA levels of other steroidogenic P450 enzymes, such as 3β-HSD and 17β-HSD in rat testis [32]. In addition, BPA inhibited the activities of 3β-HSD, CYP17A1 and 17β-HSD3 in both human and rat testis microsomes [33]. However, to date, changes in the expression of StAR and cyp11A1, or any other proteins/enzymes involved in steroidogenesis, in the adrenal gland following BPA exposure *in vivo* have not been examined.

As a first step in examining the effects of prenatal exposure to BPA on steroidogenesis in the adrenal gland, we sought changes in substrate availability by determining and comparing levels of perilipin protein between control and BPA exposed offspring. Perilipin is a protective coating protein surrounding the periphery of lipid droplets, which are stored in the adrenal gland and are associated with cholesterol ester droplets [13,34]. We found that perilipin protein content was not different between control and BPA exposed

mice in either sex, suggesting that cholesterol content, and by inference the substrate availability for steroidogenesis, is not altered in adult mouse offspring prenatally exposed to BPA. This is similar to the findings of a previous *in vitro* study, which showed that BPA had no effect on perilipin levels in human hepatocyte cells [35]. To the best of our knowledge, this is the first study to examine changes in perilipin expression following BPA exposure *in vivo*.

We then examined changes in StAR and cyp11A1, the two rate-limiting steps in steroidogenesis. We found that adrenal protein levels of both StAR and cyp11A1 were elevated in female but not male mice, suggesting that the BPA-induced increases in corticosterone levels in our female offspring are likely the result of an enhanced adrenal steroidogenesis. Importantly, these findings demonstrate that although prenatal exposure to BPA alters basal plasma corticosterone levels in both male and female offspring, its effects on adrenal steroidogenesis are sex-specific. A similar sex-dependent effect was reported by Xi, et al. [36], who showed that developmental exposure to BPA led to decreased expression of StAR and cyp11A1 in the testes, whereas no changes in StAR and an increase in cyp11A1 were detected in the ovaries. However, the lack of a corresponding increase in StAR and cyp11A1 in the adrenal gland of the male offspring begs the question of the reasons behind increased corticosterone levels in these animals. Potential mechanisms/reasons may include changes in one or more of the steroidogenic enzymes downstream of cyp11A1. Obviously, future studies will be required to address this issue.

Given that steroidogenic factor-1 (SF-1) is a key transcription factor responsible for the induction of StAR and cyp11A1 as well as other steroidogenic enzymes [37,38], we determined if changes in the expression of this transcription factor are responsible for our observed increases in levels of StAR and cyp11A1 proteins in the adrenal gland of BPA-exposed female offspring. We found that adrenal levels of SF-1 protein were similar between control and BPA-exposed mice in both males and females. This suggested that other transcription factors, such as C/EBPs, Sp1, and DAX1 [39,40], may be involved in up-regulating StAR and cyp11A1 in the adrenal gland of our BPA-exposed female offspring. Alternatively, BPA-induced phosphorylation of SF-1 could account for the increased

level of StAR and cyp11A1 protein, since phosphorylation of SF-1 at the StAR promoter is required to increase expression of StAR [41,42]. Obviously, future studies will be required to examine these possibilities. It is interesting to note that BPA exposure resulted in decreased expression of SF-1 in cultured human granulosa cells [43].

2.5 Conclusion

In conclusion, the present study demonstrates that prenatal exposure to BPA disrupts corticosterone homeostasis in the circulation without altering plasma ACTH levels in both male and female adult mouse offspring. We also provide evidence that BPA disrupts steroidogenesis independent of SF-1 in a sex-specific manner. Thus, our present findings provide novel insight into the dynamic effects of developmental exposure to BPA on the pituitary-adrenal axis development and function.

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3 BISPHENOL A INDUCES STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) EXPRESSION VIA AN UNKNOWN MECHANISM INDEPENDENT OF TRANSCRIPTION, TRANSLATION AND PROTEIN HALF-LIFE IN HUMAN ADRENAL CORTICAL CELLS[1]

[1] The material in this chapter is based on a manuscript submitted to *Steroids*: Medwid S, Guan H, Yang K. Bisphenol A induces steroidogenic acute regulatory protein (StAR) expression via an unknown mechanism independent of transcription, translation and protein half-life in human adrenal cortical cells (2017).

3.1 Introduction

In Chapter 2, I demonstrated that prenatal BPA exposure increased StAR protein expression in adult female offspring. Given that StAR is the rate-limiting step in adrenal steroidogenesis, in the following chapter, I sought to determine the molecular mechanisms underlying BPA-induced StAR expression using an *in vitro* cell model system

Bisphenol A (BPA) is a widespread endocrine disrupting chemical, and a source of major health concerns. BPA is commonly used in polycarbonate plastics and epoxy resins, such as plastic containers and the inner-lining of food cans [1-5]. BPA is present in human saliva, urine, and plasma. More concerning is the presence of BPA in placenta, cord blood, amniotic fluid and breast milk [6-9], raising serious concerns about exposure to BPA *in utero* and during critical periods of postnatal development [10]. Indeed, numerous human epidemiological studies have demonstrated an association between gestational exposure to BPA and adverse health outcomes including pregnancy complications, male genital abnormalities, childhood obesity, childhood asthma and altered neurological development in children and adults [11-13].

We recently showed that prenatal BPA exposure led to altered adrenal gland development and function in adult mouse offspring [14]. Specifically, plasma levels of corticosterone were elevated concomitant with increased adrenal levels of steroidogenic acute regulatory protein (StAR), the rate-limiting step in steroidogenesis, in adult female offspring [14]. StAR is responsible for the transport of free cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondria membrane (IMM), the first and the rate-limiting step in steroidogenesis [15]. BPA is known to alter StAR mRNA and protein levels in various reproductive tissues, and these effects appear to be species-, sex-, dose- and exposure time-specific [16-27]. However, the molecular mechanisms underlying the effects of BPA on steroidogenesis, and particularly StAR expression are largely unknown. Therefore, the present study was designed to address this important question using a human adrenal cortical cell line as an *in vitro* model system.

3.2 Methods

3.2.1 Reagents

Bisphenol A was purchased from Sigma-Aldrich Canada Ltd. (>99% purity; CAS 80-05-7; Oakville, ON) and dissolved in ethanol to prepare a 10mM stock solution, and stored at -20°C. ICI 182, 760 (ICI) was purchased from Tocris (cat. no. 1047) and dissolved in DMSO to prepare a 100mM stock, and stored at -20°C. 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol) (PPT) and 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) were purchased from Tocris (cat. no. 1426 and 1494, respectively) and dissolved in ethanol to a concentration of 5 mM and 100 mM, respectively, and stored at -20°C. Cycloheximide (CHX) was purchased from Sigma (C-0934) and dissolved in ethanol to prepare a 100 mM stock and stored at -20°C.

3.2.2 Cell Culture

The adrenocortical human cell line NCI-H295 cell line was derived from an adrenal tumor of a 48-year-old female and was first described by Gazdar et al. [28]. The NCI-H295 cell line expresses all steroidogenic enzymes present in the human fetal adrenal glands and is an established model to study adrenal steroidogenesis [29]. The subline, NCI-H295A, was further derived and characterized from the H295R cell line, and is currently the best available model of human fetal adrenal gland cells [30]. H295A cells (generously provided by Dr. Walter L. Miller) were cultured in RPMI 1640 media (Invitrogen) with 2% fetal bovine serum (FBS; Sigma), 0.1% insulin-transferrin-selenium supplement (Sigma I18884) and 100IU penicillin and 100μg/mL streptomycin (Invitrogen) at 37°C (5% CO_2). Growth medium was replaced every other day.

3.2.3 Western Blot Analysis

Levels of various proteins were analyzed using standard western blot analysis, as previously described [31]. Briefly, cells were lysed in SDS gel loading buffer (50 mM Tris-HCL, pH 6.8, 2% wt/vol SDS, 10% vol/vol glycerol, 100 mM DTT and 0.1% wt/vol bromophenol blue) to be loaded in a standard SDS-PAGE gel. Protein was then transferred to a PVDF transfer membrane (Amersham Hybond-P, cat. no. RPN303F, GE Healthcare Lifesciences, Baie D'Urfe, QC), and blocked overnight with 5% milk in TTBS (0.1% vol/vol Tween-20 in TBS). Membranes were then probed with primary antibodies

for 1-2 hours at room temperature (**Table 3.1**). Washing was done with TTBS, 3×10 minutes before labeling with horseradish peroxidase-labeled secondary antibody (**Table 3.1**) for 1 hour at room temperature. After 3×10 minute TTBS washes, proteins were detected using ECL and visualized using chemiluminescence (cat. no. WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON) and captured on the VersaDoc Imaging System (BioRad). Densitometry was performed using Image Lab Software, comparing levels of proteins expressed as percent of controls.

Table 3.1: Primary and secondary antibodies used for western blotting.

Antibody	Company	Catalog Number	Dilution Used
StAR	Santa Cruz	Sc-25806	1:4000
GAPDH	Cell Signaling	14C10	1:10000
Cyp11A1	Bioss	Bs-3608R	1:200
3β-HSD	Santa Cruz	Sc-30820	1:500
ERα	Santa Cruz	Sc-543	1:500
ERβ	Santa Cruz	Sc-8974	1:1000
SF-1	Abcam	Ab168380	1:1000
C/EBPβ	Snata Cruz	Sc-150	1:500
Sp1	Millipore	17-601	1:8000
AKAP149	Santa Cruz	Sc-377450	1:100
β-tubulin	Imgenex	IMG-5810A	1:1000
Anti-Rabbit	R&D systems	HAF008	1:3000
Anti-goat	Millipore	AP180P	1:8000
Anti-mouse	BIO RAD	170-6516	1:7500

3.2.4 Real time quantitative RT-PCR

The relative abundance of various mRNAs was determined by a two-step real time quantitative RT-PCR (qRT-PCR), as described previously [32], with the following modifications. Briefly, total RNA was extracted from cells using RNeasy Mini Kit (Qiagen Inc., Mississauga, ON) coupled with on-column DNase digestion with the RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a total volume of 20 µl using the High Capacity cDNA Archive Kit (Applied Biosystems, Forest City, CA) following the manufacturer's instructions. For every RT reaction set, one RNA sample was set up without reverse-transcriptase enzyme to provide a negative control. Gene transcript levels of GAPDH (housekeeping gene whose expression level was found to be stable across all treatment groups), and StAR were quantified separately by pre-designed and validated TaqMan® Gene Expression Assays (Applied Biosystems; GAPDH Hs02758991_g1; StAR Hs00986559_g1) following the manufacturer's instructions. Briefly, gene expression assays were performed with the TaqMan® Gene Expression Master Mix (Applied Biosystems P/N #4369016) and the universal thermal cycling condition (2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C) on the ViiA™ 7 Real-Time PCR System (Applied Biosystems).

The relative amount of StAR and GAPDH mRNA was quantified by the comparative CT method (also known as ΔΔ CT method) using the Applied Biosystems relative quantitation and analysis software according to the manufacturer's instructions. The amount of StAR mRNA was normalized to GAPDH (housekeeping gene) for each treatment group. StAR mRNA in BPA treatment is expressed relative to the amount of transcript present in the control.

3.2.5 Statistical Analysis

Results are presented as group means ± SEM for each treatment group, as indicated. Control and BPA groups were compared using a Student's *t*-test or a one-way ANOVA, followed by a Tukey's post hoc; statistical significance was set at $P<0.05$. Statistical analysis was performed using statistical software PRISM 1992-2008 GraphPAD Software.

3.3 Results

3.3.1 Concentration-dependent effects of BPA on StAR protein expression

To validate this *in vitro* model system, we determined the effects of various concentrations of BPA on StAR protein levels in H295A cells. We found that treatment with increasing concentrations of BPA (1-1000 nM) for 48 h resulted in a concentration-dependent increase in StAR protein levels (**Figure 3.1**).

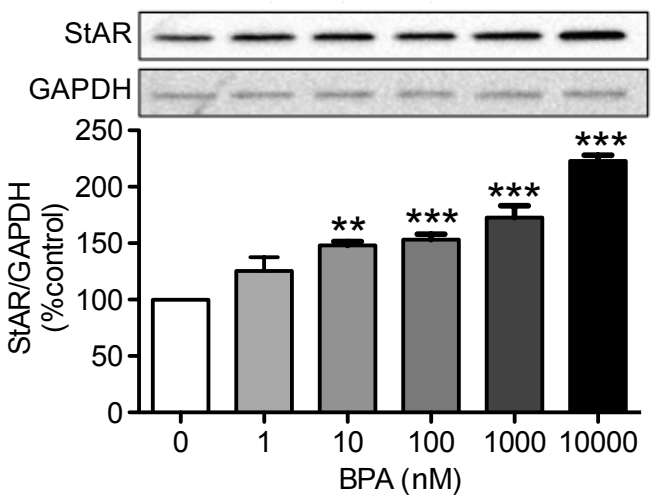

Figure 3.1: Concentration-dependent effects of BPA on StAR protein expression.
H295A cells were treated with various concentrations of BPA (1 – 10000 nM) for 48 h. At the end of treatment, levels of StAR protein were determined by western blotting. Data are presented as means ± SEM (**P<0.01, ***P<0.001; n=4 independent experiments).

3.3.2 Effects of BPA on selected key steroidogenic enzymes

To assess the effects of BPA on adrenal steroidogenesis, protein levels of the two key steroidogenic enzymes, Cyp11A1 and 3β-HSD, were measured following treatment with 10 nM BPA. We showed that protein levels of both Cyp11A1 and 3β-HSD were not different between cells treated with and without BPA (**Figure 3.2A & B**).

