Effects of Early, Elevated Prenatal Testosterone on the HPA Axis and Hormone Responsiveness in Mice

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Biomedical Science and Neuroscience

Guelph, Ontario, Canada
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ABSTRACT

EFFECTS OF EARLY, ELEVATED PRENATAL TESTOSTERONE ON THE HPA AXIS AND HORMONE RESPONSIVENESS IN MICE

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Alterations in developmental testosterone exposure can affect social and anxiety behaviour in male offspring, however, the underlying mechanisms remain unknown. We hypothesized that prenatal testosterone exposure would impact physiological development in a similar way as prenatal glucocorticoid exposure, resulting in altered hypothalamic-pituitary-adrenal axis functioning and hormone profiles in offspring. We treated dams with testosterone propionate, the synthetic glucocorticoid dexamethasone, or sesame oil during mid-late pregnancy. Testosterone was measured in male offspring on the day of birth and in adulthood. Corticosterone was measured in offspring's hair, and in plasma following restraint stress. Prenatal treatment did not affect offspring testosterone levels. While prenatal testosterone had no effect on corticosterone levels in hair, prenatal testosterone reduced corticosterone responsivity to restraint stress in males, but not females. No effects of prenatal dexamethasone on corticosterone responsivity was observed. This study may have potential implications for conditions in which testosterone or dexamethasone are elevated during pregnancy.
ACKNOWLEDGEMENTS

To Dr. Neil MacLusky, I am incredibly grateful to have had you as my MSc advisor. You've presented me with numerous opportunities that have enabled my learning and growth in many ways. Your care, dedication to, and investment in your students does not go unnoticed, and has been so very appreciated. You've been an amazing mentor. Thank you.

To Dr. Elena Choleris, I was very lucky to have gotten you as a co-advisor when I became involved with this project. Thank you for your support, guidance and willingness to indulge my love of Italy. Your insight, creativity and passion were instrumental to working through all the challenges this project presented. Grazie mille.

To Dr. Amy Newman, as an advisory committee member, you went above and beyond to welcome me into your lab and the department. Your training, guidance, and support have been essential to the work done in this thesis, and to my own academic and personal growth. Thank you for trusting me with Pinky. I am very excited to continue working with you in the future.

To Emily Martin, I would not have gotten through this degree without you. From training me in animal and laboratory work, to having an answer to practically every question I ask, you’ve been an amazing lab partner and co-mouse mother. Most importantly, I would never have known where to place my surgery tools without you. Thank you.

To the MacLusky lab family, thank you for your guidance, and friendship. Dr. Ari Mendell, you’ve been a great mentor, and our tea breaks helped keep me sane these past few years. Dr. Carolyn Creighton, Kate Nicholson, Lauren Isaacs, Emily Craig, Eric Lawton, Nariko Kuwahara, Simran Bhullar, Julia Pacosz, Jodi Hong and Akshara, you’ve all helped make these years enjoyable. Hunny, thank you, Hunny.

To the Departments of Biomedical Sciences, Psychology and Integrative Biology, and all the faculty and students, thank you for making this a welcoming and positive place to work. I will carry great memories forward of my time spent here.

To the Central Animal Facility staff, especially Theresa Martin, Janet Gugan, Michael Brunt, Annette Morrison, Mary Fowler and Cheryl Limebeer, thank you for your dedication and hard work in helping to care for the mice. Your patience, helpfulness, and accommodation for our experiments helped make the challenges of this project a little less challenging.

To all the volunteers who stepped in when we needed a hand, thank you. Your help was much appreciated.
To my family, Ma, Dad, Matt and Dan, thank you for your love and support, and for reminding me not to work so hard. To my friends, housemates, and volleyball crew, thank you for your support, encouragement, and the fun times which kept me grounded through the rollercoaster of emotions that is graduate school. Thank you to all the furry friends who helped keep my cortisol levels at bay, Maddy, Odin, Jax, Hallie and Annie. Thank you to Second Cup and Ludovico Einaudi, without which most of my writing would have never come to be.

Finally, to my partner, Giovanni, who has supported me through every step of this process. Thank you for being the person with whom I can celebrate all the successes and bear through all the challenges with. You’ve been an endless source of encouragement, validation, comfort and love these past few years. I am so grateful to have you as my best friend.
DECLARATION OF WORK PERFORMED

I declare that I have performed all the work presented in this thesis except:

Emily Martin (Choleris lab, University of Guelph), performed the treatment, sample collection, and ELISA analysis of maternal testosterone levels. Emily Martin also conducted most SRY PCR in experiments 1 and 2. Initial estradiol ELISA optimization (running unextracted and ether extracted adult female plasma samples) was also done by Emily Martin.

I also declare that throughout the course of my studies, I was assisted by other graduate and undergraduate students. Emily Martin assisted with the animal work in experiments 1, 2 and 3, including breeding, treatments, cross-fostering, weaning, lavages, surgeries, PPI, restraint stress, sacrifices and tissue collection. Michael Marcotte (MacLusky lab, University of Guelph) assisted with breeding, treatments, cross-fostering, weaning, lavages, surgeries, sacrifices and tissue collection in experiment 1. Emily Martin and Sophie Martin (Western University) assisted with estrous cycle stage reading. Dr. Ari Mendell (MacLusky lab, University of Guelph) and Dr. Carolyn Creighton (MacLusky lab, University of Guelph) assisted with tissue collection in experiments 1 and 2.
# TABLE OF CONTENTS

Abstract .................................................................................................................................................. ii

Acknowledgements .............................................................................................................................. iii

Declaration of Work Performed ........................................................................................................... v

Table of Contents .................................................................................................................................. vi

List of Tables .......................................................................................................................................... xi

List of Figures ......................................................................................................................................... xii

List of Abbreviations ............................................................................................................................ xiii

1 Literature Review ............................................................................................................................... 1

1.1 Introduction ....................................................................................................................................... 1

1.2 Animal models for studying development ...................................................................................... 4

1.3 The sex steroid hormones ............................................................................................................... 4

1.3.1 Hormone exposure during fetal development ............................................................................. 6

1.3.2 The organizational-activational hypothesis ............................................................................... 11

1.3.3 Organizational effects of sex steroid hormones ...................................................................... 12

1.3.4 Hormone exposure during postnatal life ................................................................................... 16

1.3.5 Activational effects of sex steroid hormones .......................................................................... 18

1.3.6 Prenatal and postnatal hormone interactions .......................................................................... 19

1.3.7 Developmental sex steroids and neuropsychiatric disorders .................................................. 19

1.3.8 Investigating the effects of low doses of prenatal testosterone ............................................... 23

1.3.9 Early testosterone, ASD, and schizophrenia ............................................................................ 24

1.4 Prenatal testosterone and the HPA axis ......................................................................................... 25

1.4.1 The HPA axis .............................................................................................................................. 26
1.4.2 The HPA axis influences social and anxiety behaviours

1.4.3 Sex differences in the HPA axis

1.4.4 Organizational effects of steroid hormones on the HPA axis

1.4.5 Activational effects of steroid hormones on the HPA axis and anxiety

1.4.6 Prenatal GC exposure

1.5 Rationale

1.6 Hypothesis and objectives

2 Methods

2.1 All experiments

2.1.1 Animals

2.1.2 Housing

2.2 Experiment 1

2.2.1 Breeding

2.2.2 Prenatal treatment

2.2.3 Maternal serum collection

2.2.4 Day of birth monitoring

2.2.5 Sex determination

2.2.6 Pup sacrifice and tissue collection

2.2.7 Cross-fostering

2.2.8 Weaning and pair housing

2.2.9 Vaginal lavages and estrous cyclicity

2.2.10 Surgeries

2.2.11 Ovariectomy and sham surgery

2.2.12 Castration and sham surgery
2.2.13 PD43 hair collection ................................................................. 49
2.2.14 PD58 hormone replacement surgery ........................................ 49
2.2.15 Capsules ............................................................................. 50
2.2.16 Sacrifice and tissue collection .................................................. 50
2.2.17 Maternal serum testosterone and pup testosterone analysis ........ 51
2.2.18 Hair CORT ........................................................................... 52
2.2.19 Adult male plasma testosterone analysis ................................... 54
2.2.20 Adult female plasma estradiol analysis ..................................... 56
2.3 Experiment 2 ............................................................................ 57
  2.3.1 Breeding, day of birth, and weaning ......................................... 58
  2.3.2 Prenatal treatment .................................................................. 58
  2.3.3 Prepulse inhibition of the startle response ................................ 58
  2.3.4 Restraint stress and sacrifice .................................................. 59
  2.3.5 PD0 blood testosterone analysis ............................................. 60
  2.3.6 Plasma CORT analysis ........................................................... 61
  2.3.7 Plasma testosterone analysis .................................................. 61
2.4 Experiment 3 ............................................................................ 62
  2.4.1 Breeding and prenatal treatment ............................................. 62
  2.4.2 Monitoring and sacrifice ....................................................... 62
  2.4.3 PD0 blood testosterone analysis ............................................ 63
2.5 Statistical analyses ..................................................................... 63
  2.5.1 General data handling ........................................................... 63
  2.5.2 Statistical analyses ................................................................. 63
3 Results ....................................................................................... 68
3.1 Maternal serum analysis ........................................................................................................ 68
3.2 Reproductive tissue weights ................................................................................................ 69
3.3 Day of VO and first estrus ................................................................................................... 71
3.4 Estrous cyclicity .................................................................................................................... 71
3.5 Hair CORT levels .................................................................................................................. 74
3.6 Experiment 1: PD0 testosterone levels ............................................................................... 75
3.7 Adult male testosterone levels optimization ........................................................................ 77
3.8 Adult male testosterone levels by C18 SPE ......................................................................... 79
3.9 PD43 testosterone levels ..................................................................................................... 79
3.10 PD58 testosterone levels .................................................................................................... 80
3.11 PD77 testosterone levels .................................................................................................... 81
3.12 Adult female estradiol levels .............................................................................................. 82
3.13 Prepulse inhibition of the startle response ........................................................................ 83
3.14 Plasma CORT levels .......................................................................................................... 83
3.15 Experiment 2: Adult testosterone levels ........................................................................... 86
3.16 Experiment 3: PD0 testosterone levels .............................................................................. 86
4 Discussion ................................................................................................................................ 88
4.1 Maternal testosterone levels .............................................................................................. 88
4.2 Reproductive tissue weights, VO, and estrous cyclicity ..................................................... 89
4.3 Experiment 1: PD0 testosterone levels ............................................................................... 95
4.4 Hair CORT levels ................................................................................................................ 96
4.5 Adult testosterone levels: Optimization and results ........................................................... 100
4.6 Adult female estradiol levels ............................................................................................... 103
4.7 Prepulse inhibition of the startle response .......................................................................... 103
4.8 HPA axis responsivity to restraint stress ............................................. 104
4.9 Experiment 3: PD0 testosterone levels ............................................. 111
4.10 Conclusions .................................................................................. 113
4.11 Limitations ................................................................................... 116
4.12 Future directions ........................................................................... 118
References .......................................................................................... 121
LIST OF TABLES

Table 1: Experimental timeline for experiment 1. ................................................................. 41
Table 2: PCR cycling protocol ............................................................................................ 44
Table 3: Estrous cycle stage characterization by cell type ................................................. 47
Table 4: SPE protocol ........................................................................................................ 56
Table 5: Experimental timeline for experiment 2 ............................................................. 57
Table 6: Experimental timeline for experiment 3 ............................................................. 62
Table 7: Day of VO and day of first estrus................................................................. 71
LIST OF FIGURES

Figure 1: Metabolism of testosterone to other neuroactive steroids................................. 6
Figure 2: Developmental testosterone exposure in male and female mice ...................... 11
Figure 3: Measurements of maternal testosterone taken after the E16 injection............. 68
Figure 4: Measurements of reproductive tissue weights .................................................. 70
Figure 5: Average number of days per estrous cycle ....................................................... 73
Figure 6: Percentage of time spent in each stage of the estrous cycle ......................... 73
Figure 7: Hair CORT levels ............................................................................................. 75
Figure 8: Serially diluted P0 blood in testosterone RIA .................................................... 76
Figure 9: PD0 testosterone levels 6-18 hours after birth .............................................. 77
Figure 10: Serially diluted adult plasma in testosterone RIA optimization ..................... 78
Figure 11: Serially diluted C18 SPE plasma testosterone in RIA .................................. 79
Figure 12: PD43 adult plasma testosterone levels ............................................................ 80
Figure 13: PD58 adult plasma testosterone levels ............................................................. 81
Figure 14: PD77 adult plasma testosterone levels ............................................................. 82
Figure 15: Extracted estradiol standard curve ................................................................. 83
Figure 16: Plasma CORT levels following restraint stress ............................................. 85
Figure 17: Plasma testosterone levels in adult males ....................................................... 86
Figure 18: Time-course of blood testosterone levels 1-6 hours after birth ..................... 88
LIST OF ABBREVIATIONS

ACTH  Adrenocorticotropic hormone
AFP   Alpha-fetoprotein
ANOVA Analysis of Variance
AR    Androgen receptor
ASD   Autism Spectrum Disorder
CBG   Corticosteroid binding globulin
CORT  Corticosterone
CRH   Corticotropin releasing hormone
CRH-BP CRH binding protein
CRHR1 CRH receptor 1
CRHR2 CRH receptor 2
dB    Decibels
DEX   Dexamethasone
DHEA  Dehydroepiandrosterone
DHT   5α dihydrotestosterone
E     Embryonic day
E2    17β-estradiol
EB    β-Estradiol 3-benzoate
ELISA Enzyme-linked immunosorbent assay
ERs   Estrogen receptors
ERRα  Estrogen receptor alpha
ERRβ  Estrogen receptor beta
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GDX</td>
<td>Gonadectomized</td>
</tr>
<tr>
<td>GDX-HR</td>
<td>Gonadectomized-hormone replaced</td>
</tr>
<tr>
<td>GDX-VR</td>
<td>Gonadectomized-vehicle replaced</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>GPER</td>
<td>G-protein coupled estrogen receptor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA axis</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KW</td>
<td>Kruskal-Wallis rank sum</td>
</tr>
<tr>
<td>LC-APIC-MS/MS</td>
<td>High-performance liquid chromatography mass spectrometry coupled with atmosphere pressure chemical ionization</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MPON</td>
<td>Medial preoptic nucleus</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>OVX-HR</td>
<td>Ovariectomized-hormone replaced</td>
</tr>
<tr>
<td>OVX-VR</td>
<td>Ovariectomized-vehicle replaced</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PD0</td>
<td>Day of birth</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PPI</td>
<td>Prepulse inhibition of the startle response</td>
</tr>
<tr>
<td>PS</td>
<td>Prenatally stressed</td>
</tr>
</tbody>
</table>
PVN  Paraventricular nucleus of the hypothalamus
RIA  Radioimmunoassay
SBGI Sex binding globulin inhibitor
SO  Sesame oil
SPE Solid phase extraction
SRH Scheirer-Ray-Hare
TP Testosterone propionate
VO Vaginal opening
WRST Wilcoxon rank sum test
1 Literature Review

1.1 Introduction

During development, males and females are exposed to a differing combination of genetic, environmental and epigenetic factors, which together influence the organization of the brain (Fagiolini, Jensen, & Champagne, 2009; Stiles, 2017). These factors permanently program neural pathways, and lead to the expression of sex-typical behaviours later in life (McCarty, 2012). Elucidation of the mechanisms involved in sexual differentiation of the male and female central nervous systems is critical to understanding sex differences in normal physiology and behaviour, as well as the mechanisms underlying diseases that occur at different rates in males and females (Cahill, 2006; McCarthy & Arnold, 2011).

The logic of sexual differentiation becomes clear when the concept is examined within an evolutionary context. The principle of natural selection states that phenotypes are developed and persist in a population when they improve the chances of an individual’s survival and reproduction. As the biological strategies for survival and fitness often differ between the sexes, males and females have developed markedly different neural pathways, which program different survival and reproductive traits or behaviours (Maklakov & Lummaa, 2013; Morrow, 2015).

Although these traits have evolved from ecological and biological pressures to survive and reproduce in the wild, the environment of today’s society is very different from that of our ancestors. Thus, our biological programming could be less beneficial, or
even maladaptive, for coping with modern challenges (McEwen, 2000). For example, while the “fight or flight” stress response may have been necessary for our ancestors to escape immediate and fleeting dangers like predators, today we are unlikely to encounter immediate, life-threatening dangers. Instead, stressors are often psychological, long-term challenges, which the “flight or flight” system is unsuited for (Hadany, Beker, Eshel, & Feldman, 2006). Long-term activation of the stress system can result in allostatic load, whereby the constant presence of stress hormones results in detrimental effects on the brain and body (McEwen, 2000). Furthermore, since males and females differ in normal stress physiology, consequences of allostatic load might affect one sex to a greater degree than another (Goel, Workman, Lee, Innala, & Viau, 2014).

Sex differences are common in the presentation and incidence of many stress-related neuropsychiatric disorders, including depression, anxiety, schizophrenia and developmental disorders (Altemus, Sarvaiya, & Eppers, 2014; Gogos et al., 2015; Halladay et al., 2015). While the causes of many of these disorders are still not fully defined, these sex differences may offer clues to uncovering etiology and potential treatment options.

Primary factors underlying sex differences in the brain are the sex steroid hormones. Sex steroid hormone exposure during both prenatal and postnatal life differs between the sexes, and contributes to differential programming of brain pathways, and differential physiology and behavioural expression throughout life (Maclusky & Naftolin, 1981). Additionally, sex steroid hormones are suggested to be involved in the etiology of
numerous sexually differentiated, neurological disorders (Manson, 2008; Solomon & Herman, 2009).

Arguably, one of the most important factors involved in steroid hormone mediated sexual differentiation is the timing of hormone exposure. The development of male-typical and female-typical brain pathways occurs during tightly regulated, sensitive periods of development, when hormone exposure differs between males and females (McCarthy, 2012). Typically, in mammals, males are exposed to elevated androgens during two periods of prenatal and postnatal development (Clarkson & Herbison, 2016). If timing of androgen exposure is altered, normal sexual differentiation phenotypes could be affected (Herman, Jones, Mann, & Wallen, 2000).

Although sex steroid hormones are unquestionably involved in the etiology of many sexually differentiated neurological disorders, the potential mechanisms involved remain to be determined. We hypothesize that one critical factor may be alterations in the timing of steroid hormones exposure during development, which may disrupt neural and behavioural programming in offspring. Furthermore, we hypothesize that developmental consequences of altered timing of hormone exposure may be sex specific. To examine the relationship between steroid hormones, sex, timing, and neurological disorders, we examined the effects of an early elevation in prenatal testosterone on the reproductive physiology, hormone profiles, and stress responsivity in male and female mice.
1.2 Animal models for studying development

Due to ethical limitations for manipulating hormone levels in humans, most research on the causal effects of sex steroid hormones across development has been conducted in animal models. Mice and rats are often the models of choice, as they have many anatomical and physiological similarities to humans (Bodenreider, Hayamizu, Ringwald, De Coronado, & Zhang, 2005; Perlman, 2016), they have short gestational periods and lifespans so they can efficiently produce answers to scientific questions (Rabadán-Diehl & Nathanielsz, 2013), and their development is well-characterized in relation to that of humans (Otis & Brent, 1954). This study was done using a mouse model, thus where possible literature on mice is described, otherwise literature on rats or other mammals is referenced.

1.3 The sex steroid hormones

The sex steroid hormones, which include testosterone and estrogens such as estrone, estriol and estradiol, are powerful signaling molecules which play a principal role in the determination of sex differences in the brain. Through a series of enzymatic reactions, testosterone is synthesized from cholesterol in reproductive tissues and the brain (McCarthy, 2012). Testosterone can exert its influences directly by binding to the androgen receptor (AR), or through metabolism to other steroid compounds, which can have their own neuroactive effects (Figure 1; Sherbet & Auchus, 2007). Two key metabolites of testosterone include the more potent androgen, $5\alpha$-dihydrotestosterone (DHT), which is synthesized from testosterone by the enzyme $5\alpha$-reductase, and $17\beta$-estradiol (E2), which is synthesized from testosterone by the P-450 enzyme, aromatase.
DHT exerts its androgenic actions by binding to ARs, while E2 activates estrogen receptors (ERs; Sherbet & Auchus, 2007), including estrogen receptor alpha, (ERα), estrogen receptor beta (ERβ) and the G-protein coupled estrogen receptor (GPER). Testosterone can be further converted to numerous other steroid hormones, some of which possess weak androgenic ability, such as androstenedione and androsterone (Greene, Burrill, & Ivy, 1939). While some work has suggested that progesterone, another sex steroid hormone, may play a role in sexual differentiation, it’s primary function is in adult female reproduction, and thus its actions will not be discussed in this review (McCarthy, 2012; Wagner, Nakayama, & De Vries, 1998).

ARs, ERα and ERβ belong to a subset of the nuclear steroid receptor superfamily. Once activated, these receptors primarily regulate transcription of a number of genes by binding to response elements in regions of the DNA that drive the transcription of those genes. Thus, by activating their receptors, the steroid hormones can exert long-lasting effects on a wide range of biological processes, such as cell proliferation, development, metabolism and reproduction (Sever & Glass, 2013).
Figure 1. Metabolism of testosterone to other neuroactive steroids. Testosterone exerts effects in the brain directly, by binding to ARs, or by metabolism to other steroid hormones that bind to ARs, ERs and the GABA<sub>A</sub> receptor.

1.3.1 Hormone exposure during fetal development

Male and female fetuses naturally undergo differential exposure to sex steroid hormones from numerous sources during prenatal and neonatal development, resulting in a sexually differentiated profile of steroid hormone exposure in the developing brain. The primary source of steroid hormones during early development is the gonads. Sex hormone exposure in males in utero primarily arises from testicular testosterone (Motelica-Heino, Castanier, Corbier, Edwards, & Roffi, 1988; Pointis, Latreille, Mignot, Janssens, & Cedard, 1979). The male testes, which develop under direction of the SRY gene on the Y chromosome, secrete testosterone during gestation. In male mice, testosterone secretion begins shortly after the testes are developed, on approximately
embryonic day (E) 12.5-13 (O’Shaughnessy et al., 1998; Pointis et al., 1979). In male mice, plasma testosterone levels naturally peak twice during development, with the first peak occurring on embryonic day 16-17 (McCarthy, 2016; Pointis et al., 1979), and the second peak occurring within 2 hours after birth (Figure 2; Corbier, Edwards, & Roffi, 1992; Motelica-Heino et al., 1988). During these peaks, testosterone levels are higher in males than in females (Pointis et al., 1979; Weisz & Ward, 1980). Some reports suggest testosterone is elevated in males compared to females across prenatal development, while others suggest testosterone levels are not significantly different between males and females except for at the times of the male testosterone peak (Pointis et al., 1979; Weisz & Ward, 1980). Without SRY influence, the female ovaries develop. Although the ovaries are the main source of estradiol in females, secretion of estradiol from the ovaries in rats remains very low or undetectable prior to birth (Weniger, 1993). Additionally, even though the male Sertoli cells contain aromatase, basal production of estradiol in the testes is not detectable in the fetal rat prior to birth (Weniger, 1993; Weniger, Chouraqui, & Zeis, 1993). The female ovary can secrete androgen, and plasma testosterone has been reported in female rats during prenatal development (Weisz & Ward, 1980). On the day of birth, plasma testosterone levels are low and unchanging in female mice (Corbier et al., 1992; Motelica-Heino et al., 1988).

While the gonads are the primary source of fetal steroid hormone exposure, evidence also suggests de novo synthesis of sex steroid hormones may act as a source of androgens and estradiol in the neonatal brain (Amateau, Alt, Stamps, & McCarthy, 2004; Bean, Seckl, Lathe, & Martin, 2001). Indeed, plasma hormone levels do not
necessarily correlate with brain hormone levels (Konkle & McCarthy, 2011). At 2 hours after birth, male and female rats were observed to have no significant difference in plasma estradiol, however, males had higher levels of estradiol than females in the frontal cortex, hypothalamus and preoptic area, but not the hippocampus, brainstem or cerebellum (Amateau et al., 2004). This may have functional implications to influence sexual differentiation of specific brain regions involved in later life sexually differentiated behaviours. For example, morphology of the medial preoptic nucleus (MPON), a sexually differentiated brain structure, is influenced by sex hormone exposure during neonatal development (Gorski, Gordon, Shryne, & Southam, 1978). Preventing testosterone aromatization to estradiol during prenatal and neonatal development resulted in reduced volume of the male MPON and reduced expression of male typical reproductive behaviours in adulthood (Houtsmuller et al., 1994). Overall, prenatal and postnatal testosterone and estradiol levels in the brain vary substantially by sex and region examined, and these differences result in functional implications for morphology, physiology and behaviour in the offspring (Konkle & McCarthy, 2011).

The fetus may also be exposed to exogenous sex steroid hormones in utero, which can contribute to steroid hormone influence on development. Maternal sex steroid hormones may affect offspring development. Injections of high doses of testosterone to pregnant dams can result in physical and behavioural masculinization of female offspring (Phoenix, Goy, Gerall, & Young, 1959; S. vom Saal & Bronson, 1980; Wolf, Hotchkiss, Ostby, LeBlanc, & Gray Jr., 2002). However, maternal testosterone levels generally do not correlate with fetal testosterone in rats and mice, as the majority of
maternal testosterone is thought to be metabolized by the placenta (Despres, Rigaudiere, & Delost, 1984; Sathishkumar et al., 2011; Wolf et al., 2002). Testosterone metabolism to other neuroactive steroids, such as androsterone and 5α-androstane-3α-17β-diol, occurs in the placenta, and this has been theorized to contribute to masculinizing effects of maternal testosterone in the fetus (Greene et al., 1939; Sathishkumar et al., 2011; Wolf et al., 2002). Maternal and placental estrogens are prevented from affecting the developing fetus in mice and rats by the glycoprotein, alpha-fetoprotein (AFP). AFP is found in the fetus at high levels in late gestation and in pups during their early postnatal life, where it binds to and sequesters estrogens, thus preventing the majority of circulating estrogens from affecting fetal development (Bakker et al., 2006). Plasma estradiol levels are elevated in both male and female rats on the day of birth, however the source of this estradiol is suggested to be from maternal circulation, which quickly gets cleared by AFP (Döhler & Wuttke, 1976; Konkle & McCarthy, 2011). However, Montano and colleagues (1995) demonstrated when radiolabelled estradiol was administered to 2-day old rats, free circulating plasma estradiol reached adult physiological levels, and some of the radiolabelled estradiol was recovered bound to brain cell nuclei. Therefore, AFP may not prevent all circulating estrogens from reaching the brain (Montano, Welshons, & vom Saal, 1995). Fetal offspring may also be exposed to sex steroid hormones secreted by their male siblings in utero (Gandelman, vom Saal, & Reinisch, 1977; vom Saal & Bronson, 1978). Male and female who were in closer proximity to male siblings in utero have been shown to have slightly more masculinized anatomy, physiology, and behaviour compared to
offspring who were not in such proximity to males (Clark, vom Saal, & Galef, 1992; Gandelman et al., 1977). Taken together, while there are protective mechanisms in place to prevent exogenous steroid hormones from reaching the fetal brain, these mechanisms may not always be completely effective, and thus exogenous hormones may contribute to sex steroid hormone influence on development.

While the adrenal glands are another significant source of androgens in humans, most research investigating adrenal production of androgens suggest that the adrenal glands of mice and rats do not produce biologically significant levels of androgens (Keegan & Hammer, 2002; van Weerden, Bierings, van Steenbrugge, de Jong, & Schröder, 1992). The C19 steroid dehydroepiandrosterone (DHEA), however, has been demonstrated to be synthesized in one species of mice by the adrenal gland (Quinn et al., 2013). DHEA can activate ARs and ERs by conversion to other androgens and estrogens in vivo, and potentially by its own binding, thus adrenal DHEA could act as another contributor to sex steroid hormone exposure during prenatal development in certain strains of male and female mice (Arnold & Blackman, 2005; Labrie et al., 1998).

As the fetal brain can be exposed to sex steroids from numerous potential sources at different times during pregnancy, it remains difficult to precisely define the profile of sex steroid hormone exposure in males and females across development. The general understanding and consensus regarding overall prenatal hormone exposure is that the fetal male brain is exposed to elevated and varying levels of androgens and estradiol (via testosterone aromatization) during mid to late prenatal and early neonatal development, while the fetal female brain is exposed to low and relatively unchanging
levels of these hormones. The times at which testosterone peaks in the male have been suggested to be critical for initiating sexual differentiation mechanisms, leading to differential brain development in males and females (Rhees, Kirk, Sephton, & Lephart, 1997; Ward et al., 2003).

**Figure 2:** Developmental testosterone exposure in male and female mice.

### 1.3.2 The organizational-activational hypothesis

The sexually differential exposure of sex steroid hormones in males and females influences the programming of brain pathways and the expression of behaviour.

Phoenix *et al.* (1959) first defined the “Organizational-Activational Hypothesis”, which
described the influences of sex steroid hormones on neural circuitry and behaviour across development. Programming of brain pathways by sex steroid hormones during fetal development was termed “Organizational”, where effects are typically permanent and lifelong. The term “Activational” referred to the influences of circulating endogenous sex hormones on neuroendocrine function and behaviour, and these hormones must be present in circulation to exert their effects.

1.3.3 Organizational effects of sex steroid hormones

The sex steroid hormones can have organizational effects on the male and female brain during prenatal development and early postnatal life, programming sex-typical neural pathways and resulting in later sex-typical behaviour. Sexually differentiated effects of steroid hormones in the brain have been classically demonstrated by exposing animals to an excess or an absence of hormones during critical periods of development and examining effects on behaviour in adulthood. Taken together, numerous studies have characterized how critical steroid hormones are in producing a masculinized, defeminized or feminized brain. The concepts of masculinization, defeminization and feminization may be best understood by examining steroid hormone effects on reproductive behaviour.