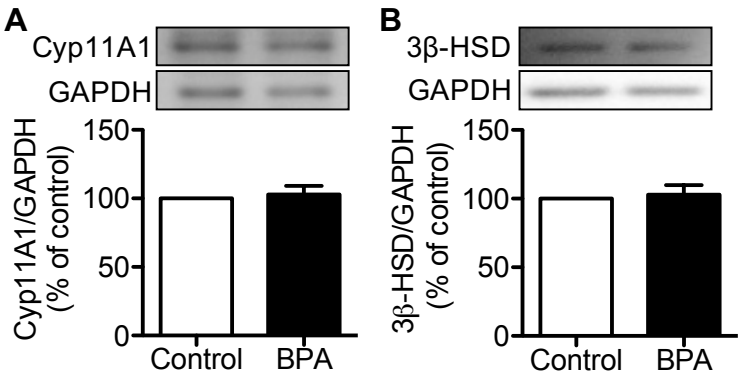

Figure 3.2: Effects of BPA on selected key steroidogenic enzymes.

H295A cells were treated with 10 nM of BPA for 48 h. At the end of treatment, protein levels of Cyp11A1 (A) and 3β-HSD (B) were measured by western blotting. Data are presented as means ± SEM (n=4 independent experiments).

3.3.3 Effects of BPA on ERα and β protein expression

To determine the effects of BPA on ER expression, protein levels of ERα and ERβ were assessed following BPA treatment. We found that neither ERα (**Figure 3.3A**) nor ERβ (**Figure 3.3B**) protein levels were altered by BPA.

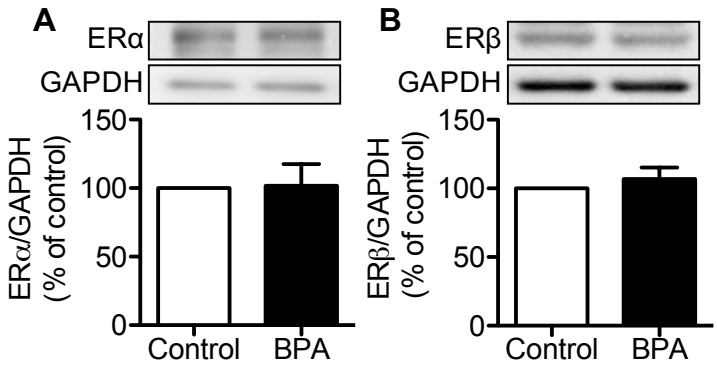

Figure 3.3: Effects of BPA on ERα and β protein expression.

H295A cells were treated with 10 nM of BPA for 48 h. At the end of treatment, protein levels of ERα (A), and ERβ (B) were measured by western blotting. Data are presented as means ± SEM (n=4 independent experiments).

3.3.4 Effects of ER agonists and antagonist on StAR protein expression

BPA has been shown to activate both ERα and ERβ [33-35]. As a first step in determining if ER is involved in mediating the effects of BPA on StAR expression, we assessed changes in StAR expression following treatment with the ERα selective agonist PPT and the ERβ selective agonist DPN, respectively (**Figure 3.4A**). We found that both PPT and DPN increased StAR protein levels. We then examined the effects of the ER antagonist ICI 182, 780 (ICI) on BPA-induced expression of StAR. We showed that ICI completely prevented BPA-induced increases in levels of StAR protein (**Figure 3.4B**).

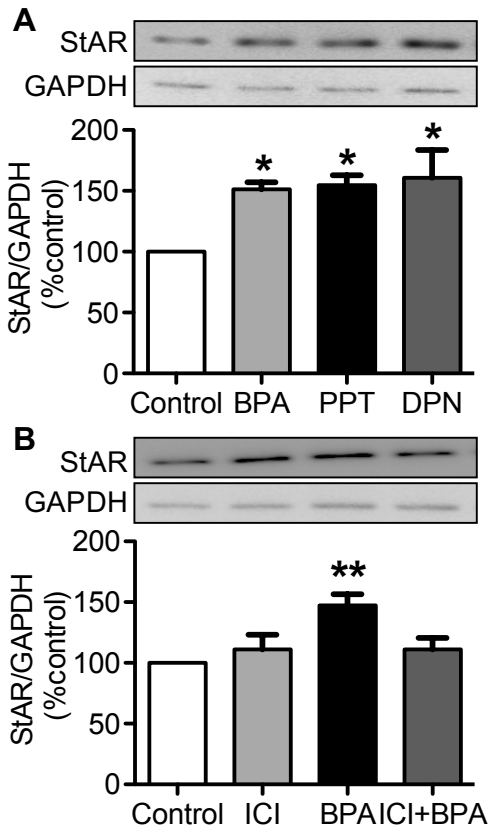

Figure 3.4: Effects of ER agonists and antagonist on StAR protein expression.

H295A cells were treated with 10 nM PPT, 10 nM DPN, 10 nM BPA, 100 nM ICI 182,780 (ICI), or BPA plus ICI for 48 h. At the end of treatment, levels of StAR protein were measured by western blotting (A&B). Data are presented as means ± SEM (*P<0.05, **P<0.01; n=5 independent experiments).

3.3.5 Effects of BPA on key StAR transcription factors and StAR mRNA levels

To explore the molecular mechanisms underlying BPA-induced StAR protein expression, we determined the effects of BPA on the expression of key StAR transcription factors [15,36] and StAR mRNA. Although BPA did not alter SF-1 protein levels (**Figure 3.5A**), it increased protein levels of C/EBPβ (**Figure 3.5B**) and Sp1 (**Figure 3.5C**). Interestingly, BPA had no effect on StAR mRNA (**Figure 3.5D**).

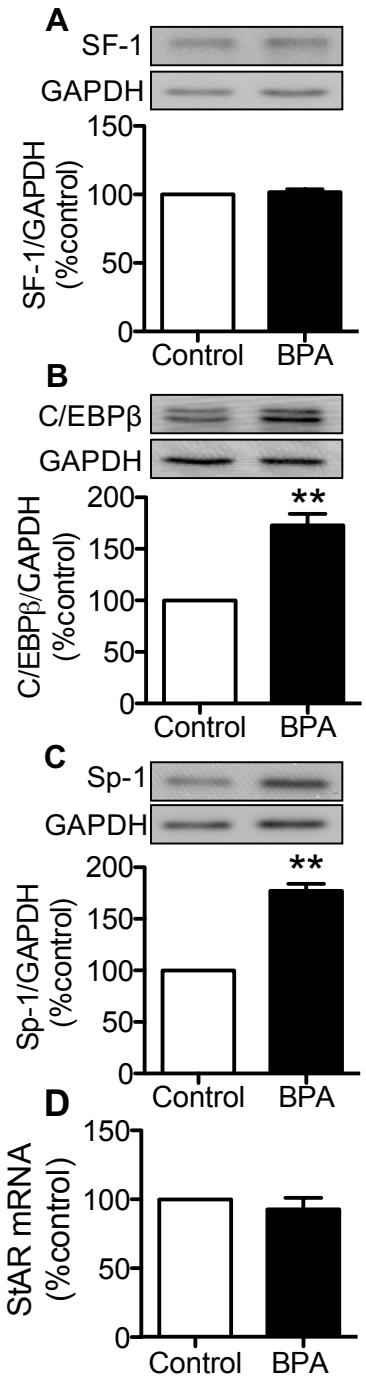

Figure 3.5: Effects of BPA on key StAR transcription factors and StAR mRNA levels.

H295A cells were treated with 10 nM of BPA for 48 h. At the end of treatment, protein levels of SF-1 (A), C/EBPβ (B) and Sp-1 (C) as well as levels of StAR mRNA (D) levels were measured by western blotting and qRT-PCR, respectively. Data are presented as means ± SEM (**P<0.01; n=4 independent experiments).

3.3.6 Effects of BPA on key StAR translation protein and StAR pre-protein

We then investigated the possibility that BPA may influence the translation of StAR protein by examining changes in the key kinase involved in recruiting StAR mRNA and translating it, namely A-Kinase anchoring protein (AKAP) 149 [37,38] as well as the 37-kDa StAR pre-protein [39]. Treatment with BPA did not alter levels of either AKAP149 protein (**Figure 3.6A**) or 37-kDa StAR pre-protein (**Figure 3.6B**).

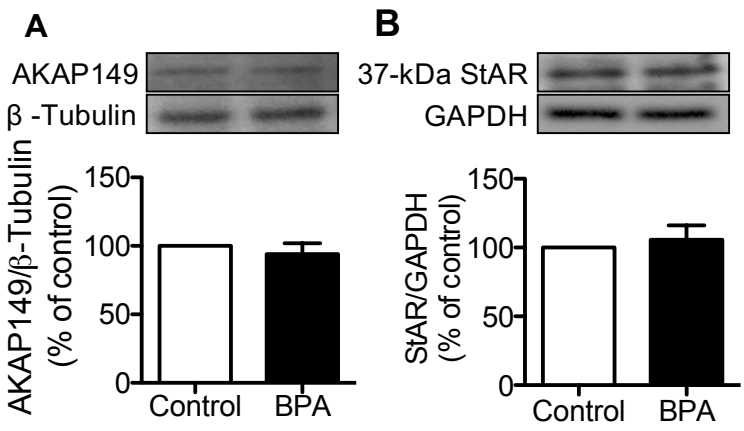

Figure 3.6: Effects of BPA on key StAR translation protein and StAR pre-protein. H295A cells were treated with 10 nM of BPA for 48 h. At the end of treatment, levels of AKAP149 protein (A) and 37-kDa StAR pre-protein (B) were measured by western blotting. Data are presented as means ± SEM (n=4 independent experiments).

3.3.7 Effects of BPA on half-life of StAR protein

To determine if BPA alters the half-life of StAR protein, cells were treated with BPA for 48 h, followed by co-treatment with the translation inhibitor cycloheximide (CHX) for 2 and 4 hours prior to harvesting for StAR protein measurement. We found that BPA did not affect the half-life of StAR protein (Control=3.1 h; BPA=3.0 h; **Figure 3.7**).

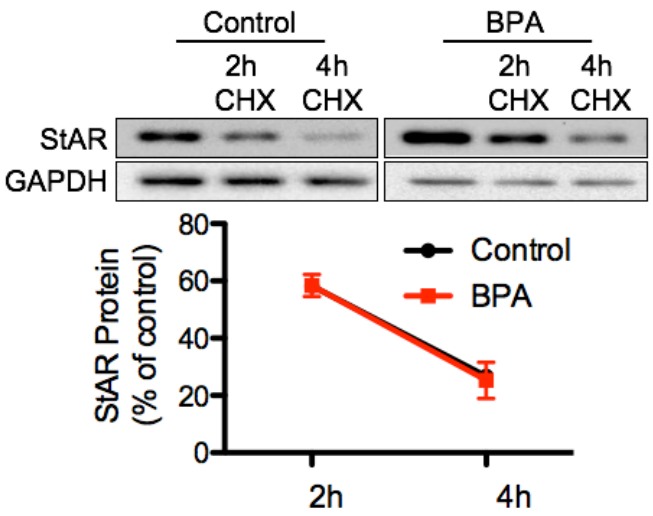

Figure 3.7: Effects of BPA on half-life of StAR protein.

H295A cells were treated with 10 nM of BPA for 48 h, and then 10 μM cycloheximide (CHX) was added for 2 h and 4 h prior to harvest. StAR protein levels were measured by western blotting, and its half-life determined by standard method. Data are presented as means ± SEM (n=4 independent experiments).

3.4 Discussion

Adrenal steroidogenesis and most notably glucocorticoid production is critically controlled by the rate-limiting protein StAR [15,40]. We recently demonstrated that prenatal exposure to BPA resulted in altered adrenal development and function leading to increased plasma corticosterone levels in adult mouse offspring [14]. Furthermore, the increased plasma corticosterone levels were concomitant with elevated adrenal levels of StAR protein in female but not male adult offspring [14]. This gender-specific changes in StAR expression is intriguing and begs the question of the molecular mechanisms that underlie BPA-induced adrenal StAR expression in female offspring. Thus, the present study was designed to address this important question using the H295A cell line, which is currently the best available *in vitro* model of human fetal adrenal cortical cells. Using this model, we have provided evidence indicating that BPA induces StAR protein expression via an ER-mediated novel molecular mechanism that does not appear to involve StAR gene transcription, translation or protein half-life.

The concentration range of BPA (1-10000 nM) used in the present study was consistent with that used previously *in vitro* [41], and was lower than what was previously shown to alter the steroidogenic pathway in non-adrenal cells [16-19]. Importantly, the 10 nM BPA concentration (equivalent to 2.28 ng/ml) used throughout the present study is well within the range previously reported in urine (0.16-43.42 ng/ml) [42] and plasma (0.5-22.3 ng/ml) [43] of pregnant women.

Since we previously demonstrated that prenatal BPA exposure led to increased StAR protein levels in adrenal glands of female offspring [14], we sought first to validate our *in vitro* model system. Indeed, we found that BPA increased StAR protein levels in a concentration-dependent manner in H295A adrenal cortical cells. Previously, numerous studies have examined the effects of BPA treatment on StAR protein levels in reproductive organs with varying effects [22-27]. *In vivo* studies demonstrated decreased StAR protein levels in the testes and ovaries of rodents after acute BPA exposure [22-25,27]. In contrast, Qiu et al. [26] observed increased StAR protein levels in the testes of male rats after chronic BPA treatment. Thus, the effects of BPA exposure on StAR protein appear to be organ-, tissue- and dose-specific. However, to date no studies have been reported to

examine the molecular mechanisms by which BPA induced StAR expression in any steroidogenic tissues or cells.

BPA has been shown to affect other key steroidogenic enzymes involved in adrenal glucocorticoid production, including cyp11A1 and 3β-HSD [14,44,45]. We also examined changes in these key enzymes, and found that protein levels of both cyp11A1 and 3β-HSD were not altered by BPA treatment. It is interesting to note that we previously reported that prenatal BPA exposure led to increased adrenal levels of cyp11A1 protein in female but not male adult mouse offspring [14]. Furthermore, Lan et al. [45] used the adrenal mouse cell line, Y1, as well as male mice treated with acute doses of BPA, and demonstrated an increase in adrenal cyp11A1 protein levels. However, in H295R adrenal cortical cells, only very high concentrations of BPA (10 μM) were shown to decrease mRNA levels of cyp11A1 and 3β-HSD [44]. Thus, these discrepancies in BPA-induced changes in cyp11A1 and 3β-HSD may be explained by differences in cell lines, treatment regime, BPA doses/concentrations and exposure times. Taken together, these findings suggest that BPA affects adrenal steroidogenesis primarily through altered StAR protein expression.

BPA is a known agonist for ERβ, and has dual agonistic and antagonistic actions for ERα, with a higher binding affinity to ERβ than to ERα [34,35]. Additionally, human H295R adrenal cortical cells have been shown to express both ERα and ERβ, with the latter being the dominant receptor present [46]. Therefore, we first examined the effects of BPA on ERα and ERβ protein expression. We found that levels of both ERα and ERβ protein were not changed after 48 hours of BPA treatment. ERα null mice showed increased StAR levels in fetal and adult testes [47,48]. Additionally, male transgenic mice expressing human aromatase, resulting in higher than normal levels of estrogen, had decreased StAR levels [47,48].However, whether a similar phenotype is present in the adrenal glands is unknown. We then examined the effects of ERα- and ERβ-specific agonists, PPT and DPN, on StAR protein. We found that both PPT and DPN increased StAR protein levels, indicating that both ERα and ERβ are involved in regulating StAR expression. To determine if BPA signals through ER to increase StAR expression, cells were treated with BPA in the presence and absence of the ER antagonist ICI. We found that ICI

abrogated the stimulatory effects of BPA on StAR protein levels. Taken together, these results suggest that BPA induces StAR protein expression via ERα and/or ERβ in adrenal cortical cells.

StAR is thought to be largely regulated transcriptionally, with numerous transcription factors binding to the StAR promoter [15,37,40,49]. SF-1 is a master regulator of adrenal steroidogenesis, since SF-1 null mice exhibited diminished basal levels of StAR [15,37,40,50]. Other key transcription factors involved in StAR regulation include C/EBPβ and Sp1 [15,37,40]. Therefore, we investigated the effects of BPA on these three key transcription factors. We found that protein levels of SF-1 were unchanged by BPA, which is consistent with what we and others have previously demonstrated in experimental animal models [14,51]. In contrast, we found protein levels of C/EBPβ and Sp1 were increased after 48 hours of BPA treatment. Increased levels of C/EBPβ were also observed in 3T3-L1 pre-adipocyte cells after BPA treatment [52]. C/EBPβ expression was decreased in livers of male, but not female, offspring after prenatal BPA exposure [53]. Previously, Sp1 was shown to be decreased in resorbed embryos but unchanged in viable embryos [54]. Taken together, these findings suggest that BPA alters the expression of specific transcription factors known to regulate StAR transcription.

As a first step in determining if BPA affects StAR transcription, we measured StAR mRNA levels. StAR mRNA was found to be unchanged after 48 hours of BPA treatment. This is consistent with a previous study in H295R adrenal cortical cells, which showed that low concentrations of BPA (0.1-1 μM) did not affect StAR mRNA levels, while higher concentrations of BPA (10 μM) decreased StAR mRNA [44]. Other studies in reproductive tissues also demonstrated no significant changes in StAR mRNA after BPA treatment [20,21]; however, StAR protein levels were not measured in those studies. In contrast, numerous studies in reproductive cells showed altered StAR mRNA levels, but results varied greatly in direction and magnitude of change [16-19]. Interestingly, StAR protein levels have been shown to be altered, independent of changes in StAR mRNA, after exposure to oxysterols [55], pesticides [56], endotoxins [57] and prostaglandins [58]; however, the mechanisms behind these effects were not examined. Collectively, these findings suggest that BPA regulate StAR primarily at post-transcriptional levels.

Recent evidence has pointed to a potential post-transcriptional regulation of StAR [37,40]. One potential player is AKAP149, a mitochondrial anchoring protein, which recruits StAR mRNA to the OMM [37,38]. AKAP149 at the OMM promotes the translation of StAR by binding the 3' untranslated region of StAR mRNA to the K homology (KH) RNA-binding motif [37,38,59]. Therefore, we investigated the effects of BPA on AKAP149 protein levels, which we found to be unaltered after 48 hours of BPA treatment. However, we recognized that the activity of AKAP121/149 as well as protein levels and the activity of other key translational regulators were not examined in this study. StAR mRNA is translated into a 37-kDa pre-protein at the OMM [37,60]. Thus, to ascertain the effects of BPA on StAR translation, we determined the level of the 37-kDa StAR pre-protein. We did not observe any changes in the 37-kDa StAR pre-protein levels after treatment for 48 hours with BPA. Taken together, these results suggest that BPA does not affect the translation of StAR protein in adrenal cortical cells.

To further investigate the molecular mechanisms underlying BPA-induced StAR protein expression, we determined the effects of BPA on StAR protein degradation. The degradation of the mature StAR protein occurs in the mitochondrial matrix by the two proteases, LON and AG132, as well as one or more unknown mitochondrial proteases [61,62]. As an indicator of protein degradation, the half-life of 30-kDa StAR protein was determined following treatment with BPA. No differences in the half-life of StAR protein were observed between control and BPA-treated cells. Taken together, these results indicate that BPA does not alter the degradation of StAR protein in H295A cells.

Collectively, the present findings suggest that BPA does not regulate StAR protein levels through changes in transcription, translation or degradation. However, the regulation of StAR is extremely complex and is not yet fully understood [37,49,63]. Thus, BPA may be acting through an unknown mechanism to modulate StAR expression, and obviously future studies will be required to examine this possibility. Additionally, due to the short half-life of both StAR pre- and mature protein [64], transcription and translation markers were only examined at 48 hours, however it is possible that changes in mRNA or 37-kDa StAR pre-protein could happen at earlier time points. Furthermore, we did not examine

the effects of BPA on the cleavage of StAR or its transportation from the OMM to the IMM. However, since we found no changes in the levels of 37-kDa StAR pre-protein, it is unlikely that BPA affects either of these two steps. Furthermore, this study did not measure the 32-kDa isoform of StAR protein, which, if changed, may provide a potential explanation for BPA-induced changes in mature StAR protein levels [60,64].

In conclusion, the present study provides strong evidence that BPA signals through ERα and/or ERβ to increase StAR protein levels in H295A cells via an unknown mechanism independent of StAR gene transcription, translation and protein half-life.

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4 BISPHENOL A STIMULATES ADRENAL CELL PROLIFERATION THROUGH ERβ-MEDIATED ACTIVATION OF THE SONIC HEDGEHOG SIGNALING PATHWAY[1]

[1] The material in this chapter is based on a manuscript submitted to *Journal of Steroid Biochemistry and Molecular Biology*: Medwid S, Guan H, Yang K. Bisphenol A stimulates adrenal cortical cell proliferation via ERβ-mediated activation of the sonic hedgehog signaling pathway (2017).

4.1 Introduction

In Chapter 2, I observed an increase in adrenal gland weight in adult mouse offspring prenatally exposed to BPA, with no changes in basal plasma ACTH levels. In this chapter, I sought to determine the cellular and molecular mechanisms that underlie BPA-induced aberrant adrenal gland developmental phenotype using an *in* vitro cell model system.

Bisphenol A (BPA) is one of the most well-known and prevalent endocrine disrupting chemicals, and has gained universal attention due to its adverse effects in humans and experimental animal models [1]. BPA is widely used in the production of polycarbonate plastics and epoxy resins, such as food and beverage storage containers and thermal paper receipts [1,2]. Biomonitoring studies have detected BPA in human saliva, milk, serum and urine collected globally [2]. More alarming is the presence of BPA in human fetal blood, placental tissue and amniotic fluid [2,3]. This has raised serious concerns about the impact of BPA exposure on the developing fetus during the critical period of organ maturation. Indeed, numerous studies have shown that BPA exerts adverse effects on many fetal organ systems, including the brain [4,5], lungs [6], liver [7], pancreas [8], heart [9], adrenal gland [10,11], mammary gland [12,13], and ovary [14,15].

We recently showed that prenatal exposure to BPA resulted in altered adrenal gland structure and function in adult mouse offspring [10]. Specifically, absolute and relative adrenal gland weight was increased in both male and female adult offspring [10]. Similarly, Panagiotidou et al. reported adrenal hyperplasia in juvenile female rat offspring following exposure to BPA during pregnancy and lactation [11]. Alterations in adrenal weight and structure is normally associated with changes in plasma levels of adrenocorticotrophic hormone (ACTH). However, we did not observe an increase in basal plasma levels of ACTH, and concluded that BPA may directly affect adrenal gland weight independent of plasma ACTH in our prenatally BPA exposed mouse model [10]. BPA has previously been shown to increase cell proliferation in various tissues, including breast cancer [16-18], ovarian cancer [19,20], neuroblastoma [21], Hela [22], prostate cancer [23], seminoma [24] and sertoli cells [25]. However, the effects of BPA on adrenal cortical cell proliferation has never been examined.

Sonic hedgehog (shh) signaling pathway is a key mediator of embryonic development, as well as cell maintenance and tissue repair in adults [26,27]. Specifically, the shh pathway is found to be activated during development, as well as in various forms of cancer due to its role in promoting cell proliferation through direct transcriptional activation of proliferation factors cyclin D1 and cyclin D2 [26]. Shh signaling components (shh, Gli, Patched 1) have been detected in human adrenal cortical cell lines, human fetal and adult adrenal glands, as well as both pediatric and adult adrenal tumors [28,29]. Evidence of shh involvement in adrenal cell proliferation is demonstrated by the presence of an adrenal cortex hypotrophy phenotype in shh null mice [30,31]. Thus, the present study was undertaken to determine ***(1)*** if BPA promotes adrenal cell proliferation, which may help explain the increased adrenal gland weight phenotype we reported in our previous study [10]; and ***(2)*** if so, whether the stimulatory effects of BPA on adrenal cortical cell proliferation are mediated through ERβ-mediated activation of the shh pathway using a human adrenal cortical cell line as an *in vitro* model system.

4.2 Methods

4.2.1 Reagents

Bisphenol A was purchased from Sigma-Aldrich Canada Ltd. (CAS 80-05- 7; Oakville, ON) and dissolved in ethanol to prepare 10 mM stock solution, and stored at -20°C. Cyclopamine was purchased from Toronto Research Chemicals (C988400; Toronto, ON), dissolved in ethanol to prepare 10 mM stock solution and stored at -20°C. 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were purchased from Tocris Bioscience (cat. no. 1494; Minneapolis, MN) and Abcam (ab145148; Toronto, ON), respectively, dissolved in ethanol to a concentration of 100 mM, and stored at -20°C.

4.2.2 Cell Culture

The adrenocortical human cell line NCI-H295 cell line was derived from an adrenal tumor of a 48-year-old female and was first described by Gazdar et al. [32]. The NCI-H295 cell line expresses all steroidogenic enzymes present in the human fetal adrenal glands

and is an established model to study adrenal steroidogenesis [33]. The subline, NCI-H295A, was further derived and characterized from the H295R cell line, and is currently the best available model of human fetal adrenal gland cells [34]. H295A cells (generously provided by Dr. Walter L. Miller) were cultured in RPMI 1640 media (Invitrogen) with 2% fetal bovine serum (FBS; Sigma), 0.1% insulin-transferrin-selenium supplement (Sigma I18884) and 100IU penicillin and 100µg/mL were starved in serum-free media 24 h before treatment, and cultured in 0.2% FBS media throughout treatments.

4.2.3 Western Blot Analysis

Levels of various proteins were analyzed using standard western blot analysis, as previously described [35]. Briefly, cells were lysed in SDS gel loading buffer (50mM Tris-HCL, pH 6.8, 2% wt/vol SDS, 10% vol/vol glycerol, 100mM DTT and 0.1% wt/vol bromophenol blue) to be loaded to a standard SDS-PAGE gel. Protein was then transferred to a PVDF transfer membrane (Amersham Hybond-P, cat. no. RPN303F, GE Healthcare Lifesciences, Baie D'Urfe, QC), and blocked overnight with 5% milk in TTBS (0.1% vol/vol Tween-20 in TBS). Membranes were then probed with primary antibodies for 1-2 hours at room temperature (**Table 4.1**). Washing was done with TTBS, 3×10 minutes before labeling with horseradish peroxidase-labeled secondary antibody (**Table 4.1**), for 1 hour at room temperature. After 3×10 minute TTBS washes, protein were detected using ECL and visualized using chemiluminescence (cat. no. WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON) and captured on the VersaDoc Imaging System (BioRad). Densitometry was performed using Image Lab Software, comparing levels of proteins expressed as percent of controls.

Table 4.1: Primary and secondary antibodies used for western blotting.

Antibody	Company	Catalog Number	Dilution Used
PCNA	Cell Signalling	2586	1:5000
GAPDH	Imgenex	IMG-5567	1:10000
Cyclin D1	Santa Cruz	Sc-717	1:500
Cyclin D2	Cell Signalling	3741	1:1000
ERβ	Santa Cruz	Sc-8974	1:1000
Gli1	Abcam	ab49314	1:500
Shh	Santa Cruz	Sc-365112	1:200
β-tubulin	Imgenex	IMG-5810A	1:1000
Lamin B1	Abcam	Ab16048	1:20000
Anti-Rabbit	R&D systems	HAF008	1:3000
Anti-mouse	BIO RAD	170-6516	1:7500

4.2.4 Cell Number Assessment

Cells were seeded in 2% FBS-RMPI 1640 culture medium and were incubated overnight. After 24 h serum starvation, the medium was changed to 0.2% FBS RMPI 1640 containing 10 nM BPA. After 72 h incubation, the cells trypsinized, added in equal volumes to trypan blue stain 0.4% (Invitrogen T10282) and counted with Countess Automated cell counter (Invitrogen C10277).

4.2.5 Real-time quantitative RT-PCR

The relative abundance of various mRNAs was determined by a two-step real time quantitative RT-PCR (qRT-PCR), as described previously [36], with the following modifications. Briefly, total RNA was extracted from cells using RNeasy Mini Kit (Qiagen Inc., Mississauga, ON) coupled with on-column DNase digestion with the RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a total volume of 20 µl using the High Capacity cDNA Archive Kit (Applied Biosystems, Forest City, CA) following the manufacturer's instructions. For every RT reaction set, one RNA sample was set up without reverse-transcriptase enzyme to provide a negative control. Gene transcript levels of GAPDH, GLI1 and SHH were quantified separately by pre-designed and validated TaqMan® Gene Expression Assays (Applied Biosystems; **Table 4.2**) following the manufacturer's instructions. Briefly, gene expression assays were performed with the TaqMan® Gene Expression Master Mix (Applied Biosystems P/N #4369016) and the universal thermal cycling condition (2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C) on the ViiATM 7 Real-Time PCR System (Applied Biosystems).