Masculinization is an active process where neural networks for male typical behaviours are programmed, and is thought to occur via exposure to androgens and estradiol during critical periods of prenatal development (McCarthy, 2008). Typical male sexual behaviour in mice and rats includes the approach of a sexually receptive female and attempting to copulate with her (McCarthy, 2012). Pioneering work by Phoenix et al.
(1959) demonstrated masculinization of sexual behaviour could occur via exposure to testosterone during mid-late prenatal development, and this was confirmed by numerous other studies (Gerall & Ward, 1966; Nadler, 1969; Popolow & Ward, 1979). In 1977, McEwen et al. (1977) suggested that masculinizing effects of testosterone, however, occurred primarily through aromatization of testosterone to estradiol. Many studies have since confirmed that estradiol’s actions on ERs in the brain are involved in masculinization of sexual behaviour. Female mice that did not produce a functional AFP proteins demonstrated a greater frequency of mounting, intromissions and pelvic thrusting behaviour compared to wildtype females (Bakker et al., 2006). Male mice without functional aromatase enzymes had prolonged latencies to mount and a decreased mounting frequency in a 30 minute sexual behaviour test (Honda, Harada, Ito, Takagi, & Maeda, 1998). Male mice lacking ERα did not express any male sexual behaviours, including mounts, intromissions or ejaculations (Ogawa et al., 2000). However, ER activation is not the only means to program male sexual behaviour during development. Activation of ARs by pre- and peri-natal androgens can also contribute to the expression of typical male sexual behaviour in adulthood. Male mice that lack functional ARs demonstrated no male sexual behaviour, including mounting, intromissions or ejaculations, and behavioural recovery was not observed following DHT treatment in 30 and 90 minute sexual behavioural tests (Sato et al., 2004). Treatment with E2 appeared to restore mounting and intromittive behaviour to about 50% frequency of what was observed for wildtype males (Sato et al., 2004). In a 10h sexual behaviour test, 100% of control males demonstrated mounting, intromission, pelvic
thrusts and ejaculation, while only 55% of males lacking functional ARs demonstrated mounting, intromission and thrusts, and 33% reached ejaculation (Raskin et al., 2009). Indeed, male mice deficient in AR functionality in the brain demonstrated a lower probability of initiating mating compared to controls in a 30 minute behavioural test, however once sexual behaviour was initiated, the pattern of expression was similar to that of control male mice (Juntti et al., 2010). Perinatal DHT treatment resulted in a high frequency of female wildtype mice exhibiting mounting and intromissions when administered DHT or E2 (Sato et al., 2004). Overall, it has been suggested that while the AR may play some role in developmental programming of masculinized behaviour, it is not necessarily critical for its expression: the primary masculinizing effects of testosterone occurs through aromatization to estradiol and activation of ERs (Juntti et al., 2010; Schwarz & McCarthy, 2008; Zuloaga, Puts, Jordan, & Breedlove, 2008).

Defeminization occurs when the ability to demonstrate female typical behaviour is lost, by either prevention of female typical programming, or overriding already programmed circuits (McCarthy, 2008). Early studies have also demonstrated defeminizing effects of testosterone, as female rats exposed to prenatal injections of androgens experienced a reduction in sexual receptivity behaviour in adulthood (Hoepfner & Ward, 1988; Huffman & Hendricks, 1981; Popolow & Ward, 1979; Rhees et al., 1997). These effects also may be attributed to aromatized estradiol exposure during development, which has been demonstrated to have defeminizing effects on offspring. Female mice with mutated AFP proteins demonstrated no female sexual behaviour, even when administered estradiol and progesterone before exposure to a sexually
active male (Bakker et al., 2006). Female mice treated neonatally with estradiol also show a reduction in typical female sexual receptivity when primed with estrogen and progesterone administration compared to vehicle treated females (Wu et al., 2009). Specifically, activation of ERβ appears necessary for defeminization, as castrated ERβ knockout (KO) mice treated with estradiol and progesterone demonstrate lordosis behaviour and allow mounting and pelvic thrusting (Kudwa, Bodo, Gustafsson, & Rissman, 2005).

Brain feminization has been classically described as a default process, where feminization occurred if androgen and estrogen exposure is absent during development, predisposing the offspring to exhibit typical female behaviours upon the influence of later activational hormones (McCarthy, 2008). Female sexual behaviour is observed as a willingness to mate, typically characterized by the lordosis posture, which enables male mounting and intromission (McCarthy, 2012). Male rats that received gonadectomy on the day of birth demonstrated lordosis to an equivalent frequency to that of normal female rats and female rats that received gonadectomy at birth when administered estradiol benzoate and progesterone (Whalen & Edwards, 1967). ARs did not appear necessary for female sexual behaviour, as female mice lacking a functional ARs still demonstrated lordosis as frequently as wildtype mice, and did not exhibit any male sexual behaviours (Sato et al., 2004). However, a deficiency in ARs does not directly program lordosis behaviour, as male mice without functional ARs did not demonstrate lordosis, even under administration of E2 and progesterone (Sato et al., 2004).
Recent findings have shown that feminization may be more than simply a passive default process occurring due to an absence of sex steroid hormones during development. For example, brain feminization of the preoptic area required active suppression of masculinization by DNA methylation (Nugent et al., 2015). There may also be a critical period for the organizing actions of estrogens on brain feminization, which occurs between birth and puberty (Bakker & Baum, 2008). For example, administering estradiol to aromatase-KO female mice between postnatal day (PD) 15 and 25, but not between PD 5 and 15, enhanced the ability of these mice to perform lordosis when primed with progesterone in adulthood (Brock, Baum, & Bakker, 2011). Thus, the traditional view whereby feminization results from an absence of steroid hormone exposure during development may need to be re-evaluated.

1.3.4 Hormone exposure during postnatal life

After birth, males and females continue to be exposed to differentiated levels of steroid hormones, which can have both organizational and activational effects on the brain and behaviour. In female mice, serum estradiol remained negligible until PD 7, when ovarian secretions began (Mannan & O'Shaughnessy, 1988). Estradiol secretions, however, remained relatively low until reproductive maturation at puberty (Bell, 2018). Across multiple strains of female mice, testosterone levels remained low from PD0 to PD10, and a peak in testosterone occurred at PD21 (Bell, 2018). In male mice, testosterone levels fell after the day of birth and remained low until approximately PD30 (Bell, 2018). Levels began to rise on PD 30, peaked on approximately PD40, then declined to level off by approximately PD60 (Bell, 2018). In males and females, puberty
begins when hypothalamic neurons release bursts of gonadotrophin releasing hormone, which results in pulses of luteinizing hormone (LH) release from the anterior pituitary gland (Hiort, 2002; Sisk, Richardson, Chappell, & Levine, 2001). Puberty has been observed to occur at ~PD44 in male, and PD32 in female CD1 mice (Varney, Varney, Hemken, Zavos, & Siegel, 1991). In males, LH stimulates Leydig cells of the testes to produce testosterone. Testosterone then remains elevated from the time of puberty until older adulthood, when it begins to decline (Machida, Yonezawa, & Noumura, 1981). In females, LH stimulates thecal cells of the ovary to produce androgens (Barbieri, 2014; Hiort, 2002). Granulosa cells in the ovary contain aromatase and convert androgens to estradiol (Barbieri, 2014). Just prior to first ovulation, estradiol, progesterone and testosterone levels have been observed to rise in female rats (Parker & Mahesh, 1976). Levels of sex steroid hormones then fluctuate in females throughout their ovarian cycles, until the time of reproductive senescence (Nilsson et al., 2015). In mice and rats, the ovarian cycle is called the estrous cycle, which completes a full cycle within 4-5 days. The estrous cycle consists of 4 stages: proestrus, estrus, metestrus and diestrus. In mice, estradiol is elevated during proestrus, low during estrus and metestrus, and begins to rise again during diestrus (Nilsson et al., 2015). Thus, beginning in adolescence and persisting throughout adult life, sex steroid hormones continue to remain present in the circulation and are differentially expressed in males and females.
1.3.5 Activational effects of sex steroid hormones

Circulating levels of sex steroid hormones can also influence sexually differentiated physiology and behaviour in adulthood. Activational effects of sex steroid hormones are often examined using GDX and hormone replaced animal models. Removing the testes of males and the ovaries of females eliminates the primary sources of circulating steroid hormones in adult animals. Hormone replacement can then directly test the effect of specific hormones on physiology and behaviour. Many studies have demonstrated how activational effects of sex steroid hormones drive behaviour in sex-typical directions.

Castration of male rats reduced copulatory and ejaculatory sexual behaviour, and administration of testosterone propionate (TP) restored these behaviours (Feder, Naftolin, & Ryan, 1974; Larsson, 1967; Stone, 1939). Daily injections of either estradiol or testosterone resulted in early demonstrations of male sexual behaviour in pre-pubertal male rats (Baum, 1972). Castrated male rats given DHT and high doses of estradiol benzoate demonstrated all male copulatory behaviours (mounting, thrusting, intromission and ejaculation) as well as lordosis (Feder et al., 1974). Ovariectomy reduced the frequency of female sexual behaviours, and administration of estradiol alone, estradiol and progesterone, or progesterone and testosterone significantly recovered these behaviours (Pfaff, 1970). Estradiol administration to ovariectomized females was also sufficient to elicit an increased frequency of male sexual behaviours including mounts and thrusts (Pfaff, 1970). Overall, circulating sex steroid hormones in adulthood can play an important role in the presentation of male-typical and female-typical behaviours.
1.3.6 Prenatal and postnatal hormone interactions

The interaction of organizational and activational effects of sex steroid hormones becomes even more complex when examining the effects that early hormone exposure has on later hormone profiles. Prenatal hormone exposure may influence reproductive system development in male and female offspring, resulting in altered secretion of steroids from the gonads later in life. Female rats treated prenatally with a high dose of androgens had irregular estrous cycles and elevated plasma testosterone and estradiol in adulthood (Mannerås et al., 2007; Wu & Shah, 2011). Treatment with flutamide, an AR antagonist, from birth to PD5 led to increased testosterone levels in adult rats (McCormick & Mahoney, 1999). Male rats exposed to increased TP during prenatal development had a delayed onset of puberty, reduced number of Sertoli cells, and reduced plasma testosterone in adulthood (Dela Cruz & Pereira, 2012; Tehrani et al., 2013). Thus, by influencing the profile of steroid hormones later in life, prenatal hormone exposure can indirectly affect activational effects of adulthood hormones on physiology and behaviour.

1.3.7 Developmental sex steroids and neuropsychiatric disorders

Most research on the influence of steroid hormones on the brain and behaviour has manipulated hormone exposure to high levels, far beyond what would occur physiologically. The need for research which exposes subjects to biologically relevant concentrations of steroid hormones is made clear when examining the consequences of steroid hormone action in human health and diseases. There is a natural range of normal steroid hormone levels and receptor expression profiles across individuals, as
well as changes in circulating steroid hormone levels which occur throughout aging, resulting in a wide variation in hormone profiles from individual to individual. Naturally elevated steroid hormone profiles have hypothesized to be involved in the etiology of various sexually differentiated brain disorders, like Autism Spectrum Disorder (ASD). ASD describes a group of neurodevelopmental disorders which are characterized by social impairment, restricted interests and repetitive behavioural patterns. While the etiology of ASD appears multifactorial, and likely emerges from the interaction of genetic, environmental and epigenetic risk factors, one of the most prominent observations is that ASD affects males four times more frequently than females (Halladay et al., 2015; Tordjman et al., 2014). Additionally, both males and females with ASD have been described as having male typical cognitive characteristics (Baron-Cohen, 2002). As there are no genetic factors that completely predict ASD, hypotheses surrounding other factors associated with maleness have been developed to try to explain the link between males and ASD. Baron-Cohen coined the “Extreme male brain theory of autism”, as one such hypothesis. This theory is based on the premise that females and males have unique cognitive profiles. Typical females demonstrate higher levels of empathizing than males, while typical males demonstrate higher levels of systemizing than females. Empathizing refers to the drive to understand and respond to another person’s emotions. Systemizing refers to the drive to understand the rules and variables which control a system. Regardless of sex, individuals with ASD show a strong preference for systemizing over empathizing, or an “extreme male” profile (Baron-Cohen et al., 2014). Since testosterone is responsible for masculinizing the
male brain during development, Baron-Cohen hypothesized that an elevation in prenatal testosterone might predispose offspring to an ASD phenotype (Baron-Cohen, 2002).

Indeed, many studies have suggested altered testosterone to be a factor in ASD. Both salivary and plasma androgens have been correlated with ASD diagnoses in children and adults (Majewska et al., 2014; Ruta, Ingudomnukul, Taylor, Chakrabarti, & Baron-Cohen, 2011). Females with ASD have higher androgen levels, and females with congenital adrenal hyperplasia, a condition which exposes them to high levels of androgens during prenatal development, have higher levels of traits typically associated with ASD (Knickmeyer et al., 2006; Schwarz et al., 2011). Mothers of children with ASD were found to have higher rates of other testosterone related disorders, such as polycystic ovarian syndrome or severe acne (Ingudomnukul, Baron-Cohen, Wheelwright, & Knickmeyer, 2007). Additionally, a significant positive relationship was found between fetal testosterone levels and traits typically associated with ASD in toddlers (Auyeung, Taylor, Hackett, & Baron-Cohen, 2010). Baron-Cohen and colleagues (2015) further investigated the hypothesis that prenatal androgen exposure is involved in ASD development by examining steroid hormone levels in amniotic fluid samples in individuals who later developed ASD. Testosterone levels were found to be elevated in amniotic fluid samples from individuals who developed ASD compared to control individuals, further supporting the hypothesis that prenatal androgen may be a risk factor for the development of ASD (Baron-Cohen et al., 2015). How prenatal androgen may be predisposing the brain to develop in a way that increases the risk of ASD remains to be determined.
Another factor to consider regarding the etiology of ASD is the importance of timing of risk factor exposure. The first trimester may be a critical time for the development of ASD, as the window of vulnerability may be especially heightened during this time when organogenesis, including neurogenesis, occurs. Indeed, many other factors, including maternal exposure to immune system activators, maternal nutrition, and pesticide exposure, have been observed to influence risk of ASD in offspring during the first trimester of pregnancy (Lyall, Schmidt, & Hertz-Picciotto, 2014). Perhaps it is not only androgen exposure during development which heightens the risk of ASD, but also the timing which this exposure occurs.

In human males, the prenatal period for sexual differentiation occurs from approximately week 8 to week 24, during which time testosterone levels are elevated in males compared to females (Hines, Constantinescu, & Spencer, 2015). The only study which has directly investigated the link between elevated prenatal androgen and ASD is that by Baron-Cohen et al. (2015), however, as amniocentesis samples are typically taken in the second trimester of pregnancy, between weeks 15 to 20 of pregnancy, it is unclear how long the elevation in testosterone persisted in those individuals sampled. Other work does however suggest that testosterone may be elevated earlier in development in individuals with ASD. The second (2D, index finger) to fourth (4D, ring finger) digit ratio (2D:4D) can be used as a measure of prenatal androgen exposure, as 2D:4D is negatively correlated with prenatal testosterone. It has been shown that individuals with ASD, as well as their siblings, fathers and mothers, had lower 2D:4D ratios than control individuals (Manning, Baron-Cohen, Wheelwright, & Sanders, 2001).
Furthermore, the critical period for finger digit development occurs from approximately the 8th to 13th week of pregnancy, with adult bone-to-bone proportions for finger digits determined by the 13th week (Garn, Burdi, Babler, & Stinson, 1975), suggesting that for androgens to influence the 2D:4D ratio, they must do so relatively early in pregnancy.

The association between prenatal testosterone, 2D:4D ratio and ASD led us to hypothesize that it is not only androgen elevation during development that presents as a risk factor for ASD, but that timing is also critical, and an early elevation in testosterone may influence the development of the brain when it is highly vulnerable, increasing the risk of ASD.

1.3.8 Investigating the effects of low doses of prenatal testosterone

The first stage in testing the hypothesis that an early elevation in prenatal androgen was a risk factor to developing ASD was investigating the influence of an early elevation in prenatal testosterone on the expression of behaviours which are typically disrupted in ASD. Since it was ethically challenging to investigate this hypothesis in humans, mice were used as a study system so steroid hormone levels could be altered prenatally (Howes, 2016; Wasson, 2017). This study pregnant mice were exposed to three low doses of TP (10μg/injection) during the 12th, 14th, and 16th days of pregnancy and offspring behaviours were investigated, both before and after adolescence (Howes, 2016; Wasson, 2017). Pregnant dams injected with TP were confirmed to have plasma testosterone levels which were significantly higher than control dams, with levels falling at the upper end of physiological range, mimicking testosterone levels seen in amniotic fluid samples in individuals who later developed
ASD in humans (Wasson, 2017). After birth, offspring were run through a series of behavioural tests aimed to target sociability, learning, and anxiety behaviour. In adolescence, TP male and female offspring demonstrated higher anxiety compared to control mice. TP males also displayed an increase in dominance behaviour. In adulthood, TP male mice had increased anxiety-like behaviour compared to control males. Social learning was impaired in GDX-TP treated males and females compared to control mice. TP males demonstrated more non-social behaviour, reduced dominance, and increased aggression compared to control males. Overall these results demonstrated that most behavioural effects of elevated TP occurred in adult males, specifically regarding social and anxiety-like behaviours. This study was one of the first which demonstrated that low doses of prenatal testosterone given early in development were sufficient to induce lifelong, sexually differentiated effects on offspring behaviour. Furthermore, this study suggested that an early increase in prenatal testosterone disrupted behaviours that are often affected in ASD, specifically in males.