The relative amounts of various gene-specific mRNAs in each RNA sample was quantified by the comparative CT method (also known as ΔΔ CT method) using the Applied Biosystems relative quantitation and analysis software according to the manufacturer's instructions. For each experiment, gene specific mRNAs were normalized to the housekeeping gene GAPDH. The amount of various gene-specific mRNAs under different treatment conditions is expressed relative to the amount of transcript present in the untreated control.

Table 4.2: TaqMan® gene expression assays for the human genes analyzed.

Gene Name	Assay ID
SHH	Hs00179843_m1
GLI1	Hs00171790_m1
GAPDH	Hs02758991_g1

4.2.6 Statistical Analysis

Results are presented as group means ± SEM of four independent experiments, as indicated. Data was analyzed using a Student's t-test or a one-way ANOVA, followed by a Tukey's post hoc; statistical significance was set at $P<0.05$. Statistical analysis was performed using statistical software GraphPad Prism Version 5 Software.

4.3 Results

4.3.1 Time- and concentration-dependent effects of BPA on cell proliferation.

As a first step in determining the effects of BPA on cell proliferation, protein levels of PCNA, a universal marker of cell proliferation, were assessed over time. Levels of PCNA protein were unchanged at 24 and 48 h, but were significantly elevated at 72 h following treatment with 10 nM of BPA (**Figure 4.1A**). A similar trend of change was observed in cell number following BPA treatment (**Figure 4.1B**). We then treated cells with increasing concentrations of BPA (1-1000 nM) for 72 h, and showed that this treatment resulted in a concentration-dependent increase in PCNA protein levels such that the maximal effect was observed at 10 nM BPA (**Figure 4.1C**).

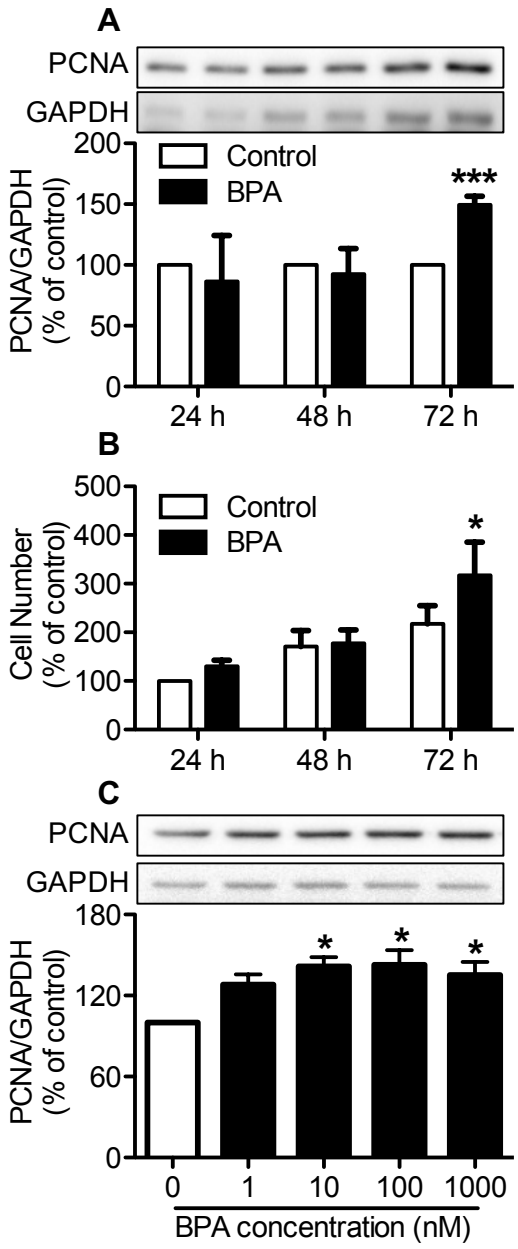

Figure 4.1: Time- and concentration-dependent effects of BPA on cell proliferation. H295A cells were treated with 10 nM of BPA for various times (24-72 h) or increasing concentrations (1-1000 nM) of BPA for 72 h. At the end of treatment, levels of PCNA (a universal marker of proliferation) (**A, C**) and cell number (**B**) were determined by western blotting and cell counting, respectively. Data are presented as mean ± SEM (*P<0.05, ***P<0.001 vs. control; n=4 independent experiments).

4.3.2 Effects of BPA on the expression of key cell proliferation factors.

To further examine the effects of BPA on cell proliferation, protein levels of the two key proliferation factors, cyclin D1 and cyclin D2, were determined. Although levels of both cyclin D1 and cyclin D2 proteins were unchanged after 48 h of BPA treatment (**Figure 4.2A**), they were significantly increased after 72 h of BPA treatment (**Figure 4.2B**).

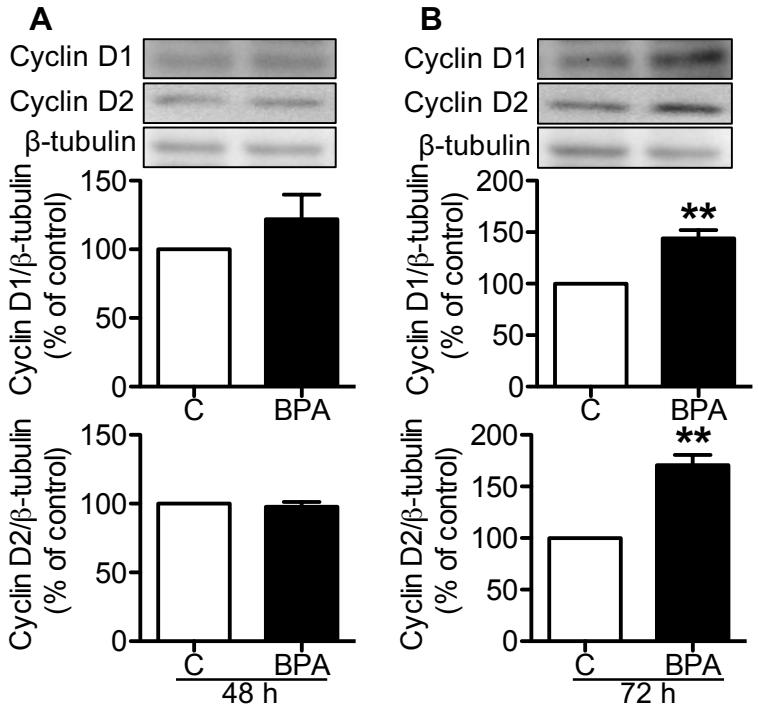

Figure 4.2: Effects of BPA on key cell proliferation factors.

H295A cells were treated with 10 nM of BPA for 48 h (A) or 72 h (B). At the end of treatment, levels of the two key cell proliferation factors, cyclin D1 and cyclin D2 were determined by western blotting. Data are presented as mean ± SEM (**P<0.01 vs. control; n=4 independent experiments).

4.3.3 Effects of BPA on selected components of the shh signaling pathway.

Shh signaling is known to be essential for adrenal development and proliferation. Adrenal specific shh knockout mice display severe adrenal hypoplasia, specifically an underdeveloped cortex in fetal and adult mice [30,37]. To explore the role of shh signaling in mediating BPA-induced cell proliferation, changes in key shh signaling pathway components were examined. Levels of shh mRNA, but not Gli1 mRNA, were increased at 48 h post BPA treatment (**Figure 4.3A&B**). In contrast, protein levels of both shh and Gli1 were elevated following 48 h of BPA treatment (**Figure 4.3C&D**), which returned to control levels at 72 h (**Figure 4.3E&F**).

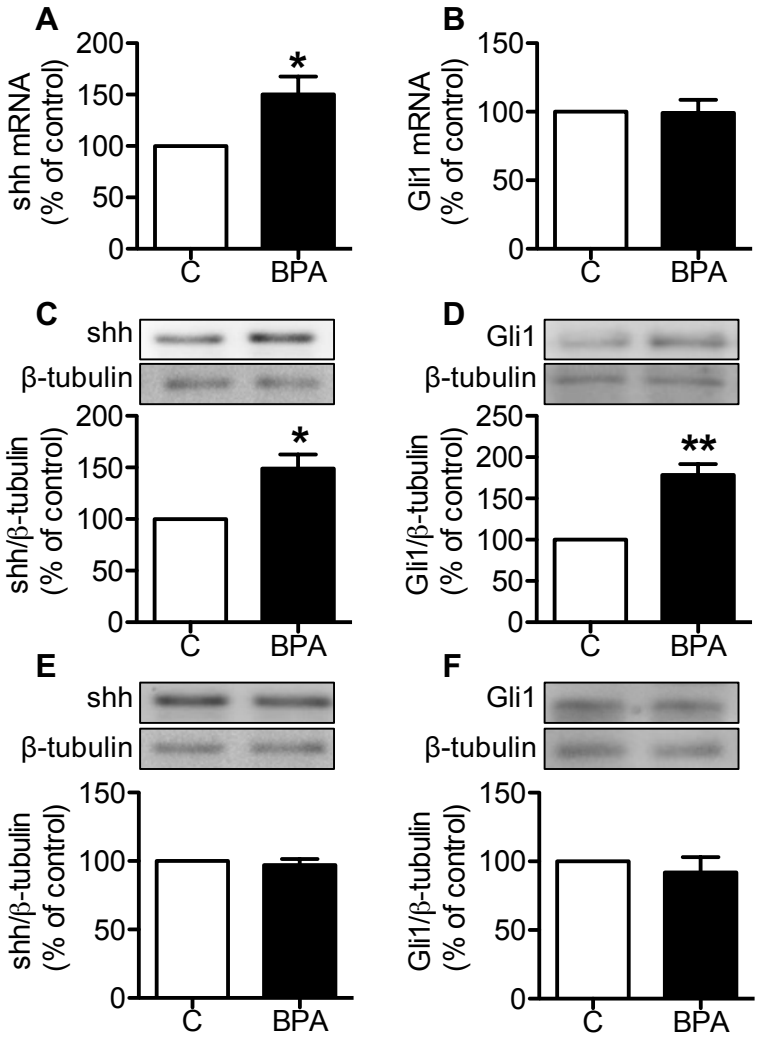

Figure 4.3: Effects of BPA on selected components of the shh signaling pathway.
H295A cells were treated with 10 nM of BPA for 48 h. At the end of treatment, levels of shh mRNA (A) and Gli1 mRNA (B) were determined by qRT-PCR. Levels of shh protein (C&E) and Gli1 protein (D&F) at 48 h (C&D) and 72 h (E&F) were determined by western blotting. Data are presented as mean ± SEM (*P<0.05, **P<0.01, vs. control; n=4 independent experiments).

4.3.4 Effects of BPA on activity of the shh signaling pathway.

Activation of the shh signaling pathway involves translocation of the shh transcription factor Gli1 from cytoplasm to the nucleus where it acts as an activator of shh target genes [38,39]. To determine if BPA activates the shh signaling pathway, we measured Gli1 protein levels in cytosolic and nuclear fractions following treatment with BPA for 48 h. BPA treatment significantly increased Gli1 protein in nuclear but not cytosolic fraction (**Figure 4.4A&B**). To ascertain if BPA activation of the shh signaling pathway is ligand-dependent, we used cyclopamine (Cyc), which blocks the shh pathway at the SMO receptor. We treated cells with BPA in the presence and absence of Cyc, and examined changes in Gli1 protein. We found that Cyc prevented BPA-induced increases in Gli1 protein levels (**Figure 4.4C**).

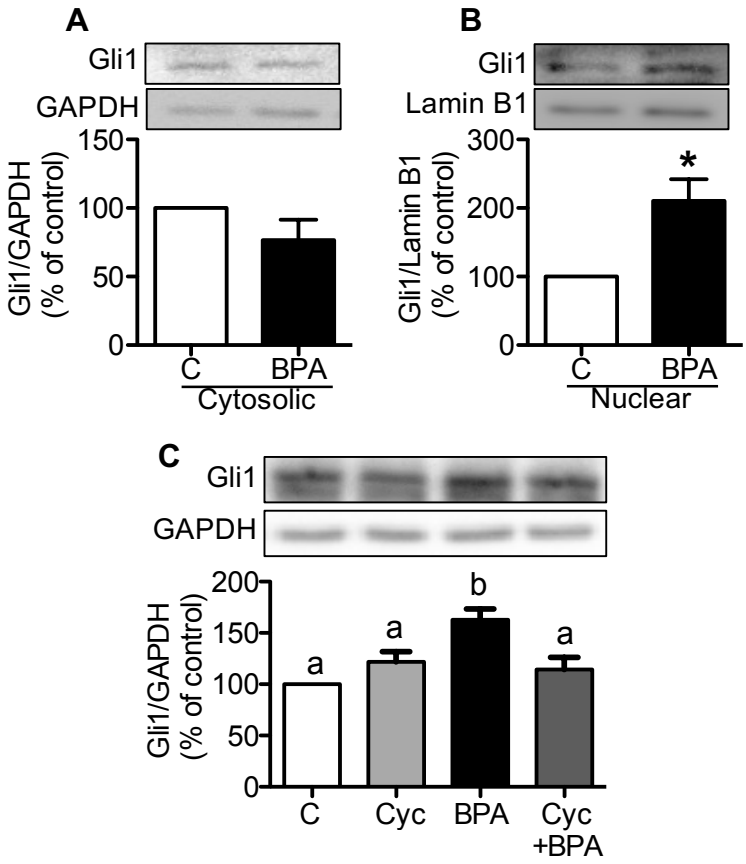

Figure 4.4: Effects of BPA on activity of the shh signaling pathway

H295A cells were treated with 10 nM BPA for 48 h. At the end of the treatment, levels of Gli1 protein in cytosolic (A) and nuclear (B) extracts were determined by western blotting. Alternatively, H295A cells were treated with either 10 nM BPA, 10 μM cyclopamine (Cyc) or both for 48 h. At the end of treatment, levels of Gli1 protein were determined by western blotting (C). Data are presented as mean ± SEM (*P<0.05 vs. control; different letters indicate statistically significant differences among groups; n=4 independent experiments).