1.3.9 Early testosterone, ASD, and schizophrenia

While prenatal TP injected offspring were investigated for behaviours typically altered in ASD in Howes (2016) and Wasson (2017), most effects of prenatal testosterone on behaviour did not occur until after adolescence. ASD typically develops in the first few years of life, thus the prenatal testosterone exposed mouse model did not appear to closely represent an ASD phenotype. There are etiological similarities between ASD and other neurological conditions where behavioural changes have a later age of onset, such as schizophrenia. Males are affected by schizophrenia ~1.4
times more commonly than females, and females have a later onset, and a better prognosis (Abel, Drake, & Goldstein, 2010). Androgens have been proposed as a potential factor underlying these sex differences. Schizophrenia diagnoses peak in males at the time when testicular androgen secretion begins during puberty (Abel et al., 2010), and females with schizophrenia were observed to have higher androgen levels (Yildirim, Dogan, Semiz, & Kilicli, 2011). Though symptoms of schizophrenia first emerge in adolescence and early adulthood, increasing evidence has suggested schizophrenia has neurodevelopmental origins (Owen, O'Donovan, Thapar, & Craddock, 2011). A number of studies have implicated prenatal factors to increase the risk of schizophrenia in offspring, such as maternal adverse life events (Khashan et al., 2008), complications during pregnancy and delivery (Cannon, Jones, & Murray, 2002) and maternal infections during pregnancy (Brown, 2006). Consistent with the neurodevelopmental hypothesis, some papers have suggested that prenatal hormone exposure in the brain could play a role in schizophrenia etiology (Markham, 2012). Thus, the mouse model developed by Howes (2016) and Wasson (2017) may also be valuable for investigating mechanisms which may be involved in schizophrenia.

1.4 Prenatal testosterone and the HPA axis

It is currently unknown how early elevations of prenatal testosterone resulted in sexually differentiated changes in offspring social behaviour and anxiety (Howes, 2016; Wasson, 2017). While numerous physiological mechanisms have been implicated in the expression of these behaviours, the hypothalamic-pituitary-adrenal (HPA) axis is a strong candidate pathway that may underly the behaviour changes seen in Howes
(2016) and Wasson (2017). The HPA axis a neuroendocrine pathway which results in the release of steroid hormones that play a primary role in stress responsivity and regulation, otherwise known as the glucocorticoids (GCs). The HPA axis regulates both anxiety and social behaviour, is normally sexually differentiated and is influenced by sex steroid hormone exposure (Dunn & Berridge, 1990; Goel et al., 2014; Wooddell et al., 2017). Prenatal HPA axis activation additionally affects later-life stress responsivity, the expression of anxiety and social behaviour, and is also a risk factor toward ASD development (Jafari, Mehla, Kolb, & Mohajerani, 2017; Lee, Brady, Shapiro, Dorsa, & Koenig, 2007; Patin, Lordi, Vincent, & Caston, 2005; Ward, 1990).

1.4.1 The HPA axis

The HPA axis regulates circulating levels of GCs under basal conditions, and produces a rapid upregulation of GCs, in response to stress. Regardless of stress conditions, the main pathway which executes GC release begins with corticotropin releasing hormone (CRH) secretion from the paraventricular nucleus of the hypothalamus into hypophysial portal vessels in the external lamina of the median eminence. CRH is transported through hypophysial vessels to the anterior pituitary gland, where by binding to its receptors (CRHR1 and CRHR2), it stimulates the production of proopiomelanocortin (POMC) mRNA. The POMC protein is then cleaved into various compounds, including adrenocorticotropic hormone (ACTH). ACTH enters the main bloodstream and travels to the adrenal cortex, where it causes the release of GCs, primarily cortisol in humans and corticosterone (CORT) in rodents. GCs exert their actions by binding to both the glucocorticoid receptors (GRs) and mineralocorticoid
receptors (MRs). Localization of GR in the rat brain is observed in numerous brain regions, including the hippocampus, lateral septum, paraventricular nucleus of the hypothalamus (PVN), amygdala, locus coeruleus and raphe area, while MR expression has been localized primarily in the hippocampus and lateral septum (Reul & De Kloet, 1986). MRs also have a ten-fold affinity for GCs compared to GRs (Reul & De Kloet, 1985). Thus, GC binding to GRs and MRs can lead to differential outcomes, depending on GC concentration and localization.

During unstressed conditions, natural fluctuations in circulating GCs occur, which play a main role in maintaining homeostasis. These fluctuations in GCs result, in part, from signals from the suprachiasmatic nucleus of the hypothalamus influencing the release of CRH (Spiga, Walker, Terry, & Lightman, 2014). Under basal conditions, when levels of circulating GCs are relatively low, GCs primarily bind MRs in the brain (Reul & De Kloet, 1985). Therefore, CORT binding to MR in the hippocampus has been suggested to provide tonic, inhibitory control on the HPA axis, maintaining baseline CORT levels (Reul & De Kloet, 1986). CORT binding to GRs primarily occurs during a stress response, thus, these effects will be discussed below. GC actions extend to numerous biological systems, such as cardiovascular, immune, and cognitive systems, such that regulation of baseline GC levels can have widespread influences on physiology and behaviour (Burford, Webster, & Cruz-Topete, 2017; De Quervain, Schwabe, & Roozendaal, 2016; D. J. Walker & Spencer, 2018)

During a real or perceived stressor, the HPA axis becomes activated, resulting in an increase in circulating GCs in the body. The increased GCs stimulate the
sympathetic nervous system, resulting in activation of the “fight or flight” response, which functions to prepare the body to cope with the stressor. This includes the mobilization of energy through upregulating readily available glucose, increasing cardiovascular output and blood pressure, and suppressing other physiological drives, such as appetite (Sapolsky, Romero, & Munck, 2000). When GCs levels are elevated, they bind in substantial quantities to GRs in the hypothalamus, pituitary, and in other brain regions involved in regulating the HPA axis, such as the hippocampus (Reul & De Kloet, 1985). There, activation of GRs, as well as MRs, proceeds as a form of negative feedback to reduce HPA axis activation and prevent long-term upregulation of GCs (O’Brien, 1997).

1.4.2 The HPA axis influences social and anxiety behaviours

Components of the HPA axis are involved in the expression of social and anxiety behaviours. Social behaviour in mice can be investigated by observing the animals in their approach or avoidance of social stimuli, their interest in investigating social stimuli and their demonstration of affiliative or aggressive behaviour (Ervin et al., 2015; Howes, 2016). GCs and CRH have been implicated in the expression of both affiliative and agonistic behaviours. Stereotaxic injections of cortisol into the anterior hypothalamus of golden hamsters resulted in heightened aggressive behaviour (Hayden-Hixson & Ferris, 1991). Additionally, a positive feedback loop between the HPA axis and aggressive behaviour has been established in rats, where HPA axis activation increases aggression, which further increases HPA axis activation (Kruk, Meelis, Halász, & Haller, 2004). Female rhesus monkeys who had higher levels of social affiliation had lower
levels of hair GCs (Wooddell et al., 2017). Intracerebroventricular injections of CRH decreased active social interactions in rats (Dunn & File, 1987). Anxiety in mice produces a number of behavioural responses, such as increased vigilance, freezing, avoidance of potentially threatening scenarios, and suppressed food consumption (Lezak, Missig, & Carlezon, 2017; D. L. Walker, Toufexis, & Davis, 2003). Several behavioural tests have been developed to evaluate the expression of these anxiety-like behaviours (Lezak et al., 2017). CRH has been demonstrated to have anxiogenic effects in various behavioural tests of anxiety (Dunn & Berridge, 1990; File, Johnston, & Baldwin, 1988). Mice overexpressing CRH demonstrate increased anxiety behaviours (Stenzel-Poore, Heinrichs, Rivest, Koob, & Vale, 1994), and mice deficient in the CRH-R1 have reductions in anxiety behaviour (Timpl et al., 1998). In humans, CRH dysregulation has been proposed as a central mechanism in the etiology of anxiety disorders (Reul & Holsboer, 2002; Risbrough & Stein, 2006). Additionally, corticosteroids themselves have anxiogenic effects. An single dose of high physiological levels of CORT was sufficient to increase anxiety responses in the elevated plus maze in male rats (Mitra & Sapolsky, 2008). Overall, previous work has suggested that CRH and corticosteroids could play an underlying role in the mechanisms behind social and anxiety behaviour.

1.4.3 Sex differences and the HPA axis

It has been well documented that neuroendocrine responses to stress are sexually differentiated. In the absence of a stressor, basal CORT levels are sexually differentiated in rats and mice. CORT levels follow a circadian pattern, rhythmically
changing throughout the day, and peaking at the beginning of the active period (Atkinson & Waddell, 1997). Female rats consistently demonstrate higher resting levels of CORT, as detected in plasma, adrenal or fecal analyses (Atkinson & Waddell, 1997; Babb, Masini, Day, & Campeau, 2013; Barker, Bobrovskaya, Howarth, & Whittaker, 2017; Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Kitay, 1963; Lu et al., 2015; Luo et al., 2013; Weinstock, Razin, Schorer-Apelbaum, Men, & McCarty, 1998). It has been additionally shown that female CORT levels are consistently elevated compared to males, regardless of the time the measurements were taken (Watts & Swanson, 1989). Higher basal ACTH levels have also been observed in female rats compared to males (Gagliano, Nadal, & Armario, 2014; Leśniewska, Nowak, & Malendowicz, 1990).

HPA axis responses to various acute stressors are observed to be consistently elevated in female rats and mice. Responses included elevated plasma CORT and ACTH, and CORT and ACTH rising more rapidly compared to males (Aloisi, Ceccarelli, & Lupo, 1998; Babb et al., 2013; Goel & Bale, 2008; Haleem, Kennett, & Curzon, 1988; Handa, Burgess, Kerr, & O'Keefe, 1994; Heinsbroek, Van Haaren, Feenstra, Endert, & Van de Poll, 1991; Iwasaki-Sekino, Mano-Otagiri, Ohata, Yamauchi, & Shibasaki, 2009; Kant et al., 1983; Kitay, 1963, 1961; Le Mevel, Abitbol, Beraud, & Maniey, 1979; Livezey, Miller, & Vogel, 1985; Lu et al., 2015; McCormick, Linkroum, Sallinen, & Miller, 2002; Rivier, 1993, 1999; Sterrenburg et al., 2012; Viau, Bingham, Davis, Lee, & Wong, 2005; Weinstock et al., 1998). In these studies, the source of the stressor did not appear to influence HPA axis activation, as females demonstrated heightened HPA axis
responses regardless of whether the stressor was ether anesthesia, alcohol, a novel environment, footshock, restraint, or forced running.

The duration of time which HPA axis metabolites stay active following a stressor has also been observed to be sexually differentiated, but results from different studies have not been consistent. Some studies have shown females to metabolize plasma CORT faster than males (Glenister & Yates, 1961; Kitay, 1961, 1963; Lin et al., 2008). However, female rats were observed to have a longer duration of CORT response to footshock stress compared with males (Heinsbroek et al., 1991).

The elevation in basal and stress induced CORT in females may be partially countered by a simultaneous elevation in corticosteroid binding globulin (CBG). CBG binds to CORT in circulation, rendering it biologically inactive. Thus, only free CORT is available to bind to MRs and GRs to exert physiological effects and negative feedback. Females have been observed to have higher CBG levels compared to males (Gala & Westphal, 1965; McCormick et al., 2002; Minni et al., 2014). Similarly, CRH-binding protein (CRH-BP) binds to CRH and prevents CRH from stimulating the pituitary. Female mice demonstrated higher levels of CRH-BP compared to males, both basally and following restraint stress (Speert, McClennen, & Seasholtz, 2002; Stinnett, Westphal, & Seasholtz, 2015). Thus, while female rats and mice consistently demonstrate elevated levels of CORT and greater HPA axis responsivity compared to males, elevations in CBG and CRH-BP in females may reduce the availability of these GCs to bind to receptors, and therefore potentially lessen the functional implications of elevated GCs in females.
1.4.4 Organizational effects of steroid hormones on the HPA axis

Neonatal steroid hormones can have organizational effects on the HPA axis. Male rats that received gonadectomy on the day of birth had increased basal CORT and CORT responses to acute stress (Bingham & Viau, 2008; McCormick, Furey, Child, Sawyer, & Donohue, 1998), even when hormone replacement was given 14 days prior to stress (McCormick & Mahoney, 1999). Neonatal gonadectomy with testosterone or estradiol restored CORT levels to those seen in controls (Bingham & Viau, 2008; McCormick et al., 1998). Rats exposed to the AR antagonist, flutamide, either prenatally or neonatally (between birth and PD5) had increased CORT responsivity in adulthood (McCormick & Mahoney, 1999). Exposure to flutamide or the aromatase inhibitor, ATD, during early development was shown to raise basal and stress induced CORT levels in adult male rats (Bingham, Wang, Innala, & Viau, 2012; Seale, Wood, Atkinson, Lightman, & Harbuz, 2005). In female rats, TP exposure on the day of birth (80 μg/dose) resulted in reduced basal and stress induced CORT responsivity in adulthood compared to control females (Seale, Wood, Atkinson, Harbuz, & Lightman, 2005). In female mice, however, the same results were not observed, since mice receiving TP exposure on the day of birth (100 μg/dose) did not differ in CORT responsivity compared to control females (Goel & Bale, 2008). Thus, prenatal and neonatal sex steroid hormone exposure can program the development of the HPA axis and can influence stress responsivity later in life.
1.4.5 Activational effects of steroid hormones on the HPA axis and anxiety

Effects of the sex steroid hormones on stress responsivity extend into adulthood when sex steroid hormones are continuously present in circulation. Overall, estrogens have been shown to increase HPA axis activation, whereby testosterone attenuates stress responsivity (Goel et al., 2014).

Activational effects of sex steroid hormones on stress responsivity are classically observed by examining the effect of gonadectomy and hormone replacement. In male rats, castration led to increased basal CORT and CORT responsivity to acute stress (Handa et al., 1994; Kitay, 1963; Seale, Wood, Atkinson, Bate, et al., 2004; Seale, Wood, Atkinson, Harbuz, & Lightman, 2004). GDX male rats also demonstrate increased CRH mRNA in the PVN (Seale, Wood, Atkinson, Bate, et al., 2004; Seale, Wood, Atkinson, Harbuz, et al., 2004). Testosterone or DHT replacement following castration has been shown to reduce CORT and ACTH responsiveness to acute stressors (Handa et al., 1994; Lund, Munson, Haldy, & Handa, 2004; Seale, Wood, Atkinson, Harbuz, et al., 2004; Viau & Meaney, 1996). Castration with estradiol replacement increased both basal and stress induced free, functional CORT levels to a greater degree than the effects of castration alone (Handa et al., 1994; Lund et al., 2004). Ovariectomized female rats had lower basal and stress induced CORT compared to intact females (Babb et al., 2013; Seale, Wood, Atkinson, Bate, et al., 2004; Seale, Wood, Atkinson, Harbuz, et al., 2004). Estrogen replacement returned CORT levels back to baseline (Seale, Wood, Atkinson, Harbuz, et al., 2004). Ovariectomy reduced CRH mRNA in the PVN, compared to sham and estradiol
replaced animals (Seale, Wood, Atkinson, Harbuz, et al., 2004). Ovariectomy and estrogen replacement additionally led to higher CORT responses to an acute stressor and prolonged recovery to CORT and ACTH responses (Burgess & Handa, 1992). Ovariectomy with testosterone injections in female mice decreased CORT responsiveness to restraint stress to levels which were observed in males (Goel & Bale, 2010).

The effects on sex steroid hormones on stress responsiveness become even more complex when taking into consideration the female estrous cycle. Indeed, since estrogens have a stimulatory effect on the HPA axis, female stress responses have been shown to vary over the estrus cycle. Basal levels of CORT and ACTH may change throughout the estrus cycle in females (Atkinson & Waddell, 1997; Iwasaki-Sekino et al., 2009; Weinstock et al., 1998). Plasma and adrenal CORT levels are typically highest in proestrus, when estradiol levels are highest (Critchlow et al., 1963; Iwasaki-Sekino et al., 2009; Raps, Barthe, & Desaulles, 1971; Viau & Meaney, 1991).

Thus, both prenatal and postnatal sex steroids can independently influence HPA axis responsivity in offspring. Additionally, since prenatal steroid hormone exposure can influence postnatal steroid hormone levels, it is important to consider this interplay when examining sex differences in the dynamics of the HPA axis.

1.4.6 Prenatal GC exposure

There are various mechanisms by which a fetus can become exposed to GCs in utero. Animal models can experimentally test GC exposure through stressing the
mother during pregnancy. Dams may be subjected to a physical or psychological stressor, or injected with GCs, such as the synthetic GC dexamethasone (DEX). In humans, synthetic GCs are often given to pregnant women who are at risk of preterm birth, as fetal lung maturation occurs in the final stage of pregnancy, and synthetic GC exposure reduces the chance of offspring developing respiratory distress syndrome (Liggins & Howie, 1972). Regardless of the mechanism of exposure, prenatal GCs can produce organizational effects on the fetal brain and HPA axis development, that can result in long-term changes in stress responsivity and behaviour (Viltart & Vanbesien-Mailliot, 2007).

Prenatal stress has sexually differentiated effects on HPA axis development and functionality in offspring. Female offspring of dams subjected to physical stressors during pregnancy consistently demonstrate elevated GC levels and responses compared to males and control offspring. Prenatally stressed (PS) females had elevated basal plasma CORT levels compared to controls and PS males (Montano, Wang, & vom Saal, 1993; Szuran, Plíška, Pokorny, & Welzl, 2000). PS female rats have also demonstrated greater CORT responsiveness to acute stressors (Ježová, Juránková, Mosnárová, Kriška, & Škultétyová, 1996; McCormick, Smythe, Sharma, & Meaney, 1995; Szuran et al., 2000; Weinstock, Matlina, Maor, Rosen, & McEwen, 1992). PS female rats had a prolonged elevation in CORT after acute stress compared to PS males and control females (Szuran et al., 2000; Weinstock et al., 1992). Unlike females, the effects of prenatal stress on male CORT is not as consistent. Males exposed to prenatal stress or GCs have been shown to either show increased basal
CORT or CORT responsivity (Hauser, Feldon, & Pryce, 2009; O'Regan, Kenyon, Seckl, & Holmes, 2004; Shoener, Baig, & Page, 2006), no changes in CORT (McCormick et al., 1995; O'Regan et al., 2004; Szuran et al., 2000; Weinstock et al., 1992), or reduced basal and responsive levels of CORT (Liu, Li, & Matthews, 2001). Prenatal stress has been demonstrated to affect male CRH expression. PS males had reduced CRH mRNA expression in the PVN, while no changes were observed in PS females (Liu et al., 2001). PS males had increased steady state levels of CRH in the amygdala (Cratty, Ward, Johnson, Azzaro, & Birkle, 1995). Overall, prenatal stress has been shown to affect both male and female offspring, but outcomes differ between the sexes.

Some evidence has also suggested prenatal stress to be a risk factor for ASD. Retrospective studies in humans have correlated maternal stress during pregnancy with heightened risk of having a child with ASD (Beversdorf et al., 2005; Ward, 1990). Indeed, individuals with ASD often experience symptoms of anxiety and have alterations in the HPA axis feedback mechanisms. Serum CRH was found to be elevated in children with ASD (Tsilioni et al., 2014). Children with ASD were also seen to have an elevated peak in serum cortisol after an external stressor, as well as a longer recovery time for cortisol to return to baseline (Spratt et al., 2012). Prenatal stress may also disrupt behaviours that are often affected in ASD, as, superficially, the changes in behavior observed in Howes (2016) and Wasson (2017) resemble the changes seen in mice and rats exposed prenatally to stress. When exposed to prenatal stress, male rats and mice demonstrated a reduced expression of active social interactions, reduced social drive and increased anxiety (Jafari et al., 2017; Lee et al., 2007; Patin et al.,
2005). Taken together, these observations suggest that an early elevation in prenatal testosterone may be influencing the development of the brain via similar mechanisms to that which prenatal stress employs.

1.5 Rationale

Beginning in early development and extending into adulthood, the sex steroid hormones are crucial players in determining physiological and behavioural differences in males and females. While steroid hormones are involved in normal brain sexual differentiation, natural variation in hormone levels occur in humans, and elevation in testosterone levels to the upper end of physiological range has been suggested as a risk factor to developing ASD in humans. Although studies have correlated ASD with increased prenatal testosterone (Baron-Cohen et al., 2015a), and elevations in prenatal testosterone have demonstrated to affect social and anxiety behaviour in male mice (Howes, 2016; Wasson 2017), mechanisms underlying the dysregulation which could result from this testosterone exposure remain unclear. The current study was conducted to explore physiological mechanisms which might explain how an early elevation in prenatal testosterone could cause long term, sexually differentiated effects on anxiety and social behaviour.

1.6 Hypothesis and objectives

Since the HPA axis is involved in regulating social and anxiety behaviours, is normally sexually differentiated, and is influenced by steroid hormone exposure, we hypothesized that low doses of early prenatal testosterone affected the development of
the stress system and led to altered HPA axis functioning in offspring. We hypothesized that HPA axis activity would be particularly disrupted in males, since the elevation of prenatal steroid hormone exposure during critical periods of development is crucial for normal male development. Two potential mechanisms were investigated:

(1) As prenatal sex steroid hormone exposure can influence neonatal and postnatal reproductive physiology and hormone profiles, and sex steroids can have activational effects on sexually differentiated behaviour, such as anxiety and social behaviour, we predicted that slightly elevated prenatal testosterone would affect sexual differentiation outcomes, leading to changes in hormone profiles in offspring, which would indirectly result in altered HPA axis responsivity. Furthermore, we predicted that hormone profiles and stress responsivity would be disrupted in males more than females. Pregnant CD1 mice were assigned to a vehicle control group or an experimental group receiving TP. The influence of early testosterone on sexual differentiation was investigated by examining offspring’s reproductive physiology and sex steroid hormone profiles. Importantly, the testosterone surge on the day of birth was characterized, as this surge is a critical event for masculinization. The influence of early testosterone on the HPA axis was investigated by examining basal CORT levels. As the male testes are the primary source of adult testosterone levels, and circulating testosterone can impact GC levels, we predicted that an early elevation in prenatal testosterone would alter testicular weight in adult males and alter testosterone levels in male offspring on the day of birth and in adulthood (Goel et al., 2014). We predicted that alterations in testosterone would also be inversely correlated with CORT levels in male
mice. We predicted that female ovarian weight, uterine weight, estrous cyclicity, and estradiol profiles would remain unaltered by prenatal testosterone exposure.

(2) Like fetal GC exposure, fetal sex steroid hormone exposure can affect HPA axis development and produce long-term changes in stress responsivity. Furthermore, prenatal GC exposure can produce behavioural changes in social behaviour and anxiety, similar to those seen in Howes (2016) and Wasson (2017). Therefore, we predicted that slightly elevated prenatal testosterone would alter HPA axis development via similar mechanisms as prenatal GC exposure. Since an early elevation in testosterone has been previously shown to affect anxiety behaviour specifically in males, we predicted that male offspring who received an early elevation in testosterone or exposure to GCs would demonstrate an altered profile of GC responsivity to stress. Pregnant CD1 mice were assigned to a vehicle control or an experimental group receiving TP or an experimental group receiving DEX, as a model of prenatal GC exposure. Offspring were examined for stress responsivity profiles in adulthood. Furthermore, offspring were run through a behavioural paradigm to investigate the early TP mouse model as a potential model for researching schizophrenia. We predicted that HPA axis responses to restraint stress would be reduced in prenatal TP and prenatal DEX exposed male offspring. We also predicted that TP treated male mice would demonstrate increased behaviours often associated with schizophrenia.
2 Methods

2.1 All experiments

Three sets of experiments were performed. Experiment 1 followed directly from the work of Howes (2016) and Wasson (2017), using similar methodology.

2.1.1 Animals

CD1 male and female mice were obtained from Charles River Laboratories (St. Constance, QC, Canada). Mice arrived at 3 months of age and were used for breeding between 3-4 months of age. All procedures were approved by the University of Guelph Animal Care and Use Committee and were in accordance with the guidelines set by the Canadian Council on Animal Care.

2.1.2 Housing

Mice were housed at the University of Guelph, Central Animal Facility. Male and female mice were double- or triple-housed with mice of the same sex upon arrival to the animal facility. Animals were kept on a 12-hour reverse light/dark cycle where the lights would be off from 8:00 to 20:00. Any activity within the colony room during these hours would occur under red light, as to not disrupt the mice’s circadian cycle. Housing consisted of clear polyethylene cages (26cm x 16cm x 12cm) with metal grated lids, corncob bedding, paper nesting material and a plastic house or paper cup. Mice had access to rodent chow (soy free, 14% Protein Rodent Maintenance Diet, Harlan Teklad, WI) and tap water ad libitum. A high protein diet (18% Protein Rodent Maintenance Diet, Harlan Teklad, WI) was provided to dams and litters from the day of breeding until
one week after weaning (PD27). Mice would be transferred to a clean cage once per week. Room temperature was kept at 21±1°C.

2.2 Experiment 1

**Table 1: Experimental timeline for experiment 1**

<table>
<thead>
<tr>
<th>Timeline</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Mating occurred</td>
</tr>
<tr>
<td>E12, E14, E16</td>
<td>Prenatal treatment injections</td>
</tr>
<tr>
<td>PD0</td>
<td>Day of birth and cross fostering</td>
</tr>
<tr>
<td>PD20</td>
<td>Weaning, vaginal opening checks and lavages begin</td>
</tr>
<tr>
<td>PD43</td>
<td>Gonadectomy or sham surgery</td>
</tr>
<tr>
<td>PD58</td>
<td>Hormone replacement surgery</td>
</tr>
<tr>
<td>PD77</td>
<td>Sacrifice</td>
</tr>
</tbody>
</table>

E – Embryonic day; PD – Postnatal day.

2.2.1 Breeding

After transport to the animal facility, mice were left undisturbed for a minimum time of one week to settle into their new housing conditions and recover from any stress associated with transport before being paired to mate. Breeding pairs were set up on a continuous basis, with approximately one litter being born per week. A sexually naive male and female mouse were paired together in a clean cage for breeding. Vaginal plug checks were done daily at 20:00h until a vaginal sperm plug was observed, or until the female had gone through two cycle stages, then the male would be moved to a new
cage. The observation of a sperm plug would indicate mating had occurred. Two mating pairs would be formed on the same day. The first pair to mate was assigned as the cross-foster litter and the second pair to mate was assigned as the experimental litter.

2.2.2 Prenatal treatment

At 08:00h on E12, E14 and E16, experimental dams were injected subcutaneously with 10μg of TP (Galenova, Saint-Hyacinthe, QC, Canada) in 0.05ml of sesame oil (SO; Sigma-Aldrich, Oakville, ON, Canada), or with 0.05ml SO. The dose of testosterone was chosen with the aim of elevating maternal testosterone to the upper end of normal physiological range. A few drops of liquid bandage (Nexcare™) were applied to the injection site to prevent effluence of the treatment. Cross-foster dams remained untreated throughout pregnancy.

2.2.3 Maternal serum collection

A subset of dams (n=37) were deeply anesthetized with an intraperitoneal injection of avertin (340mg/kg) 24 hours after the E16 injections, and blood was collected by cardiac puncture for later analysis of plasma testosterone levels. Blood was centrifuged at 12,000 RPM for 5 minutes, and serum was aspirated off and stored at -80°C until later hormone analysis.

2.2.4 Day of birth monitoring

Pregnant dams were monitored every 3h from 08:00h to 20:00h from E18 until birth (PD0). From the time labor was observed, dams were left undisturbed for at least 1h, to ensure completion of birth before pups were removed from their mother.
2.2.5 Sex determination

Visual identification of sex is challenging during early postnatal life, particularly in animals that have been treated prenatally with androgens, which may masculinize the external appearance of females (Hotchkiss et al., 2007). To the best of our knowledge, it is unknown whether the dose of testosterone used in this study masculinizes the anogenital distance of female mice. Thus, SRY gene (317 bp) detection by PCR amplification was performed to determine the genetic sex of the pups in the experimental litters. IL3 was used as a control. Toe clippings were taken from the newborn pups and Clotisol (Creative Science, Ballwin, MO, USA) was applied to the site where tissue was removed to limit bleeding. Tissue clippings from each mouse were put into sterile 1.5mL tubes and immediately placed onto dry ice. DNA extraction and genotyping were performed using the KAPA mouse genotyping kit (Sigma-Aldrich). Each tissue sample was combined with 88μL UltraPure™ distilled water (Invitrogen, Waltham, MA USA), 10μL 10X KAPA Express Extract Buffer, and 2μL of 1U/μL KAPA Express Extract Enzyme, then lysed at 75°C for 10 minutes using a Thermomixer (Eppendorf, Mississauga, ON, Canada). Enzyme inactivation then occurred at 95°C for 5 minutes. DNA extract was then diluted 10X with UltraPure™ distilled water. PCR reactions were set up by combining 1μL DNA extract with 12.5μL of 2X KAAPA2G Fast (HotStart) Genotyping Mix with dye, 0.7μL of 10μM SRY forward primer (mSRY Fwd; Invitrogen), 0.7 μL of 10μM SRY reverse primer (mSRY Rev; Invitrogen), 0.5μL of 10μM IL3 forward primer (mIL3 Fwd; Invitrogen) 0.5μL of 10μM IL3 reverse primer (mIL3 Rev; Invitrogen), 0.5μL of MgCl² (Applied Biosystems, Waltham, MA USA) and
8.6μL UltraPure™ distilled water. Samples were loaded into a MyCycler™ thermocycler (BIO-RAD, Hercules, CA, USA) and cycled through the steps listed below (Table 2). 12μL of each sample was loaded into an agarose gel beside a 100bp ladder (Invitrogen) and run under gel electrophoresis using a PowerPac Basic™ power supply (BIO-RAD) for 45 minutes at 100V. Bands at ~402kd were indicative of the SRY gene and these samples were classified as male (Lambert et al., 2000). In the rare instances where PCR failed to give clear SRY bands distinguishing between males and females, pups were sexed based on visual inspection of anogenital distance (Deeney, Powers, & Crombleholme, 2016), with sex confirmed anatomically at weaning.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>57.7</td>
<td>15 seconds</td>
<td>33</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>2 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

### 2.2.6 Pup sacrifice and tissue collection

After pups were genotyped, four males and four females from the experimental litters would be selected at random for cross-fostering. When experimental litters contained more than eight pups, additional pups were euthanized by decapitation for
tissue collection. All pups from cross-foster litters were euthanized. Trunk blood was collected, heads frozen immediately in liquid nitrogen, and body tissue frozen on dry ice. Tissue was stored at -80°C for future analysis. Sacrifice of experimental litters occurred between 6 and 18 hours after birth.