4.3.5 Effects of shh pathway inhibition on BPA-induced cell proliferation markers.

Shh signaling is known to induce cell proliferation in a variety of tissues [26,40,41]. Specifically, the transcription factor Gli1 is known to directly stimulate the transcription of cyclin D1 and D2 genes, CCND1 and CCND2 [26]. To provide functional evidence for the involvement of the shh signaling pathway in mediating BPA-induced cell proliferation, we assessed changes in protein levels of PCNA, cyclin D1 and D2 following treatment with BPA in the presence and absence of the shh pathway inhibitor Cyc. Cyc completely blocked BPA-induced increases in protein levels of PCNA (**Figure 4.5A**), as well as cyclin D1 and D2 (**Figure 4.5B**).

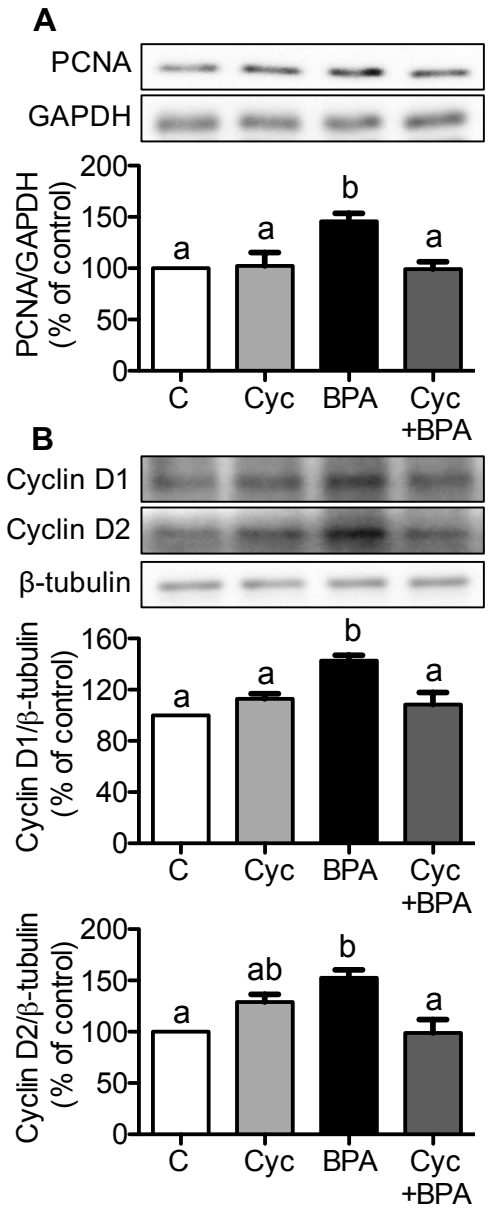

Figure 4.5: Effects of shh inhibition on BPA-induced cell proliferation markers. H295A cells were treated with 10 nM BPA, 10 μM cyclopamine (Cyc) or both for 72 h. At the end of treatment, levels of PCNA (A), cyclin D1 and cyclin D2 (B) were determined by western blotting. Data are presented as mean ± SEM (Different letters indicate statistically significant differences among groups; n=4 independent experiments).

4.3.6 Effects of BPA on estrogen receptor β expression and activity

The translocation of ER from cytosol to the nucleus is essential for transcriptional activation of estrogen target genes [42-44]. To examine if BPA activates ERβ, we measured protein levels of ERβ in total cell lysates as well as cytosolic and nuclear fractions following BPA treatment for 48 h. Although BPA treatment did not alter total ERβ protein levels (**Figure 4.6A**), it decreased cytosolic while increasing nuclear levels of ERβ protein (**Figure 4.6B&C**).

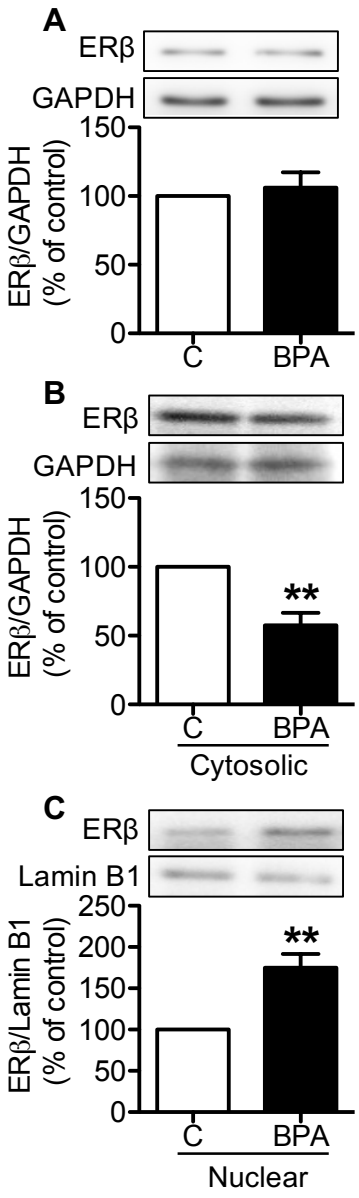

Figure 4.6: Effects of BPA on estrogen receptor β expression and activity.

H295A cells were treated with 10 nM of BPA for 48 h. At the end of treatment, levels of ERβ protein in total (A) cytosolic (B) and nuclear (C) extracts were subjected to western blotting. Data are presented as mean ± SEM (**P<.0.01 vs. control; n= 4 independent experiments).

4.3.7 Effects of DPN and PHTPP on BPA-induced cell proliferation markers.

We then investigated the involvement of ERβ in BPA-induced cell proliferation using the ERβ specific agonist DPN and the ERβ specific antagonist PHTPP. Treatment with DPN significantly increased protein levels of PCNA (**Figure 4.7A**) as well as cyclin D1 and cyclin D2 (**Figure 4.7C**) at 72 h. Furthermore, pretreatment with PHTPP completely prevented BPA-induced increases in levels of PCNA (**Figure 4.7B**), cyclin D1 and cyclin D2 (**Figure 4.7D**) proteins.

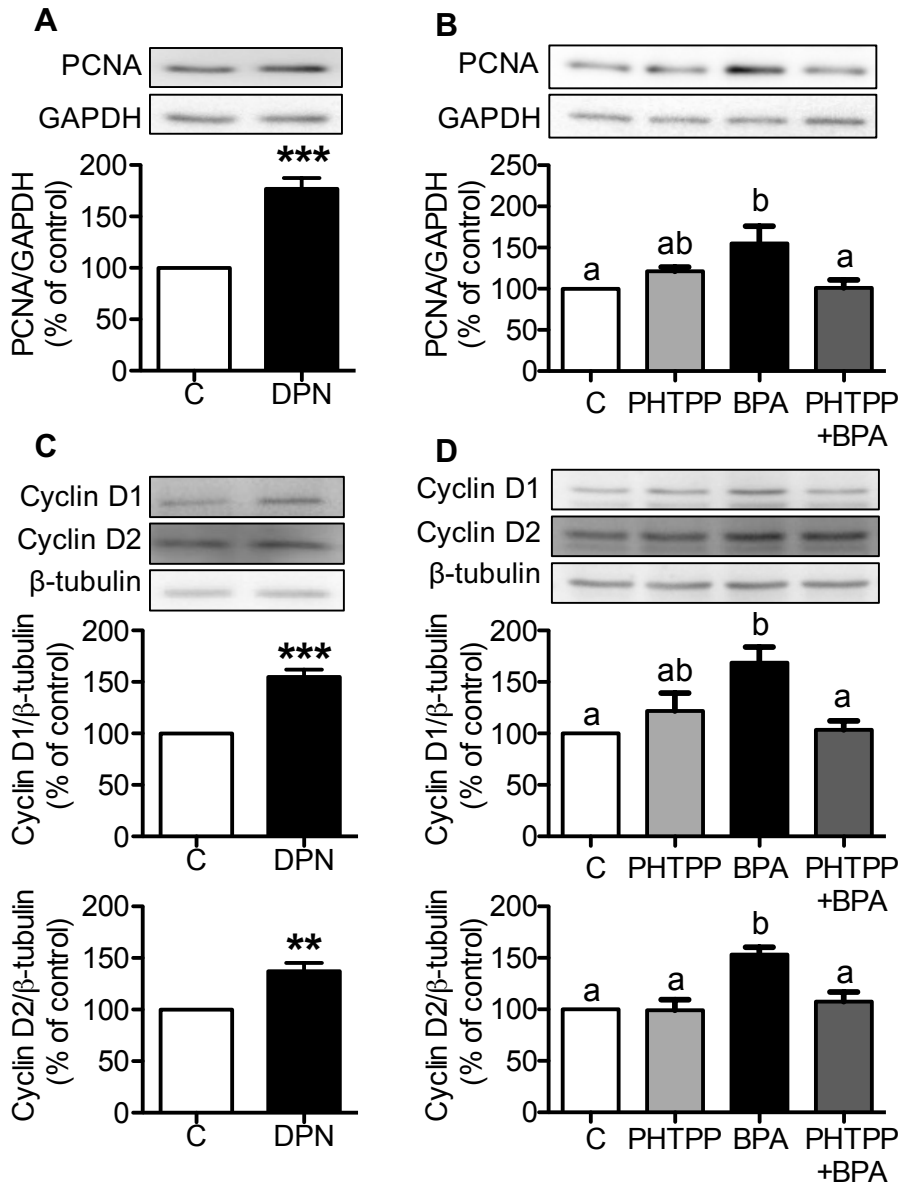

Figure 4.7: Effects of DPN and PHTPP on BPA-induced cell proliferation markers. H295A cells were treated with 10 nM DPN, 10 nM BPA, 100 nM PHTPP, or both PHTPP and BPA for 72 h. At the end of treatment, levels of PCNA protein (A&B), cyclin D1 and cyclin D2 protein (C&D) were determined by western blotting. Data are presented as mean ± SEM (**P<0.01, ***P<0.001 vs. control; different letters indicate statistically significant differences among groups; n=4 independent experiments).

4.3.8 Effects of DPN and PHTPP on BPA-induced activation of the shh signaling pathway.

ERα has been shown to increase shh activity in breast [45,46] and gastric [47] cancer cells. However, this effect has yet to be shown with ERβ. Therefore, we tested the hypothesis that BPA acts through ERβ to activate the shh signaling pathway. We showed that the ERβ specific agonist DPN increased protein levels of both shh and Gli1 after 48 h treatment (**Figure 4.8A&C**). Importantly, the ERβ specific antagonist PHTPP completely blocked BPA-induced increases in both shh and Gli1 protein levels (**Figure 4.8B&D**).

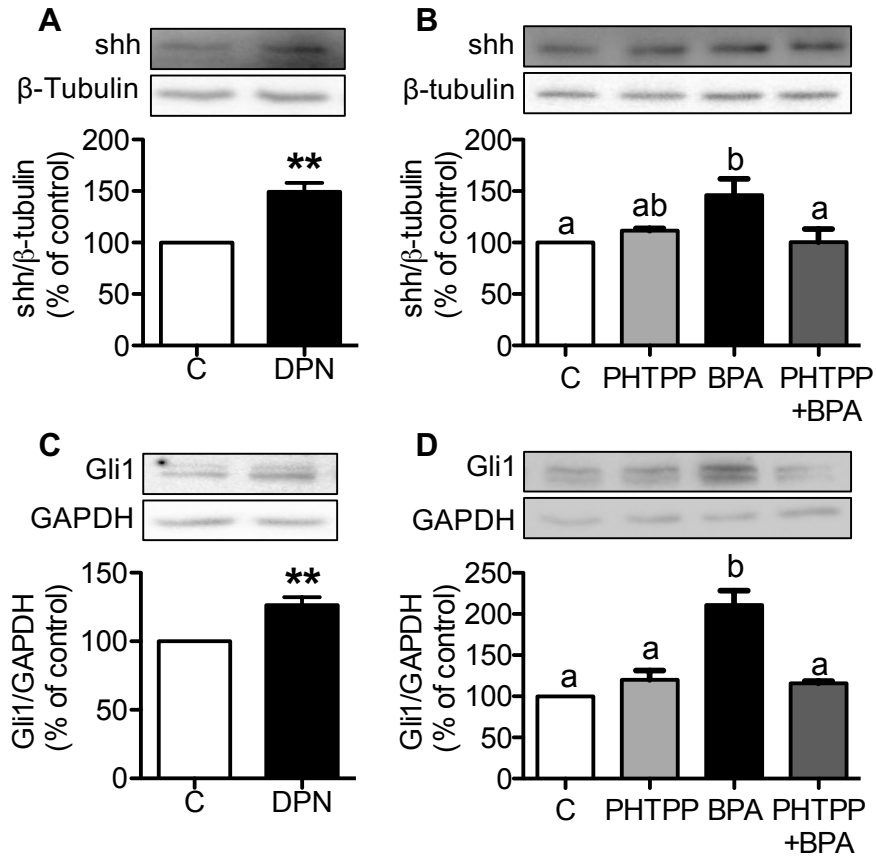

Figure 4.8 Effects of DPN and PHTPP on BPA-induced shh pathway activation. H295A cells were treated with 10 nM DPN, 10 nM BPA, 100 nM PHTPP, or both PHTPP and BPA for 48 h. At the end of treatment, levels of shh protein (A&B) and Gli1 protein (C&D) were determined by western blotting. Data are presented as mean ± SEM (**P<0.01 vs. control; different letters indicate statistically significant differences among groups; n=4 independent experiments).