2.2.7 Cross-fostering

The remaining pups were then cross-fostered, because circulating steroid hormones may influence maternal behaviour, and it is currently undetermined whether TP injections altered maternal estradiol and progesterone levels (Stolzenberg & Champagne, 2016). Pups were placed in bedding from the cross-foster mother’s cage for 10 minutes, then placed into the cross-foster mother’s cage. Pups were monitored for 24 hours to ensure adequate maternal care, including the presence of milk spots and pup retrieval. Offspring remained with the cross-foster mother until weaning. If the cross-foster litter was born more than 5 days before the pair-matched experimental litter, the experimental litter would remain with their own mother until weaning. Three litters were unable to be cross fostered in Experiment 1 and Experiment 2.

2.2.8 Weaning and pair housing

Offspring were weaned from their cross-foster mother on PD20. Offspring were double-housed with a same-sex sibling until PD43. If sex-ratios were uneven as a result of incorrect sex identification at birth, offspring were triple-housed. Females would be single housed for 1 week following surgeries (PD 43-PD49, and PD58-PD64), then repaired with the sibling(s) she had been paired with after weaning. At the time of repairing, both females would be transferred to a clean cage and monitored briefly to
ensure successful cohabitation. Males would be singled housed following gonadectomy surgery until the time of sacrifice (PD77), as CD1 males may demonstrate aggression toward other male cage-mates (Kappel, Hawkins, & Mendl, 2017).

2.2.9 Vaginal lavages and estrous cyclicity

Beginning on PD20, at 20:00h females were monitored daily for vaginal opening (VO) to determine the start of the females reproductive period (Ekambaran, Kumar, & Joseph, 2017). Once VO occurred, females were monitored for estrous cyclicity through daily vaginally lavages at 20:00h. Females were briefly placed on top of their cage and 20uL of Milli-Q water (Millipore Canada Ltd., Ontario, Canada) was dispensed via pipette into the vaginal canal. Water was aspirated and dispensed into the pipette tip 4-8 times. The fluid was dispensed onto a glass slide and allowed to dry at room temperature. Slides were examined under light microscopy to determine vaginal cytology. Estrous cycle stages were classified for each day by the primary cell types present in the vaginal smear (Table 3; Caligioni, 2009).
Surgeries were performed on PD43 and PD58. Two hours before all surgeries, mice were injected subcutaneously with of 50 mg/kg of carprofen (Pfizer Canada Inc, Kirkland, QC, Canada) at 10 ml/kg. One hour before surgery, mice were gently restrained in a 50mL falcon tube and ~200uL of blood was drawn from the saphenous vein for later hormone analysis. Blood collection was done before surgeries as HPA axis activation can be triggered by anesthetization using isoflurane (Bekhbat, Merrill, Kelly, Lee, & Neigh, 2016). Blood was centrifuged at 12,000 RMP for 10 minutes, plasma aspirated off, and stored at -80°C. Mice were anesthetized using isoflurane gas (Benson Medical Industries, Markham, ON, Canada). Surgical sites were shaved using an electric razor and sterilized using Germi-Stat Gel 4% Antimicrobial soap, 70% Isopropyl alcohol, and tincture of Savlon. Animals were injected subcutaneously with 0.5ml of warm saline and tear gel (Bausch + Lomb, Vaughan, ON, Canada) was placed on the eyes to prevent dehydration. A mixture containing 0.67% lidocaine (Alveda

| **Table 3. Estrous cycle stage characterization by cell type** |
|-----------------|----------------------------------|
| **Cycle Stage** | **Primary Cell Types Present** |
| Proestrus       | Nucleated epithelial cells       |
| Estrus          | Cornified squamous epithelial cells |
| Metestrus       | Leukocytes and fragments of cornified squamous epithelial cells |
| Diestrus        | Primarily leukocytes, may also have cornified squamous or nucleated epithelial cells |

2.2.10 Surgeries

Surgeries were performed on PD43 and PD58. Two hours before all surgeries, mice were injected subcutaneously with of 50 mg/kg of carprofen (Pfizer Canada Inc, Kirkland, QC, Canada) at 10 ml/kg. One hour before surgery, mice were gently restrained in a 50mL falcon tube and ~200uL of blood was drawn from the saphenous vein for later hormone analysis. Blood collection was done before surgeries as HPA axis activation can be triggered by anesthetization using isoflurane (Bekhbat, Merrill, Kelly, Lee, & Neigh, 2016). Blood was centrifuged at 12,000 RMP for 10 minutes, plasma aspirated off, and stored at -80°C. Mice were anesthetized using isoflurane gas (Benson Medical Industries, Markham, ON, Canada). Surgical sites were shaved using an electric razor and sterilized using Germi-Stat Gel 4% Antimicrobial soap, 70% Isopropyl alcohol, and tincture of Savlon. Animals were injected subcutaneously with 0.5ml of warm saline and tear gel (Bausch + Lomb, Vaughan, ON, Canada) was placed on the eyes to prevent dehydration. A mixture containing 0.67% lidocaine (Alveda
Pharmaceuticals, Toronto, ON, Canada), and 0.17% bupivacaine (Hospira Inc.,
Montreal, QC, Canada) was applied at all incision sites. Incision sites were closed with
EZ Clip surgical staples (Stoelting Co., Illinois, USA). All mice received either
gonadectomy or sham surgery on PD43. Sham surgeries were performed in the same
manner as gonadectomy, however no manipulation of the gonads occurred. Mice who
received gonadectomy surgery then received capsule implantation surgery on PD58.

2.2.11 Ovariectomy and sham surgery

For ovariectomy, a 1cm incision was made in the skin of the lower back, slightly
below the midline along the rostro-caudal plane. The blunt end of a pair of surgical
scissors were used to break the connective tissue which separates the dorsal muscles
from the skin. A 0.5cm incision was made in the dorsal muscle over each ovary and the
ovary carefully drawn through the incision. The oviduct was clamped, and the ovary and
surrounding fat pad removed. The clamp was left for 1-2 minutes to limit bleeding. The
oviduct was placed back through the incisions, below the dorsal muscles, and the
incision in the skin closed with surgical staples. For sham surgery in females, a midline
incision in the skin was made, and the dorsal muscles were separated from the skin (as
in ovariectomy), but no further incision was made into the dorsal muscles. The incision
in the skin was then closed.

2.2.12 Castration and sham surgery

A 1cm incision was made in skin of the scrotum along the dorsal axis. The testis
was located within the tunica and was carefully surfaced via a small incision in the
tunica. The blood vessels and spermatic cord within the tunica were ligated using a
heated hemostatic clamp, and the testes removed. The incision in the skin was closed with surgical staples. For sham surgery in males, a scrotal incision was made and then closed.

2.2.13 PD43 hair collection

Hair shaved from the surgical site of the females was kept for later hair CORT analysis. Hair was additionally shaved from the males at the same location during surgery. The shaved area extended in the rostro-caudal plane, approximately 3cm from the midline to just below the hips, and in the dorsoventral plane, approximately 2cm across the midline. Hair was stored in zip-top bags at room temperature until time of extraction.

2.2.14 PD58 hormone replacement surgery

GDX mice were implanted with capsules on PD58. Mice who received sham surgery on PD43 received carprofen injections and had their blood collected, as the mice who received gonadectomy, but did not receive further surgical manipulations on PD58. Hormone replaced males received capsules containing pure crystalline testosterone (Galenova), and control males received capsules containing cholesterol (Sigma-Aldrich). Capsules were placed in 1% bovine serum albumin (Sigma-Aldrich) for 24 hours prior to surgery to initiate hormone release from the capsules. A 1cm incision was made at the base of the neck. The blunt end of a pair of surgical scissors was used to separate the skin from underlying muscles along the lateral side of the mouse and create a subcutaneous pocket for the capsule. The side of insertion was alternated randomly. The capsule was washed with 100% ethanol (Commercial
Alcohols, Brampton, ON, Canada), then physiological saline, then inserted through the incision into the subcutaneous pocket. The incision was closed with surgical stapes.

### 2.2.15 Capsules

Male capsules were made using 12mm pieces of silastic tubing (1.57 mm internal diameter, 3.18 mm external diameter; Dow Corning, Midland, MI, USA) which were sealed on each end with 1mm of silicone medical adhesive, leaving 10mm of the tube to be filled with testosterone or cholesterol. Hormone replaced females received capsules containing 12.5 ug β-Estradiol 3-benzoate (EB; Sigma-Aldrich) in SO (201.62ug/ml), and control females received capsules containing SO. Female capsules were made using 30mm pieces of silastic tubing (1.98 mm inner diameter, 3.18 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) which were sealed on each end with 5mm of silicone medical adhesive (silicone type A, Dow Corning Corporation), resulting in an internal volume of 0.062mL to be filled. EB filled capsules contained a concentration of 202.98μg/mL EB in SO, and SO filled capsules contained 0.062mL SO. These capsules were designed following previously used protocols to produce hormones at physiological levels, and the capsules have been demonstrated to provide continuous release of hormones for 35 days (Clipperton-Allen, 2011; Ribeiro, Pfaff, & Devidze, 2009).

### 2.2.16 Sacrifice and tissue collection

Mice were sacrificed by cervical dislocation between PD77 and PD82 to collect tissue for later analysis. Females were sacrificed when vaginal cytology indicated they were in metestrus or diestrus, when circulating estrogen levels are low (Nilsson et al., 2009).
Trunk blood was collected for later hormone level analysis. Blood was centrifuged at 12,000 RPM for 10 minutes, then plasma was collected and stored at -80°C. Brains were extracted within 1 minute of sacrifice and flash-frozen in the vapour of liquid nitrogen. Testes from intact males, ovaries from intact females, and uteri from all females were extracted and weighed. Hair was shaved on the entire dorsal side of each mouse, from neck to tail, and stored in zip-top bags at room temperature until time of extraction.

2.2.17 Maternal serum testosterone and pup testosterone analysis

Maternal serum samples were analyzed previously by enzyme-linked immunosorbent assay (ELISA; Wasson 2017), however due to known challenges for obtaining accurate hormone measures by immunoassays (Sturgeon & Viljoen, 2011), testosterone levels in a subset of samples were remeasured by radioimmunoassay (RIA).

Testosterone levels in maternal serum were analyzed using I^{125} Testosterone Double Antibody RIA Kit (MP Biomedicals Ltd., Solon, OH, USA). Each sample was prepared and analyzed in duplicate and compared to a set of 8 standards ranging from 0.3125 to 250 pg/tube. Serum samples were combined with steroid diluent (phosphosaline gelatin buffer; MP Biomedicals Ltd.) to reach a combined volume of 175µl. 50µl of sex binding globulin inhibitor (SBGI) solution was added to each tube, followed by 200µl of Anti-Testosterone primary antibody. Tubes were left at room temperature for 4h, then 200µl of Testosterone I^{125} tracer was added to each tube. After
overnight incubation at room temperature, 50μl of secondary antibody was added to each tube, and tubes were placed in an incubator for 1h at 37°C and shaking at 90 RPM. Tubes were centrifuged at 3200 RPM for 30 minutes at 4°C, then decanted, and hormone levels determined using a Wizard² 2-Detector Gamma Counter (PerkinElmer, Waltham, MA, USA).

Day of birth testosterone levels were measured in pups from experiment 1 and experiment 2 at the same time. See Experiment 2: PD0 blood testosterone analysis for methods.

**2.2.18 Hair CORT**

The choice of collection timepoint was done in attempt for this method to be minimally invasive. As shaving a mouse could not be done without restraint or anesthetization, both of which are highly stressful events, we chose to collect hair on the day of surgery, when mice were already being anesthetized, and immediately after final sacrifice. Hair collected on PD43 was therefore previously unshaven, while hair on PD77 would be a combination of original hair and hair which had regrown since PD43. Indeed, these timepoints also dictated the amount of hair which we were able to collect. On PD43, we aimed to take a minimum amount of hair, to ensure mice did not have an added thermal stress of hair loss during surgery recovery. However, as this was the first time attempting the method of hair CORT extraction followed by RIA in mouse hair, the initial sample of hair required was suspected to be at least 20mg (Erickson, Browne, & Lucki, 2017). Therefore, for the sample of hair collected on PD77, we aimed to collect as much hair as possible, shaving the entire dorsal side of the mouse. While it was
undesirable to have this sample contain both shaven and unshaven hair patches, the possibility existed that hair samples collected on PD43 would not be large enough to be analyzed.

CORT was extracted from hair prior to analysis with RIA. Hair samples of 1mg weight were combined with 5mL methanol (Caledon Laboratories Ltd., Georgetown, ON, Canada), then placed in a sonicating water bath for 30 minutes. Samples were incubated at 50°C for 16 hours. Vacuum filtration was used to remove hair and filter the methanol extract in scintillation vials. Vials were left under a fume hood for 3 days, until all methanol had been evaporated. Steroid residues were reconstituted in 100% ethanol (Commercial Alcohols; 5% total volume) and steroid buffer (MP Biomedical; 95% total volume) to reach a final volume of 50μL. Reconstituted samples were stored overnight at -20°C before analysis.

The optimum sample weight (1mg) was determined prior to extracting individual samples. This was a small enough amount of hair that we could obtain this sample size from our PD43 collection timepoint, and therefore these samples were used, as they only contained unshaven hair. A pool of male and female hair was extracted and reconstituted in steroid buffer. A serial dilution of the extracted pool was prepared, and CORT levels in the serial dilution were determined by RIA (see methods below). The optimum sample weight was determined to be that which produced ~50% binding.

Hair CORT levels were measured using an 1²⁵I Corticosterone Double Antibody RIA Kit (MP Biomedicals Ltd.). The reconstituted samples were analyzed in duplicate
and compared to a set of 6 standards ranging from 12.5ng/ml to 1000ng/ml. 100\(\mu\)l of Corticosterone I\(^{125}\) tracer was added to each tube, followed by 10\(\mu\)l of Anti-Corticosterone antibody. Samples were left to incubate at room temperature for 2h. 250\(\mu\)l of precipitating solution was then added to each tube, and tubes were immediately centrifuged at 3200 RPM for 15 minutes at 4\(^o\)C. Tubes were decanted, and hormone levels determined using a Wizard\(^2\) 2-Detector Gamma Counter (PerkinElmer).

**2.2.19 Adult male plasma testosterone analysis**

An I\(^{125}\) Testosterone Double Antibody RIA Kit (MP Biomedicals Ltd.) was used in attempt to measure plasma testosterone levels in male mice, however, serially diluted samples suggested interference. To remove interfering substances, two procedures were attempted, as described below.

**Extraction using diethyl ether:** Steroid hormones were extracted from plasma using 5:1(v/v) diethyl ether (Sigma-Aldrich) to plasma ratio in 12x75mm test tubes. The sample/ether mixture was vortexed for 2 minutes, then left for 15 minutes to allow for phase separation. The bottom of the test tube was submerged in an ethanol-dry ice bath until the aqueous phase froze, then the organic phase was aspirated and dispensed to a new 12x75mm tube. The organic phase, containing the steroid hormones, was left to evaporate overnight in a fume hood. Steroid hormones were resuspended in 5% ethanol and 95% steroid buffer, as resuspension with 5% ethanol has been shown to improve recovery in comparison to reconstitution in steroid buffer alone (Newman, 2009).
**C18 Solid Phase Extraction**: Solid phase extraction (SPE) was conducted with a GX-271 ASPEC™ SolidPhase Extraction system (Gilson, Middleton, WY, USA) and Resprep SPE C18 cartridges (Restek, Bellefonte, PA, USA). Plasma pools were made from intact and GDX male serum. Standards and samples were combined with 10mL of deionized water in preparation for SPE. The SPE protocol was modified from Newman (2009) and consisted of the steps in *Table 4*. Following extraction, samples and standards were reconstituted in steroid buffer (MP Biomedical).