4.4 Discussions

Proper adrenal gland development is essential for adrenal steroidogenesis, particularly glucocorticoid production in later-life. We recently demonstrated that prenatal exposure to BPA resulted in abnormal adrenal gland development and function in adult mouse offspring, including increased adrenal gland weight independent of plasma ACTH levels [10]. However, the molecular mechanisms underlying the BPA-induced increase in adrenal gland weight remain unknown. Therefore, the present study was designed to address this important question using the best available model of fetal adrenal cortical cells, the H295A cell line. We have demonstrated that BPA stimulates adrenal cell proliferation via ERβ-mediated activation of the shh signaling pathway. Thus, our present findings reveal a plausible molecular mechanism by which BPA influences adrenal gland development and function.

The concentration of BPA used in this study (10 nM) is in line with those used in previous *in vitro* studies [48]. Importantly, this concentration (equivalent to 2.28 ng/ml) is well within the range previously reported in plasma (0.5-22.3 ng/ml) [49] and urine (0.16-43.42 ng/ml) [50] of pregnant women in North American.

BPA has been shown to influence cell proliferation in both *in vivo* and *in vitro* models. In experimental animal models, prenatal exposure to BPA led to increased cell proliferation in fetal liver [7], prostate [51], pancreas [52], and pituitary gland [53]. In contrast, offspring of rats exposed to BPA during pregnancy and lactation showed decreased proliferation in neural stem cells of the hypothalamus and sub-ventricular zone [54]. In several *in vitro* models, BPA increases cell proliferation at various concentrations [16-25]. Interestingly, in sertoli cells, nanomolar concentrations of BPA induced cell proliferation, while micromolar concentrations decreased cell proliferation, suggesting that the effect of BPA on cell proliferation is concentration-dependent [55]. To the best of our knowledge, we are the first to demonstrate that BPA, at environmentally relevant concentrations, significantly increases cell number as well as the expression of PCNA, cyclin D1 and D2, three key markers of cell proliferation, in adrenal cortical cells. This indicates that BPA stimulates adrenal cortical cell proliferation. Thus, our present study provides a plausible cellular

mechanism by which prenatal BPA exposure results in increased adrenal gland weight in adult mouse offspring [10].

Activation of the shh signaling pathway is known to increase the transcription of genes encoding both cyclin D1 and D2 genes, leading to increased cell proliferation [26]. Recently, BPA has been shown to increase levels of microRNA-107 (miRNA-107), which inhibits the expression of suppressor of fused homolog (SUFU) and GLI family zinc finger 3 (Gli3) in human endometrial cancer in RL95-2 cells [56]. Both SUFU and Gli3 are repressors of the shh signaling pathway, thus BPA-induced suppression of these proteins may potentially lead to the activation of shh signaling and consequently increased proliferation in endometrial cells [56]. Therefore, we investigated the possibility that the BPA-induced adrenal cortical cell proliferation may be mediated via activation of the shh signaling pathway. As a first step in examining this possibility, we determined the effects of BPA on shh expression, and found that levels of both shh mRNA and protein were increased after 48 hours of BPA treatment, which preceded the increase in cell proliferation we observed at 72 hours.

An increase in shh protein and secretion results in its binding to the transmembrane receptor Patched 1 (Ptch1), which prevents Ptch1 from inhibiting another transmembrane protein smoothened (SMO) [38,39]. SMO can then be released from the plasma membrane into the cytoplasm, leading to the release of a complex containing the transcription factors Gli1-3, allowing them to translocate to the nucleus to regulate transcription of target genes [38,39]. Specifically, the nuclear translocation of the positive transcriptional regulator Gli1, is considered a marker of shh signaling activation [38,39]. Therefore, we investigated the potential for BPA to alter Gli1 protein and mRNA levels. We found that although BPA did not alter Gli1 mRNA, it increased Gli1 protein levels at 48 hours. The regulation of Gli1 at post-transcriptional level is well established and could be a result of changes in translation and phosphorylation efficiency [57,58]. Furthermore, BPA significantly increased Gli1 protein levels in the nuclear fraction without altering those in the cytosolic fraction, suggesting that BPA enhanced nuclear translocation of Gli1, and consequently the activity of the shh signaling pathway. Given the observed increase in Gli1 protein levels in total cell lysates, the relatively minor and non-significant decrease seen in cytosolic Gli1 levels is consistent with our notion of an enhanced Gli1 nuclear

translocation following BPA treatment. It is known that activation of the shh signaling pathway is mediated through either the ligand-dependent or the ligand-independent pathway [59]. To determine if BPA acts through the ligand-dependent shh signaling pathway, we examined the effects of BPA on Gli1 protein levels in the presence and absence of cyclopamine. Cyclopamine is a potent inhibitor of the shh signaling pathway by preventing release and translocation of the SMO receptor. In the present study, we showed that cyclopamine blocked the effects of BPA on Gli1 protein levels, indicating that BPA activates the shh pathway through the ligand-dependent pathway.

To ascertain whether BPA-induced activation of the shh signaling pathway leads to increased cell proliferation, we treated cells with BPA in the presence and absence of cyclopamine. We found that cyclopamine completely abrogated the stimulatory effects of BPA on cell proliferation, as indicated in protein levels of PCNA, cyclin D1 and D2. Taken together, these results demonstrate the involvement of the shh signaling pathway in BPA-induced adrenal cortical cell proliferation.

It is well known that BPA acts as an ERβ agonist, with a higher affinity for ERβ than ERα [60,61]. Furthermore, ERβ is the dominant estrogen receptor expressed in human H295R adrenal cortical cells [62]. Therefore, we then investigated the role of ERβ in BPA-induced cell proliferation and shh activation. Given that a key step in ERβ activation is its rapid nuclear translocation upon binding to its ligand [63], we determined the effects of BPA on ERβ translocation at 48 h. This time point was chosen based on the BPA-induced increase in shh mRNA at 48 h. We found that levels of ERβ protein were increased in the nuclear fraction but decreased in the cytosolic fraction following BPA treatment, indicating that BPA enhanced translocation of ERβ to the nucleus in H295A cells. However, it is likely that the BPA-induced increase in ERβ nuclear translocation may have occurred earlier than 48 h.

Although estrogen has previously been shown to increase adrenal cell proliferation in both animal models [64] and the H295R cell line [62]. We then sought to determine if activation of ERβ stimulates adrenal cell proliferation using the ERβ selective agonist DPN. We showed that DPN increased protein levels of the three key proliferation markers, PCNA, cyclin D1 and D2, indicating that the activation of ERβ by DPN led to

increased cell proliferation. To provide evidence for the involvement of ERβ in mediating BPA-induced cell proliferation, we treated cells with BPA in the presence and absence of the ERβ-specific antagonist PHTPP. We found that PHTPP completely blocked the stimulatory effects of BPA on PCNA, cyclin D1 and D2 protein. Taken together, these results demonstrate that ERβ mediates BPA-induced proliferation in adrenal cells.

The ability of estradiol to activate the shh signaling pathway has previously been demonstrated in ERα positive breast and gastric cancer cells [45-47], however it remains unknown if a similar effect can be observed through ERβ. Therefore, to determine if ERβ activates the shh signaling pathway in adrenal cells, we examined the effects of ERβ specific agonist DPN on expression of the two key proteins in the shh signaling pathway. We found that DPN increased both shh and Gli1 protein levels, indicating a novel link between ERβ and shh activation. We then determined if the activation of ERβ by BPA leads to activation of the shh signaling pathway. We treated cells with BPA in the presence and absence of ERβ-specific antagonist PHTPP, and found that PHTPP abrogated the stimulatory effects of BPA on shh and Gli1 protein levels. Collectively, these results indicate that BPA stimulates adrenal cell proliferation via ERβ-induced activation of the shh signaling pathway.

In conclusion, the present study demonstrates for the first time that BPA acts on ERβ to activate the shh signaling pathway, which in turn leads to increased proliferation in H295A cells. Thus, our present study reveals a novel BPA-induced cell proliferation signalling pathway that may underlie the increased adrenal gland weight phenotype we reported previously in prenatally BPA exposed adult mouse offspring.

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5 DISCUSSION AND CONCLUSION

5.1 Summary

This thesis utilizes both *in vivo* and *in vitro* approaches aimed at determining the effects of prenatal exposure to environmentally relevant doses of BPA on adrenal gland development and steroidogenic function. Prenatal exposure to BPA resulted in altered adrenal steroidogenesis, as evidenced by increased plasma corticosterone levels. However, there were no corresponding changes in plasma levels of ACTH. The increased plasma corticosterone in female offspring was likely a result of enhanced adrenal expression of StAR, the rate limiting factor of steroidogenesis. However, adrenal StAR expression was not altered in male offspring, suggesting that BPA exerted sex-specific effects on adrenal StAR expression. Using the H295A cell line as an *in vitro* model system, I demonstrated that BPA induced StAR protein expression through an ERα- and/or ERβ-mediated unknown mechanism that was independent of transcription, translation, and protein half-life. I also provided evidence that BPA increased adrenal cortical cell proliferation *in vitro* via a novel molecular mechanism that involves ERβ-mediated activation of the Shh signaling pathway. Taken together, these findings demonstrate that prenatal BPA exposure at environmentally relevant doses disrupts adrenal gland development and steroidogenic function specifically pertaining to glucocorticoid production. They also suggest that BPA-induced aberrant adrenal gland development and steroidogenic function help explain alterations in plasma glucocorticoid levels and HPA dysfunction seen in epidemiological [1,2] and experimental animal studies [3-6].

5.1.1 Doses and concentrations of BPA used in *in vivo* and *in vitro* experiments

The dose of BPA used in Chapter 2 (25 mg BPA/kg diet; equivalent to 5 mg BPA/kg body weight) was chosen based on our previous prenatal exposure dose-response studies in which impaired fetal lung, pancreas, and liver maturation were induced without any effect on fetal body weight or litter size [7-9]. This dose is also one tenth of the LOAEL reported for rodents (50 mg/kg/day), as determined by the U.S. Environmental Protection Agency (IRIS 2012). Importantly, maternal plasma concentrations of BPA in our mouse model were determined to be 1.7 ng/ml, measured using GC–MS [7]. The concentration of BPA (10 nM) used throughout Chapters 3 and 4 is equivalent to 2.28 ng/ml, which is

consistent with previous *in vitro* studies [10]. The doses and concentrations of BPA used in both *in vivo* and *in vitro* experiments are at the lower end of the exposure range reported in the plasma (0.5-22.3 ng/ml) [11] and urine (0.16-43.42ng/ml) [1,2] of pregnant women in the North America. Additionally, pharmacokinetic studies have shown that oral administration of unconjugated BPA resulted in similar plasma levels in rodents and non-human primates [12]. Thus, the dosages of BPA as well as the mouse model and the adrenal cortical cell line used in this thesis are relevant to human exposure.

5.1.2 Prenatal BPA disrupts adrenal steroidogenesis in a sex-specific manner

Exposure to EDC during critical periods of organ maturation affect lifelong organ structure and function, leading to disease and dysfunction later in life [13,14]. Evidence suggests that EDC exposure during fetal development can lead to HPA axis programming alterations, resulting in HPA and adrenal dysfunction in adulthood [15,16]. As such, prenatal BPA exposure in humans is associated with HPA axis dysfunction in both pregnant women and 3-month old infants [1,2]. In addition, animal studies have shown that perinatal BPA exposure caused HPA dysfunction in adult offspring, resulting in changed gene expression in the hypothalamus and pituitary, and altered plasma levels of CRH, ACTH, and glucocorticoid [3-6]. Moreover, Panagiotidou, et al. [6] demonstrated that BPA exposure during pregnancy and lactation resulted in increased adrenal gland weight and abnormal adrenal cortex zone thickness. Taken together, these findings suggest that developmental exposure to BPA can disrupt the HPA axis and adrenal gland development, leading to altered adrenal function in adulthood.

In Chapter 2, the effects of developmental BPA exposure on adrenal function were assessed by examining the hypothesis that prenatal exposure to environmentally relevant doses of BPA disrupts adrenal gland development and steroidogenic function in adult mouse offspring. This study demonstrated that prenatal BPA exposure led to increased basal plasma corticosterone levels independent of ACTH levels in both male and female adult offspring. However, protein levels of StAR and cyp11A1, the two rate limiting factors of steroidogenesis, were only increased in female offspring adrenal glands, demonstrating the sex-specific effects of BPA.

These findings are supported by numerous studies demonstrating the adverse effects of other environmental chemicals on adrenal steroidogenesis. As described in **Table 5.1**, pesticides, fungicides and insecticides (atrazine, imazalil, prochloraz, lindane, and 3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichloroethene (3-MeSO$_2$-DDE)) [17-23], phytoestrogens (diadzein and geinstein) [18,21,22], organophosphates (Diethylumbelliferyl phosphate and dimethoate) [18,24,25], nicotine [26], cadmium [18], mercury [27], and nonlyphenol [28] have all been shown to alter adrenal steroidogenesis by disturbing steroidogenic enzymes and the downstream production of steroid hormone levels.

Table 5.1: Environmental chemicals that alter adrenal steroidogenesis.

Environmental chemical	Structural/ functional impact	Reference
Atrazine	Cyp19 inducer	[17]
Imazalil	Inhibit steroidogenic enzymes Decreased cortisol and aldosterone secretion	[19] [21,22]
Prochloraz	Inhibit cortisol and aldosterone secretion through inhibition of cyp17	[18]
Lindane	Increased steroidogenic enzymes and StAR promotor activity	[20]
3-MeSO_2-DDE	Inhibit cortisol secretion through interaction with cyp11B1	[18,23]
Diadzein	Inhibit activity but not expression of 3β-HSD Decreased cortisol and aldosterone secretion	[21,22] [18]
Geinstein	Inhibit activity but not expression of 3β-HSD	[21,22]
Diethylumbelliferyl phosphate	Decreased cortisol and aldosterone secretion	[18]
Dimethoate	Decreased cortisol and aldosterone secretion Inhibited StAR transcription	[18] [25]
Nicotine	Inhibit transcription of StAR	[26]
Cadmium	Decreased cortisol and aldosterone secretion	[18]
Mercury	Inhibit testosterone and progesterone levels	[27]
4-nonylphenol	Decreased progesterone and increased testosterone and 17β-estradiol levels	[28]

Importantly, evidence suggests that dysfunction of the HPA axis during fetal development results in increased glucocorticoid levels in adults, leading to an increased risk for depression and other mood disorders [29,30]. Thus, these studies provide a possible mechanism behind BPA's association with neurological and behavioural disorders such as depression and anxiety [5,31].