To investigate the ability of SPE to clear interfering compounds from mouse serum prior, standards, a pool of intact male mouse serum, and a pool of GDX male mouse serum were extracted prior to RIA.
### Table 4: SPE protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Solvation</td>
<td>Columns primed with 3mL ethanol</td>
</tr>
<tr>
<td>Equilibration</td>
<td>Columns primed with 10mL water to prepare sorbent for sample loading</td>
</tr>
<tr>
<td>Sample Loading</td>
<td>Sample passed through the column</td>
</tr>
<tr>
<td>Interference Elution</td>
<td>10mL of water passed through the column to wash out interfering polar compounds</td>
</tr>
<tr>
<td>Sample Elution</td>
<td>5mL of 90% methanol passed through the column to elute steroid compounds</td>
</tr>
<tr>
<td>Drying</td>
<td>Sample dried under stream of nitrogen gas at 40°C</td>
</tr>
<tr>
<td>Resuspension</td>
<td>Dried eluates resuspended in solution</td>
</tr>
</tbody>
</table>

#### 2.2.20 Adult female plasma estradiol analysis

A mouse/rat estradiol ELISA kit (Calbiotech, El Cajon, CA, USA) was used in attempt to measure plasma estradiol levels in female mice, however, results suggested interference (Martin, 2017; unpublished). Additionally, diethyl-ether extraction failed to remove interference (Martin 2017; unpublished). In continued attempt to remove interfering substances, SPE was done to extract steroids, and ELISA used to measure estradiol levels following extraction.

The SPE protocol for extracting estradiol was the same as that for extracting testosterone (Table 4). Following extraction, samples were resuspended in 1% bovine serum albumin (Sigma-Aldrich). To investigate the ability of SPE to clear interfering compounds from mouse serum prior to estradiol ELISA, standards, a pool of intact
female mouse serum, and a pool of GDX female mouse serum were extracted prior to ELISA.

Using a mouse/rat estradiol ELISA kit (Calbiotech), each sample was analyzed in duplicate and compared to a set of 6 standards ranging from 0 to 300 pg/ml. 25µl of each standard and sample was combined with 100µl of Estradiol enzyme conjugate for 120 minutes. Wells were then washed with 300µl of wash buffer 3 times. 100µl of TMB was added to each well for 30 minutes, then 50µl of stop solution was added to complete the colour change. Absorbance measures were read at 450nm using a Synergy™ HT Multi-Detection Reader (BioTek, Winooski, VT, USA).

2.3 Experiment 2

<table>
<thead>
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<th>Table 5: Experimental timeline for experiment 2</th>
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<tbody>
<tr>
<td><strong>Timeline</strong></td>
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<td>E1</td>
</tr>
<tr>
<td>E12, E14, E16</td>
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<tr>
<td>PD0</td>
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<td>PD20</td>
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<tr>
<td>PD25*</td>
</tr>
<tr>
<td>PD69*</td>
</tr>
<tr>
<td>PD77</td>
</tr>
</tbody>
</table>

*Prepulse-inhibition of the startle response was run, but results will not be included in this thesis.
2.3.1 Breeding, day of birth, and weaning

All methods were replicated from Experiment 1 for Breeding, Day of Birth and Weaning. As no surgeries were conducted in experiment 2, both male and female offspring were housed with a same-sex sibling from weaning until sacrifice.

2.3.2 Prenatal treatment

At 08:00h on E12, E14 and E16, experimental dams were injected subcutaneously with either 10μg of TP in 0.05ml of SO, 0.1mg/kg of dexamethasone (DEX; Sigma-Aldrich) in 0.05ml of SO or with 0.05ml SO. A few drops of liquid bandage were applied to the injection site. Cross-foster dams remained untreated during pregnancy.

2.3.3 Prepulse inhibition of the startle response

A disrupted startle response has been documented as a hallmark behaviour in individuals with schizophrenia (Mena et al., 2016). Typically, the startle response can be reduced or eliminated by the previous presentation of a weak stimulus, otherwise known as prepulse inhibition (PPI). Individuals with schizophrenia are observed to have deficits in multisensory gating, which is required for PPI (Chavez, Gogos, Jones, & Van Den Buuse, 2009). Furthermore, PPI can be examined in mice (Chavez et al., 2009). To investigate the TP injected mouse model as a potential model for behaviour associated with schizophrenia, each mouse was run through PPI once in adolescence on ~PD25, and once in adulthood on ~PD69. Testing was run between 16:00h and 20:00h and all testing occurred in the during the dark phase of the light cycle and under red light. PPI was conducted using a previously developed protocol for mice (aromatase KO x
C57B6/J129 strain; Chavez et al., 2009). Thirty minutes before testing, mice were brought to the testing room to acclimatize. Mice were transferred from their home cage directly into the automated startle chambers (SR-Lab; San Diego Instruments, San Diego, CA, USA). Background noise was set to 70 decibels (dB) throughout the entire protocol. In the startle chambers, mice were exposed to a 5-minute acclimation period. The PPI session consisted of eight pulse-alone trials of 115 dB of white noise (40-ms burst) at the beginning and end of the session. The middle trials were presented in a pseudo-randomized fashion, consisting of sixteen additional pulse-alone trials, four groups of eight prepulse-pulse trials, where the prepulse value was 72, 74, 78, or 86 dB, and eight no stimulus trials. The inter-stimulus interval between the prepulse and pulse were either 30ms or 100ms. Inter-trial intervals were randomized between 12 and 28ms to prevent habituation. Responses were measured with SR-Lab software (San Diego Instruments). A maximum of eight mice were tested at a time. If both pair-housed mice were unable to be tested at once, the second mouse was transferred to a startle chamber before the first mouse was returned to its cage, to prevent potential transfer of stress between animals. Startle chambers were cleaned and dried between all sessions.

2.3.4 RESTRAINT STRESS AND SACRIFICE

To determine stress recovery, mice were restrained for 30 minutes to induce a stress response, and were sacrificed either 10 minutes, 1 hour or 3 hours after the end of restraint by cervical dislocation. Restraint stress occurred between 12:00h and 14:00h, during which time the circadian CORT levels are relatively stable (Romero,
2002). Mice were placed in a 50mL falcon tube which had the tip removed to allow for ventilation, and a hole drilled into the cap for the tail to extend. Larger males were restrained in a similarly designed apparatus made of a plastic water bottle which was the same length, but 1cm wider than the falcon tube. Within both restraint tubes, mice could extend forward or backward, but could not turn around. This set up has been shown to be suitable to induce HPA axis responses in mice (Stewart, Roper, Young, O’Carroll, & Lolait, 2008). Following stress, mice were placed back into their home cages until the time of sacrifice by cervical dislocation. Control mice did not receive restraint stress, but were taken directly from their home cage and immediately euthanized. Trunk blood was collected for later hormone level analysis. Blood was centrifuged at 12,000 RMP for 10 minutes, and plasma was collected and stored at -80 °C. Brains were extracted within 1 minute of sacrifice and each hemisphere was separated along the rostro-caudal midline. Half of the brain (alternating between the left and right hemispheres) was frozen by liquid nitrogen, and the other half placed into Golgi-Cox solution.

2.3.5 PD0 blood testosterone analysis

The sample volume was determined prior to analyzing individual samples. A serial dilution made from a pooled sample of blood was prepared, and testosterone levels in the serial dilution were determined by RIA. Sample volume was chosen based on percent binding of sample antigen to the primary antibody in the serially diluted samples, and the volume of blood available per pup.
Testosterone levels in pup blood were analyzed using $I^{125}$ Testosterone Double Antibody RIA Kit (MP Biomedicals Ltd.). Samples consisted of whole blood, as plasma was unable to be extracted from the small volume of blood collected on PD0. The protocol used was the same as that described above in **Experiment 1: Maternal serum testosterone and pup testosterone analysis.**

### 2.3.6 Plasma CORT analysis

The optimum plasma volume was determined by running a serial dilution of pooled male and female plasma samples and determining the volume of plasma which produced ~50% binding. Plasma CORT levels were measured using an $I^{125}$ Corticosterone Double Antibody RIA Kit. The protocol used was the same as that described in **Experiment 1: Hair CORT.**

### 2.3.7 Plasma testosterone analysis

Plasma testosterone levels were measured in male offspring that did not receive restraint stress (controls). The optimum plasma volume was determined by running a serial dilution of C18 SPE extracted pooled adult male plasma and determining the volume of sample which produced ~50% binding. Plasma testosterone levels were measured using an $I^{125}$ Testosterone Double Antibody RIA Kit (MP Biomedicals Ltd.). The SPE extraction protocol used was the same as that described in **Experiment 1: Adult male plasma testosterone analysis.** The RIA protocol used was the same as that described in **Experiment 1: Maternal serum testosterone and pup testosterone analysis.**
2.4 Experiment 3

<table>
<thead>
<tr>
<th>Table 6: Experimental timeline for experiment 3</th>
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<tbody>
<tr>
<td>Timeline</td>
</tr>
<tr>
<td>E1</td>
</tr>
<tr>
<td>E12, E14, E16</td>
</tr>
<tr>
<td>PD0</td>
</tr>
</tbody>
</table>

2.4.1 Breeding and prenatal treatment

All methods were replicated from Experiment 2 for Breeding and Prenatal Treatment. Cross foster litters were not used, as all pups were sacrificed on the day of birth.

2.4.2 Monitoring and sacrifice

Pregnant dams were monitored continually from E19 until birth to ensure the precise time of birth was known. Two or more pups from each litter were then euthanized by decapitation at each of the following time points after birth: 1h, 1.5h, 2h, 2.5h, 4h, and 6h. Trunk blood was collected, and heads and tails were frozen immediately in liquid nitrogen. Tissue was stored at -80°C for future analysis. Sex of all pups was determined by internal sexing, including identifying the uterus in female pups or the testes in male pups (Deeney et al., 2016). Dams were sacrificed following the last pup sacrifices at 6h. Dams were deeply anaesthetized by an intraperitoneal injection of avertin (340 mg/kg), then euthanized by cervical dislocation. Trunk blood was collected,
and brains were extracted and immediately frozen by liquid nitrogen. Blood and brains were stored at -80°C for future analysis.

### 2.4.3 PD0 blood testosterone analysis

A time course of plasma testosterone levels for the first 6 hours after birth was determined using RIA. The protocol used was the same as that described in Experiment 2: PD0 blood testosterone analysis.

### 2.5 Statistical analyses

#### 2.5.1 General data handling

All data manipulations were performed using R (R Core Team, 2015) and all graphs were made using SigmaPlot 14.0 (Systat Software, San Jose, CA). RIA count/minute data was converted to pg/mL values using a four parameter curve fitting routine adapted from DeLean, Munson, & Rodbard (1978). Optical density values from the ELISA were run in duplicate, averaged, and fit to a four-parameter logistic curve using the “Analysis of Dose-Response Curves Package” in R (R Core Team, 2018; Ritz & Streibig, 2015).

#### 2.5.2 Statistical analyses

In general, Analysis of Variance (ANOVA) and general linear models (GLMs) were used to compare data between groups, with sex (male, female), and prenatal treatment (SO control, testosterone treated, DEX treated) as between-subject factors. The Welch two sample t-test was used to further compare means within sex and prenatal treatment groups and the Wilcoxon rank sum test (WRST) was used to
compare group medians. Non-parametric testing or parametric testing with
transformations were used when datasets did not meet assumptions of normality. For
RIA analyses, parallelism between serially diluted samples and the standard curve were
determined by testing equality of slopes using GLMs. A lack of interaction between
these curves indicated that slope lines were similar and suggested that the kit could
reliably measure the samples. Results were considered statistically significant when
p<0.05. Specific analyses used for each experimental result are described below:

**Maternal serum analysis:** The difference in plasma testosterone levels in dams
injected with testosterone compared to dams injected with SO was evaluated using the
WRST.

**Reproductive tissue weights analysis:** The effect of prenatal treatment on the
weights of the ovaries, uteri and testes was investigated using the WRST. Two-way
ANOVA and Tukey multiple comparisons test were used to explore the effect of prenatal
and adult hormone profile on uteri weight. Uteri weight values were square root
transformed prior to the ANOVA and Tukey tests, as suggested by the box-cox
transformation.

**Day of VO and first estrus analysis:** WRST was used to investigate the effect of
prenatal treatment on VO and the day of first estrus. Data were analyzed with and
without the inclusion of outliers whose values fell beyond two standard deviations of the
mean, as outlier inclusion changed the day of VO by ~0.5 days.
**Estrous cyclicity analysis:** Lavage data were split into two time periods for analysis. Data were collected for all mice from the time of VO to the time of gonadectomy or sham surgery, and this dataset is titled Pre-PD43. Lavage data were then continued to be collected from PD44 to the day of sacrifice (PD77) in animals who received sham surgery, and this dataset is titled Post-PD43. Lavage data were analyzed by examining the average length of time per cycle and the percentage of time mice were spending in each cycle stage. The average length of time per cycle was calculated after mice had progressed through at least 2 cycle stages for Pre-PD43, and 3 cycle stages for Post-PD43, and was measured as the number of days between each reoccurring estrus. The Welch two sample t-test was used to compare the effect of prenatal treatment on the average length of time per estrous cycle. Cycle length values were log-transformed for Pre-PD43 analyses, as suggested by the box-cox transformation. Cycle length values for Post-PD43 remained untransformed. The percentage of time mice spent in each cycle stage was calculated as the ratio of the number of days per cycle stage to the total number of days lavages were done. The Welch two sample t-test was used to compare the percentage of time mice were spending in proestrus, estrus, metestrus and diestrus in prenatal TP injected and prenatal SO injected female mice.

**Hair CORT analysis:** A two-way ANOVA was used to analyze the effect of prenatal treatment and sex on CORT levels retained in the mouse’s hair. Prior to ANOVA, log-transformation was performed on CORT values, as suggested by the box-cox transformation. WRST was used as post-hoc analyses to compare the effect of sex in
experimental mice and control mice separately, and to compare the effect of prenatal treatment regardless of sex.

**EXPERIMENT 1: PD0 testosterone analysis:** The effect of prenatal treatment and sex on day of birth testosterone levels were analyzed using a two-way ANOVA. The Kruskal-Wallis rank sum (KW) test and the WRST were used to further analyze the effect of prenatal treatment in each sex independently. Log-transformation of testosterone values were done for all parametric analyses, as suggested by the box-cox transformation.

**EXPERIMENT 1: Adult testosterone analysis:** WRST was used to compare the effect of prenatal treatment on PD43 adult male testosterone levels. The Scheirer-Ray-Hare (SRH) test was used to analyze the effect of prenatal treatment and adult treatment on PD58 adult male testosterone levels. Further analysis by WRST was used to compare testosterone levels between sham operated, prenatal testosterone and vehicle treated males, and to compare testosterone levels between GDX and sham males in each prenatal treatment group. The SRH test was used to compare the effect of prenatal treatment and adult treatment on PD77 adult male testosterone levels. Further analysis by the KW test was used to compare the effect of adult treatment on testosterone levels in each treatment group. In addition, WRST was used to compare testosterone levels between GDX-hormone replaced (GDX-HR), GDX-vehicle replaced (GDX-VR) and sham operated offspring.
**Plasma CORT analysis:** A general linear model (GLM) was used to analyze the effect of sex, prenatal treatment, and time after restraint stress on plasma CORT levels. A GLM was also used to analyze the effect of prenatal treatment and time after stress on plasma CORT levels in each sex separately. Correction for multiple comparisons was done using the Tukey test. Time was coded as a factor in GLM analyses. Welch’s two sample t-test was used to examine the effect of prenatal treatment at each time point after restraint stress. Log-transformation was done on CORT values prior to all analyses, as regression diagnostics of log-transformed data suggested the transformation improved heteroscedasticity.