Feedback and signaling pathways are essential for maintaining proper adrenal gland development and function, and thus any alterations in these pathways may lead to disease and dysfunction [32-35]. Furthermore, there are a variety of transcription factors and receptors that are essential for proper developmental processes and can be altered during the critical period of organogenesis [32,33]. Previous experimental animal studies have provided evidence for an important role of the following factors and signaling pathways in adrenal development: ***(1)*** steroid hormone receptors such as ER [36] and glucocorticoid receptors [33]; ***(2)*** Shh [37] and IGF signaling [38]; and ***(3)*** transcription factors, such as SF-1 [39] and DAX-1 [40]. Alterations in any of these factors result in abnormal adrenal development and can potentially lead to altered adrenal function in adult life. Therefore, it is likely that prenatal BPA disrupts fetal adrenal development by altering one or more of these factors and signaling pathways.

5.1.3 BPA stimulates StAR protein expression through estrogen receptor signaling

In Chapter 2, I demonstrated that prenatal BPA exposure increased expression of the rate-limiting factor of adrenal steroidogenesis, StAR, in adult female mouse offspring [41]. Therefore, H295A cells, the best available model of human fetal adrenal cortical cells, were used an *in vitro* model system to investigate the underlying molecular mechanisms.

As shown in Chapter 3, BPA treatment increased StAR protein levels in a concentration-dependent manner, indicating that these cells were a suitable *in vitro* model system. Chronic induction of the rate-limiting step of adrenal steroidogenesis, StAR, suggests a permanent upregulation of the steroidogenic pathway. This would result in an increase in the production of glucocorticoids, which may lead to long term diseases such as depression, anxiety, metabolic dysfunction, and glucocorticoid resistance. Additionally, this sustained increase in adrenal steroidogenesis would suggest that BPA may disrupt

HPA axis function. This is consistent with previous literature suggesting BPA affects the HPA axis at the level of the hypothalamus and pituitary gland [3,5].

Since the ER pathway has been shown to be critical for adrenal gland development, specifically for cortical development and ACTH receptor sensitivity [42], the possible involvement of ER signaling was examined with BPA treatment. The ERα and ERβ isoform specific agonists PPT and DPN, respectively, were used to determine if ERα and/or ERβ were involved in regulating StAR expression. I demonstrated that both PPT and DPN mimicked while the ER antagonist ICI blocked the stimulatory effects of BPA on StAR protein levels. These findings suggested that the BPA-induced StAR protein expression is likely mediated by ERα and/or ERβ. They also revealed a novel role of these two nuclear ERs in regulating adrenal StAR expression, and consequently adrenal steroidogenesis.

I then elucidated the precise molecular mechanism by which BPA, via ERs, induces StAR protein expression. First, I examined if BPA could be interfering with the transcription of StAR mRNA, and found that levels of StAR mRNA were unchanged after BPA treatment. This suggested that BPA did not affect StAR gene transcription. Previous studies have found that StAR protein levels can be altered independent of changes in StAR mRNA after exposure to oxysterols [43], pesticides [44], endotoxins [45] and prostaglandins [46]. However, the post-transcriptional mechanisms behind these effects were not examined.

Next, I examined if BPA altered the translation of the 37-kDa StAR pre-protein, which was found to be unchanged after BPA exposure, suggesting that BPA did not alter StAR translation. The StAR pre-protein is cleaved into the mature 30-kDa isoform at the OMM and transported to the IMM following cholesterol transport [47,48]. If BPA interfered in the processes of StAR cleavage or transport, there would not only be a change in the mature StAR protein levels but also a reciprocal change in the 37-kDa pre-protein. Therefore, since there was no change in StAR pre-protein levels, it is unlikely that BPA affected StAR cleavage or transport.

Lastly, I determined if BPA affected the half-life of StAR protein, and found that it was unchanged following BPA treatment. An increase in mature StAR protein build-up in the mitochondria is normally associated with an increase in corresponding proteases (LON and AFG3L2) responsible for StAR degradation to prevent a novel form of mitochondrial stress, termed StAR overload response (SOR) [49,50]. Thus, the BPA-induced increase in StAR protein levels independent of alterations in the half-life of StAR protein suggests that BPA may alter the SOR. This buildup of StAR protein in the mitochondria may lead to alterations in mitochondria structure and function [49].

Collectively, these findings suggest that BPA increases StAR protein levels likely through ERα and/or ERβ in adrenal cortical cells that involve an unknown novel mechanism independent of StAR gene transcription, translation, and protein half-life. Nevertheless, BPA-induced increases in StAR protein suggest the induction of adrenal steroidogenesis, and consequently an increase in glucocorticoid production. However, future research is required to decipher the precise molecular mechanisms behind these effects to help better understand the adverse effects of BPA, as well as other EDCs, on adrenal gland steroidogenesis.

5.1.4 BPA stimulates adrenal cortical cell proliferation through ERβ-mediated activation of the Shh pathway

In Chapter 2, I demonstrated that prenatal BPA exposure increased both absolute and relative adrenal gland weight without a change in plasma ACTH levels in adult mouse offspring [41]. Increased adrenal weight is normally a result of high plasma ACTH levels [51]. However, I did not observe an increase in basal plasma levels of ACTH, and concluded that BPA likely affects adrenal gland weight independent of ACTH in our prenatal BPA exposed mouse model [41]. Therefore, I addressed this possibility in Chapter 4 using the H295A cell line as an *in vitro* model system. Proliferation in the adrenal gland is essential for development and remodeling in the adult [35,52]. I demonstrated that BPA treatment of H295A cells induced proliferation, as evidenced by increased levels of PCNA (a universal proliferation marker), and key proliferation factors cyclins D1 and D2.

The Shh signaling pathway is heavily involved in the development and formation of the adrenal glands, and *Shh* knockout mice display a severe hypotrophic adrenal gland

phenotype [37,53]. Furthermore, Shh signaling has been shown to induce proliferation in other cell types, by increasing transcription of cyclin D1 and D2 genes [54]. Analysis of the Shh signaling pathway showed that BPA increased mRNA and protein levels of Shh, as well as protein levels and activity (determined by nuclear translocation) of Gli1, a key transcription factor in the Shh signaling pathway. Additionally, inhibition of the Shh pathway by cyclopamine, a well-known Shh signaling pathway antagonist, blocked BPA-induced increase in proliferation. Collectively, these results suggest that BPA activates Shh signaling to increase adrenal cortical cell proliferation. Shh signaling pathway is not only important for cell proliferation but is involved in a variety of other developmental processes that may be altered due to BPA exposure [54]. Previous studies have demonstrated altered Shh levels in adrenal tumors in both adult and pediatric patients, indicating its involvement in adrenal cancer, and suggesting that BPA may increase the risk of adrenal carcinoma tumors [55,56].

The ability of estrogen to activate Shh signaling has previously been shown in breast and gastric cancer cells [57-59]. Therefore, I examined the hypothesis that BPA increases adrenal cell proliferation through ERβ-mediated activation of the Shh pathway. As shown in Chapter 4, the ERβ-specific antagonist PHTPP blocked the stimulatory effects of BPA on PCNA, cyclin D1, and cyclin D2 levels, suggesting that ERβ is involved in mediating BPA-induced adrenal cell proliferation. Furthermore, PHTPP was also shown to prevent the stimulatory effects of BPA on Shh and Gli1 protein levels, linking ERβ to the activating the Shh signaling pathway following BPA treatment. This is consistent with previously reported findings that estrogen activates the Shh signaling pathway through ERα [57-59]. However, my findings demonstrate for the first time that ERβ, through a direct or indirect mechanism, regulates the Shh signaling pathway.

Taken together, these findings suggest that BPA acting through ERβ activates the Shh signaling pathway and results in increased adrenal cortical cell proliferation (**Figure 5.1**). Changes and dysregulation in cell proliferation have previously been associated with increased risk of cancer in numerous tissues [35,54,60]. As well, BPA has been shown to be associated with various types of cancers in both human and experimental animal studies [61-63]. Therefore, it is tempting to speculate that an increase in adrenal cell proliferation

could lead to an increased risk of adrenal cortical cancer. Moreover, higher levels of adrenal proliferation *in vitro* suggest an increased potential for steroid hormone production. If these findings could be extrapolated to humans, it is conceivable that prenatal BPA exposure could lead to chronically elevated glucocorticoid levels. In addition, due to the ubiquitous nature of both Shh and ER expression, there is the potential that this novel BPA signaling pathway induced cell proliferation may be applicable to other tissues, leading to numerous risks, including an increased risk of cancer.

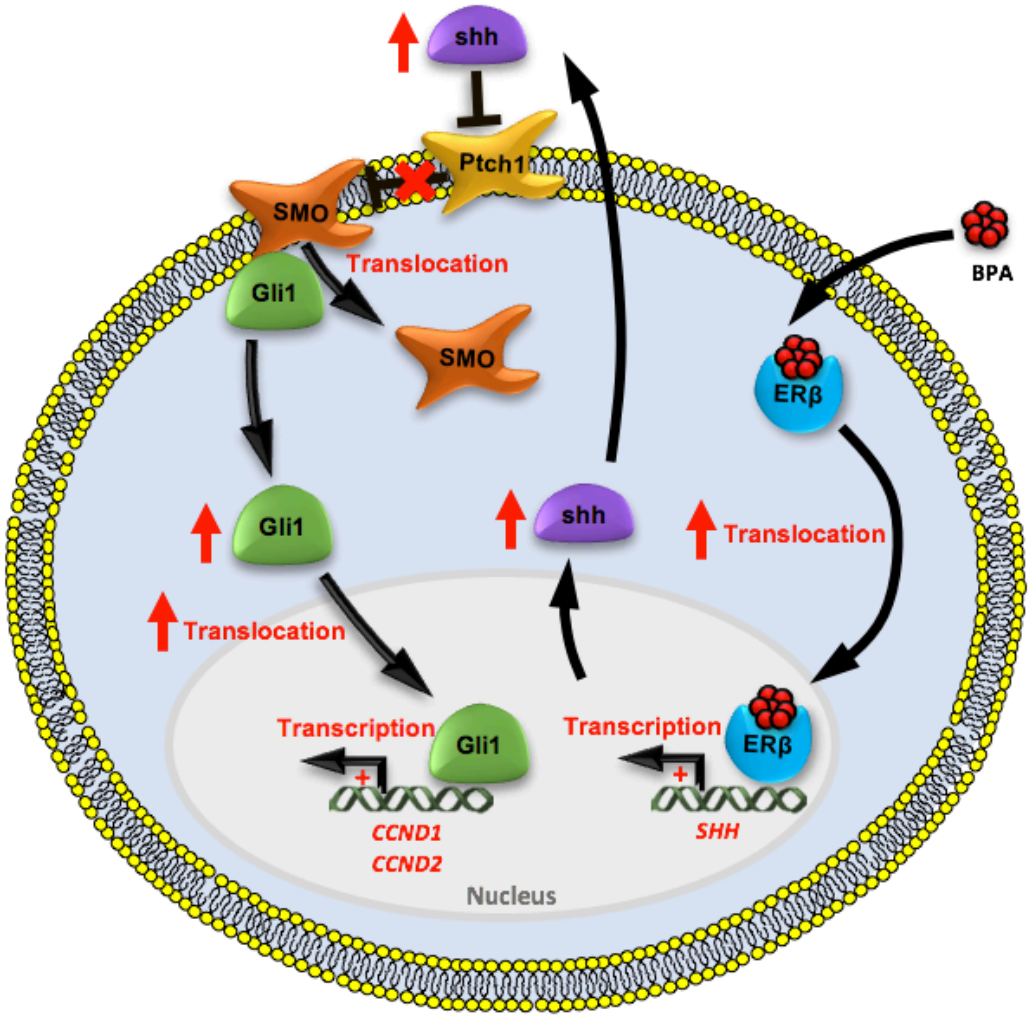

Figure 5.1: A schematic representation of the postulated molecular pathway by which BPA stimulates adrenal cortical cell proliferation.

BPA readily crosses the cell membrane into the cytoplasm where it binds to and activates ERβ. The activated ERβ translocates to the nucleus where it promotes transcription of the shh gene, leading to increased shh mRNA and protein. Shh is secreted, acts in an autocrine/paracrine fashion and binds to Ptch1 receptor, preventing Ptch1 from inhibiting SMO. SMO is then released from the plasma membrane into the cytoplasm, leading to the release of a complex containing the key shh transcription factor Gli1. Gli1 translocates to the nucleus where it binds to the promoters of key proliferation factors, such as CCND1 and CCND2, and enhances their transcription, and ultimately leading to increased cell proliferation.

5.2 Future Directions

The findings reported in this thesis reveal an important role of prenatal BPA in altering adrenal gland development and steroidogenic function in adult mouse offspring, and defines novel mechanisms of BPA actions in stimulating both adrenal StAR expression and adrenal cortical cell proliferation. However, it is important to recognize there are numerous important questions that need to be addressed, and are discussed below.

5.2.1 To study the effects of prenatal BPA on the other components of the steroidogenic pathway

The ZG is essential for the production of the steroid hormone aldosterone, which acts on the distal tubule and collecting duct of the kidney to promote sodium reabsorption [64-66]. Aldosterone is synthesized from corticosterone by the enzyme Cyp11B2, localized in the ZG, as part of adrenal steroidogenesis (**Figure 1.8**) [64-66]. The regulation of aldosterone production is not only controlled by ACTH, but is also regulated by angiotensin II and potassium levels [64,65]. Moreover, changes in aldosterone levels can result in altered blood pressure due to its actions on sodium reabsorption [64,65]. Indeed, epidemiological evidence suggests an association between high BPA levels in plasma/urine and hypertension [67,68]. However, there are currently no known studies that have examined the effects of BPA on aldosterone or Cyp11B2 levels. Thus, it is of importance to determine the potential effects of BPA on aldosterone production and regulation, and to examine the physiological consequences these effects may have.

The adrenal glands are also essential in producing precursors to sex hormones, such as DHEA from the ZR [64,66]. DHEA is not only required for the synthesis of estrogens and testosterone in the adult reproductive system, but is critical for estrogen production by the placenta during fetal development [64,66]. Previous studies using an adult adrenal gland cell line (H295R) have demonstrated that BPA treatment resulted in altered levels of progesterone, testosterone, estrone, and estradiol [69]. However, the effects of prenatal BPA on DHEA levels in the adrenal glands and the potential mechanism behind these effects are largely unknown. Therefore, further investigation into the effects and mechanism of BPA's actions on steroid hormones other than glucocorticoids in the fetal and adult adrenal gland, and the long-term consequences of these effects, is warranted.

5.2.2 To determine the precise molecular mechanism behind the effects of BPA on StAR protein levels

In Chapter 3, I was not able to completely elucidate the potential molecular mechanism underlying the effect of BPA on StAR protein levels. However, I suggest that BPA does not affect the gene transcription, translation, or protein half-life of StAR. Thus, further research is warranted to examine the effects of BPA on StAR regulation in human adrenocortical cells due to its essential role as the rate-limiting step in adrenal steroidogenesis [47,70]. The mechanisms underlying StAR regulation are not yet fully understood, and new mechanisms and pathways are being continually discovered [47,71]. As such, BPA may affect StAR protein levels through an undiscovered novel mechanism.

However, there are a few possible mechanisms that may underlie BPA-induced StAR protein levels, which include: ***(1)*** Changes in the 32-kDa isoform of StAR [72,73], which I was unable to detect by western blotting. The role of the 32-kDa isoform of StAR is not fully understood, yet it is interesting to speculate that an increase in the mature 30-kDa StAR may be complimented by a corresponding decrease in the 32-kDa StAR; ***(2)*** alterations in transport and/or cleavage of StAR protein at the OMM. It was proposed that a change in transport and/or cleavage of StAR would need to be accompanied with a change in StAR pre-protein. However, this may not be the case and further investigation to confirm this is needed; and ***(3)*** changes in multiple steps of the StAR regulation pathway. As such, if there are changes in multiple steps of the StAR regulation pathway, smaller changes in individual steps may not be sensitive enough to be detected in these experiments. Further studies need to examine each of these potential mechanisms.

5.2.3 To determine whether aspects of the signaling pathway identified *in vitro* can be observed in BPA-exposed mouse adrenal glands

In Chapter 4, I presented a novel molecular pathway by which BPA acts on ERβ to activate the Shh signaling pathway, which in turn leads to increased cell proliferation in H295A cells. However, future studies are necessary to determine whether this same molecular pathway operates *in vivo* in the adrenal glands of both fetal and adult mouse offspring following prenatal BPA exposure. These include ***(1)*** measuring key proliferation markers and factors (PCNA, cyclin D1, and cyclin D2); ***(2)*** determining the

levels (mRNA and protein) of key Shh signaling pathway factors Shh and Gli1, and their activity *in vivo* (nuclear translocation of Gli1); and ***(3)*** examining levels of ERβ and its activity (nuclear translocation) in adrenal glands of both fetal and adult offspring after prenatal BPA exposure. The foregoing experiments will ascertain whether the observed mechanism *in vitro* is also in operation *in vivo* following prenatal exposure to BPA.

5.2.4 To determine the adrenal phenotype in *ERα* and *ERβ* null mice

It has previously been shown that estrogen is essential for adrenal gland development in rhesus monkeys [36,42,74]. However, there are currently no known studies examining the effects on adrenal gland development and/or function in ERα and ERβ null mice. Additionally, experiments in this thesis have demonstrated an important role of ER in regulating adrenal steroidogenesis, as well as adrenal cortical cell proliferation via ERβ-mediated activation of the Shh signaling pathway. Thus, it is of important to characterize adrenal gland phenotypes in both fetuses and adults of adrenal-specific ERα and ERβ knockout mice. These novel *in vivo* mouse models will define the role of ERα and ERβ during the development of the adrenal gland in the fetus, and the potential long-term function of ERα and ERβ in the adult offspring.

Generation of these mouse models would be useful in the future to further examine the effects of BPA on adrenal gland development and function. Specifically, it will help to further confirm the role of BPA in regulating adrenal steroidogenesis, and provide definitive evidence to either support or refute if these effects are mediated through ERα and/or ERβ. These genetically modified mouse models will also be valuable in confirming the role of ERβ in activating the Shh signaling pathway, and consequent stimulation of adrenal cortical cell proliferation. Additionally, these mouse models will be invaluable in determining the mechanism of actions of other EDCs, acting through ER, on adrenal gland development and function.

5.2.5 To determine the effects of BPA analogues on adrenal gland development and function

The continued research into the adverse effects of BPA has led to many manufacturers discontinuing the use of BPA in their products [75,76]. As such, BPA analogues such as Bisphenol S, F, and AF (BPS, BPF, and BPAF) are being used increasingly as replacement for BPA in products due to their similar chemical properties (**Figure 5.2**) [75,76]. However, due to the structural similarities, there is potential for these chemicals to not only exert the same effects as BPA but to cause other adverse effects. Indeed, recent studies have found that exposure to BPS, BPF, and BPAF affect the reproductive [77,78], endocrine [77], neurological [79,80], cardiovascular [81], and metabolic [82,83] systems.

It has previously been demonstrated that treatment of the H295R cell line (adult adrenal gland) with increasing concentrations of BPS, BPF, and BPAF resulted in various effects on steroidogenic function differing from that of BPA [84]. Therefore, future *in vitro* and *in vivo* studies are needed to further understand the potential adverse effects of these bisphenol analogues on adrenal development and function, which will contribute to the growing literature concerning the question of whether bisphenol analogues are safer alternatives to BPA.

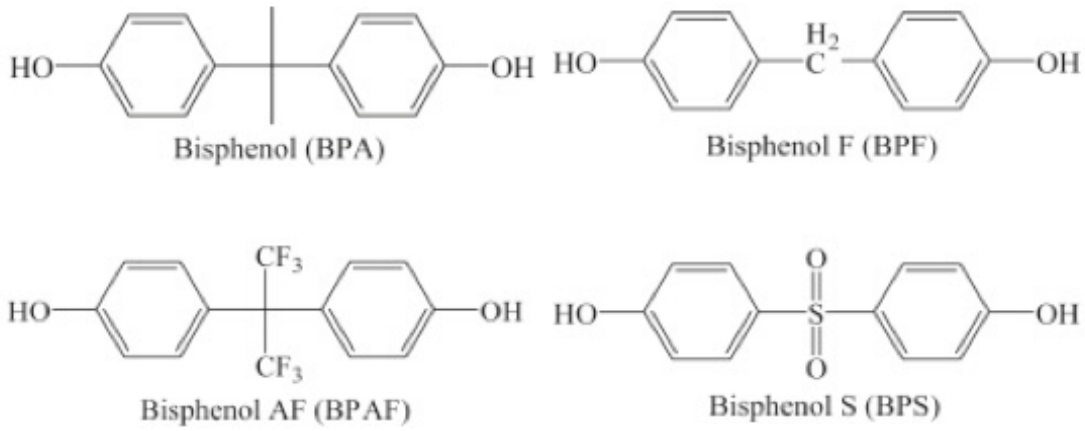

Figure 5.2: BPA and its analogues structure [84]

5.3 Conclusions

Insults during the critical period of development can often lead to long-term adverse effects, resulting in disease and dysfunction in later life [13,14]. Exposure to EDCs such as BPA during critical periods of organ development is alarming for numerous reasons. First, the presence of BPA in the environment is ubiquitous, and is detectable in the urine of over 91% of Canadians [85]. Second, BPA has been demonstrated to cross the placenta and reach fetal circulation during organ development [86,87]. Lastly, BPA is known to work through several receptors and alter numerous cell signaling pathways that are involved in organ development [61,88]. While more attention has been placed on restricting/eliminating the exposure of infants to BPA, specifically by banning BPA in baby bottles and products, this does not prevent exposure of the fetus to BPA through maternal exposure [89,90]. Epidemiological and animal studies have demonstrated the potential for prenatal BPA to cause HPA dysfunction [1-6]. However, the specific effects of prenatal BPA on the adrenal gland development and function in later life remain largely unknown.

The experiments presented in Chapter 2 demonstrates the effects of environmentally relevant doses of prenatal BPA induces adrenal steroidogenesis independent of ACTH levels in a sex-specific manner in the mouse model. The next set of experiments, described in Chapter 3, present a potential mechanism by which BPA induces StAR protein expression, the rate-limiting factor of steroidogenesis, seen in adult female mice prenatally exposed to BPA. Lastly, experiments in Chapter 4 provided evidence of BPA altering adrenal development by increasing adrenal cell proliferation via ERβ-mediated activation of the shh signaling pathway in human adrenocortical cells. A schematic of this molecular pathway through which BPA induces adrenal cell proliferation is shown in Figure 5.1. This model speculates that BPA freely crosses the cell membrane, which then binds to and activates ERβ, leading to ERβ translocation to the nucleus and consequently increasing Shh mRNA and protein levels. Shh is then secreted and acts in an autocrine/paracrine fashion to bind to Ptch1 receptor, prevent Ptch1 from inhibiting SMO. SMO is released from the plasma membrane into the cytoplasm, leading to the release of a complex containing Gli1, a key Shh transcription factor. Gli1 translocates to the nucleus where it binds to the promoters of key proliferation factors CCND1 and CCND2, and enhances their transcription and ultimately leading to increased cell

proliferation. This is a postulated model and further experiments are required to confirm if this model translates into an *in vivo* model system. The long-term consequences of increased cell proliferation in fetal adrenal glands must also be considered in the application of this model.

Thus, this thesis supports the hypothesis that prenatal BPA exposure disrupts adrenal gland development and steroidogenic function in adult offspring. These findings raise concerns about the potential for adverse effects of prenatal BPA on adrenal gland development and function, adding to the growing epidemiological evidence suggesting adverse effects of BPA on the HPA axis and adrenal gland, and which we hope will promote the continual banning of BPA in consumer products by regulatory agencies.

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Appendices

Appendix 1

Western

2012-004::3:
AUP Number: 2012-004
AUP Title: Molecular Mechanisms of Fetal Growth Restriction

Yearly Renewal Date: 06/01/2015
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2012-004 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Curriculum Vitae

Samantha Medwid

EDUCATIONAL BACKGROUND

PhD Physiology and Pharmacology

September 2013–December 2017

Western University, London, Ontario, Canada

Department of Physiology and Pharmacology

Thesis: Effects of prenatal bisphenol A on adrenal gland development and steroidogenic function

Hon. BSc Nutritional and Nutraceutical Sciences

September 2009–June 2013

University of Guelph, Guelph, Ontario, Canada

Department of Human Health and Nutritional Sciences

SCHOLARSHIPS AND AWARDS

George W. Stavraky Teaching Award 2017

Obstetrics & Gynecology Graduate Scholarship (OGGS) 2015-2016

Western Graduate Research Scholarship 2013-2017

Nomination for Graduate Student Teaching Assistant Award 2015-2016 & 2016-2017

PUBLICATIONS

Medwid S, Guan H, Yang K (2017). Prenatal exposure to bisphenol A disrupts adrenal steroidogenesis in adult mouse offspring. *Environmental Toxicology and Pharmacology*. 43:203-208

Medwid S, Guan H, Yang K (2017) Bisphenol A induces steroidogenic acute regulatory protein (StAR) expression via an unknown mechanism independent of transcription, translation and protein half-life in human adrenal cortical cells. Submitted *Steroids*.

Medwid S, Guan H, Yang K (2017). Bisphenol A stimulates adrenal cell proliferation through ERβ-mediated activation of the sonic hedgehog signaling pathway. Submitted *Journal of Steroid Biochemistry and Molecular Biology.*

Westerman C, **Medwid S**, Guan H, Yang K (2017). Effects of prenatal bisphenol A exposure on the expression of water homeostasis genes in kidneys of adult mouse offspring. In preparation.

Abou Taka M, **Medwid S**, Guan H, Yang K (2017). Prenatal bisphenol A causes sex-specific effects on liver function in adult mouse offspring. In preparation.

ABSTRACTS

Medwid S, Guan H, and Yang K. Bisphenol A stimulates adrenal cell proliferation through ERβ-mediated activation of the sonic hedgehog signaling pathway. 15th Annual Paul Harding Research Day (1st place oral presentation), London Ontario, May 2017

Medwid S, Guan H, and Yang K. Bisphenol A stimulates adrenal cell proliferation through ERβ-mediated activation of the sonic hedgehog signaling pathway. Southern Ontario Reproductive Biology Day (Poster Presentation), London Ontario, May 2017

Medwid S, Guan H, and Yang K. Bisphenol A stimulates adrenal cell proliferation through ERβ-mediated activation of the sonic hedgehog signaling pathway. London Health Research Day (Poster presentation), London Ontario, March 2017

Medwid S, Guan H, and Yang K. Prenatal exposure to bisphenol A disrupts adrenal steroidogenesis in adult mouse offspring. 14th Annual Paul Harding Research Day (2nd place poster presentation), London Ontario, May 2016

Medwid S, Guan H, and Yang K. Prenatal exposure to Bisphenol A disrupts adrenal steroidogenesis in adult mouse offspring. London Health Research Day (Poster presentation), London Ontario, March 2016

Medwid S, Guan H, and Yang K. Prenatal exposure to Bisphenol A disrupts adrenal

steroidogenesis in adult mouse offspring. 13th Annual Paul Harding Research Day (Oral presentation), London Ontario, May 2015

Medwid S, Guan H, and Yang K. Effects of prenatal Bisphenol A exposure on fetal adrenal gland development in the mouse. Developmental Biology Day (Poster presentation), London Ontario, June 2015

Medwid S, Guan H, and Yang K. Effects of prenatal Bisphenol A exposure on fetal adrenal gland development in the mouse. 12th Annual Paul Harding Research Day (Poster presentation), London Ontario, May 2015

RELATED WORK EXPERIENCE

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