**EXPERIMENT 2: Adult plasma testosterone analysis:** A one-way ANOVA was used to analyze the effect of prenatal treatment on adult male offspring plasma testosterone levels. Prior to ANOVA, log-transformation was performed on testosterone values, as suggested by the box-cox transformation.

**EXPERIMENT 3: P0 testosterone analysis:** The effect of prenatal treatment and time after birth analysed using a two-way ANOVA. The KW test was used to further analyse the effect of prenatal treatment at each time point independently. Log-transformation of testosterone values were done prior to parametric analyses, as suggested by the box-cox transformation.
3 Results

3.1 Maternal serum analysis

Dams injected with prenatal testosterone had significantly higher plasma testosterone levels compared to SO injected dams when samples were analyzed previously by ELISA (Figure 3A; Wasson, 2017). Reanalysis by RIA confirmed that prenatal TP treated dams had elevated plasma testosterone compared to SO treated dams (Figure 3B; WRST: W=79, p<0.005). Mean levels of testosterone in testosterone treated dams and SO treated dams 0.45ng/mL and 0.16 ng/mL measured with ELISA, and 0.40 ng/mL and 0.15 ng/mL measured with RIA, respectively.

Figure 3: Measurements of maternal testosterone taken after the E16 injection. A: Testosterone levels measured by ELISA (Wasson, 2017; control: n=18, testosterone: n=19). B: Testosterone levels measured by RIA (control: n=9, testosterone: n=10). Error bars ± SEM. *p<0.05, **p<0.005.
3.2 Reproductive tissue weights

There was no significant effect of prenatal treatment on ovarian weight (*Figure 4A*; WRST: \(W=361, p=0.23\)), testicular weight (*Figure 4B*; WRST: \(W=323, p=0.63\)), or uterine weight (*Figure 4C*; WRST: \(W=415, p=0.54\)). Treatment of adult offspring had a significant effect on uterine weight (*Figure 4D*; ANOVA: \(F=23.88, p<0.001\)). Uteri of gonadally intact and ovariectomized-hormone replaced (OVX-HR) mice weighed significantly more than ovariectomized-vehicle replaced (OVX-VR) mice (Tukey HSD: Intact-OVX-VR \(p<0.001\), OVX-HR-OVX-VR \(p<0.001\), OVX-HR-Intact \(p=0.20\)).
Figure 4: Measurements of reproductive tissue weights. A: Ovarian weights in prenatal TP and SO injected mice (control: n=25, testosterone: n=24). B: Testicular weights in prenatal TP and SO injected mice (control: n=27, testosterone: n=28). C: Uterine weights in prenatal TP and SO injected mice (control: n=26, testosterone: n=27). D: Uterine weights in gonadally intact, vehicle replaced, and estradiol replaced mice. Intact and estradiol replaced females had larger uterine weights than vehicle replaced females (intact: n=5-6/group, vehicle replaced: n=9-12/group estradiol replaced: n=8-9/group). Error bars ± SEM. **p<0.0001.
3.3 Day of VO and first estrus

Prenatal treatment did not have any significant effect on VO or the day of first estrus (Table 7). There was a marginal difference in VO between testosterone treated and control mice, but results were dependent on the presence of one litter, who’s values fell beyond the upper limit of outlier acceptance. If this litter was included in analysis, VO was approximately half a day earlier in the testosterone treated mice compared to control mice, however this result was not significant, regardless of whether the outliers were included.

<table>
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<th>Table 7: Day of VO and day of first estrus</th>
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3.4 Estrous cyclicity

From PD20-PD43, females were group housed. Females housed together did not appear to sync in their estrous cycles. The average length of time of the estrous
cycle measured from VO to PD42 was not significantly affected by prenatal treatment 
(*Figure 5A; Welch t-test: t=0.34, p=0.74*). The average length of the estrous cycle was 
5.04 days in testosterone treated mice, and 4.89 days in control mice.

Prenatal treatment did not affect the percentage of time mice were spending in 
each cycle stage of the estrous cycle (*Figure 6A*). From the time of VO to PD42, 
prenatal testosterone injected, and SO injected mice spent approximately the same 
amount of time in proestrus (*Welch t-test: t=-0.33, p=0.74*), estrus (*Welch t-test: t=-0.53,  
p=0.60*), metestrus (*Welch t-test: t=0.10, p=0.92*) and diestrus (*Welch t-test: t=1.28,  
p=0.20*).

From PD43-PD49 and PD58-PD64, females were single housed to recover from  
surgery. From PD50-PD57 and PD65-PD77, females were group housed. Females 
housed together did not appear to sync in their estrous cycles. Prenatal treatment did  
not affect the average length of time of the estrous cycle from PD44-PD77 (*Figure 5B;  
Welch t-test: t=0.10, p=0.92*). The average length of the estrous cycle was 5.23 days in  
testosterone treated mice, and 5.19 days in control mice.

The percentage of time mice spent in each stage of the estrous cycle was not  
affected by prenatal treatment in mice from PD44-PD77 (*Figure 6B*). Prenatal  
testosterone treated and control mice did not differ in the amount of time they spent in  
proestrus (*Welch t-test: t=0.31, p=0.77*), estrus (*Welch t-test: t=-0.72, p=0.48*),  
metestrus (*Welch t-test: t=1.80, p=0.09*) and diestrus (*Welch t-test: t=-0.69, p=0.50*).
**Figure 5: Average number of days per estrous cycle.**

A: Cycle length measured between VO and PD42 (control: n=19, testosterone: n=32). B: Cycle length measured between PD44 and PD77 (control: n=5, testosterone: n=9). Error bars ± SEM.

**Figure 6: Percentage of time spent in each stage of the estrous cycle.**

A: Time in each cycle stage measured between VO and PD42 (control: n=29, testosterone: n=36). B: Time in each cycle stage measured between PD44 and PD77 (control: n=7, testosterone: n=9). Error bars ± SEM.
3.5 Hair CORT levels

Serially diluted, extracted hair samples yielded a displacement curve which was parallel to the CORT standard curve (Figure 7A; GLM: no significant interaction, t=-1.75, p=0.11). The mass of hair determined to be the appropriate sample mass was 1mg. This value was chosen, as a mass of 1.25mg of hair produced 46% binding, and 0.625mg of hair produced 58% binding, approximating 1mg of hair to produce ~50% binding.

Sex but not prenatal treatment was a significant predictor of hair CORT levels in mice (Figure 7B; ANOVA: sex, F=50.77, p<0.001; prenatal treatment, F=0.082, p=0.78). When comparing males and females within each treatment group, female mice had higher CORT levels compared to males, in both prenatal TP and prenatal SO groups (WRST: TP, W=166, p<0.001; SO, W=112, p<0.001). When male and female data were grouped together, the effect of prenatal treatment on hair CORT levels remained nonsignificant (WRST: W=287, p=0.86).
Figure 7: Hair CORT levels. A: Regression plot comparing slopes of the CORT standard curve and sample serial dilution. B: Hair CORT levels. Females had higher levels of CORT retained in their hair compared to males (control: n=10-12/group, testosterone: n=13-14/group). Error bars ± SEM. ***p<0.001.

3.6 Experiment 1: PD0 testosterone levels

Serially diluted male blood samples yielded a displacement curve which was parallel to the testosterone standard curve (Figure 8A; GLM: no significant interaction, t=-1.48, p=0.17). Serially diluted female blood samples yielded a displacement curve which was not parallel to the testosterone standard curve (Figure 8B; GLM: significant interaction, t=-4.63, p<0.001). Therefore, while female testosterone values were included in analyses, results may not be reliable for female data. The volume of blood selected as the sample volume was 5μL. The amount of blood to produce 50% binding was >30μL, however due to the small volume of blood collected on the day of birth, it was not possible to use more than 5μL sample volumes. A sample volume of 5μL produced ~78% binding.
Figure 8: Serially diluted P0 blood in testosterone RIA. Regression plot comparing slopes of the testosterone standard curve, male blood serial dilution, and female blood serial dilution.

Testosterone levels on the day of birth appeared to be significantly affected by prenatal treatment (Figure 9: ANOVA: F=6.10, p<0.005) and sex (F=50.00, p<0.001). Further analysis by the KW test did not suggest a significant effect of prenatal treatment when comparing all treatment groups in either males or females (Males: H=4.60, df=2, p=0.10, Females: H=5.47, df=2, p=0.06). However, when comparing testosterone levels in prenatal testosterone treated and prenatal vehicle treated males, prenatal testosterone treated males had significantly lower testosterone levels than prenatal vehicle treated males (Wilcoxon rank sum: W=188, p<0.05). No significant difference in testosterone levels was seen between prenatal DEX treated males and control males (Wilcoxon rank sum: W=59, p=0.15). The same pattern was seen in females, where testosterone levels were lower in testosterone treated female pups compared to control
females (W=47, p<0.05), but no differences were seen between testosterone levels in DEX treated females compared to control females (W=49.5, p=0.14).

![Day of Birth Blood Testosterone Levels](image)

**Figure 9:** PD0 testosterone levels 6-18 hours after birth. Male testosterone values were significantly greater than females. Testosterone treated males had lower testosterone levels than SO control males. + indicates a significant effect of sex, * indicates a significant effect of prenatal treatment (female: control: n =13, testosterone: n=12, DEX: n=11; male: control n=20, testosterone n=29, DEX, n=9). Error bars ± SEM. *p<0.05, +++p<0.001.

### 3.7 Adult male testosterone levels optimization

Serially diluted plasma samples yielded a displacement curve which was parallel to the testosterone standard curve (*Figure 10A*: GLM: no significant interaction t=1.37, p=0.20). However, the amount of testosterone in pg/mL in the samples in the serial dilution were not consistent, and testosterone values were higher than expected testosterone levels in adult male mice (*Figure 10B*; Machida, Yonezawa, & Noumura, 1981). This suggested potential interference in samples. Serially diluted, ether extracted
plasma samples yielded a displacement curve which was not parallel to the
testosterone standard curve (Figure 10C: GLM: significant interaction, t=4.59, p<0.001),
thus, this method could not reliably be used to correct for interference.

Figure 10: Serially diluted adult plasma in testosterone RIA optimization. A:
Regression plot comparing slopes of the testosterone standard curve and the pooled
male plasma sample serial dilution. B: Testosterone levels in each serially diluted
sample. Testosterone levels were inconsistent and elevated compared to expected
testosterone levels. C: Regression plot comparing slopes of the testosterone standard
curve and the diethyl ether extracted pooled male plasma sample serial dilution.
3.8 Adult male testosterone levels by C18 SPE

The SPE standard curve was parallel to the SPE serial dilution (Figure 11A: GLM: no significant interaction, t=0.305, p=0.7667). Testosterone levels were consistently measured to be ~4ng/mL in 20μL to 1.25μL SPE sample volumes (Figure 11B). These results suggested that SPE effectively removed interference, thus, this method was used to extract testosterone from PD43, PD58, and PD77 adult male plasma samples.

Figure 11: Serially diluted C18 SPE plasma testosterone in RIA. A: Regression plot comparing slopes of the SPE testosterone curve and SPE serial dilution. B: Testosterone levels in each serially diluted SPE sample.

3.9 PD43 testosterone levels

Although mean testosterone levels appeared to be higher in testosterone treated males (mean= 2.18ng/mL) compared to controls (Figure 12; mean=1.15ng/mL),
testosterone levels on PD43 were not significantly affected by prenatal testosterone treatment (WRST: W=64, p=0.32).

\[ \text{Figure 12: PD43 adult plasma testosterone levels (n=10/group). Error bars ± SEM.} \]

3.10 PD58 testosterone levels

Prenatal testosterone treatment did not significantly affect testosterone levels in male offspring on PD58 (Figure 13; SRH: H=0.04, p=0.83). Mean testosterone levels in sham offspring also appeared to be elevated in prenatal testosterone treated males (mean=7.85ng/mL) compared to control males (mean=5.64ng/mL), however this result was not statistically significant (WRST: W=34, p=0.40). Gonadectomy in male offspring was successful at significantly reducing testosterone levels in offspring (SRH: H=26.43, p<0.001). The effect of gonadectomy on PD58 testosterone levels was significant in both testosterone treated (WRST: W=4, p<0.001) and control offspring (WRST: W=0, p<0.001).
Figure 13: PD58 adult plasma testosterone levels (n=9-10/group). Legend indicates prenatal treatment. Error bars ± SEM.

3.11 PD77 testosterone levels

Prenatal treatment did not have a significant effect on PD77 testosterone levels (SRH: H=0.21, p=0.65). Testosterone levels were significantly affected by adult treatment (SRH: H=23.58, p<0.001). The significant effect of adult treatment on testosterone levels was further confirmed in both prenatal testosterone treated males (KW: H=14.45, df=2, p<0.001) and control males (KW: H=8.65, df=2, p=0.01). Further analysis revealed that GDX- hormone replaced (GDX-HR) and sham offspring had significantly elevated testosterone levels compared to GDX- vehicle replaced (GDX-VR) offspring (WRST: GDX-HR x GDX-VR: W=51.5, p<0.001, sham x GDX-VR: W=37, p<0.001), and that GDX-HR offspring had PD77 testosterone levels that did not differ significantly from sham offspring (WRST: W=144, p=0.30).
**Figure 14**: PD77 adult plasma testosterone levels (n=8-10/group). Legend indicates prenatal treatment. Error bars ± SEM.

### 3.12 Adult female estradiol levels

Extracted standards produced a standard curve (Figure 15), which was used to estimate pg/mL values for estradiol in adult intact and GDX female serum. Estradiol levels were determined to be $3.93 \pm 1.30$ pg/mL in intact serum, and <0 pg/mL in GDX serum. Thus, this method appears reliable, and we will obtain estradiol levels for adult female offspring via SPE extraction followed by ELISA.
Figure 15: Extracted estradiol standard curve. Curve was used to determine pg/mL values of estradiol in intact and GDX female adult mouse serum.

3.13 Prepulse inhibition of the startle response

Meaningful results from PPI could not be obtained, as mice tested at both PD25 and PD69 failed to demonstrate appropriate startle responses. Potential pitfalls of using this technique with CD1 mice will be highlighted in the discussion.

3.14 Plasma CORT levels

Serially diluted plasma samples yielded a displacement curve which was parallel to the CORT standard curve (Figure 16A; GLM: no significant interaction, $t=-0.87$, $p=0.41$). The volume of plasma determined to be the appropriate sample volume was 0.3125 µl, as this volume produced 48% binding.

CORT responsivity was significantly affected by sex ($t=-5.15$, $p<0.001$), time at 10 minutes after stress ($t=13.79$, $p<0.001$), and time at 60 minutes after stress (Figure 16B; $t=3.52$, $p<0.001$; GLM: $F_{6,128}=53.11$, $p<0.001$). GLM analysis of males alone
revealed a significant effect of prenatal treatment in testosterone treated males (t=-2.54, p=0.02), but not DEX treated males (Figure 16C; t=-0.85, p=0.40). Significance of this effect remained when accounting for multiple comparisons (Tukey: t=-2.45, p=0.04). A significant effect of time at 10 minutes after stress (t=11.19, p<0.001) and 60 minutes after stress (t=6.15, p<0.001) also remained (GLM: F_{5,58}=36.06, p<0.001). The effect of prenatal TP treatment on CORT levels in male mice was only significant when examining all time points together, as prenatal TP did not significantly affect CORT values when examining each time point alone (Welch's t-test: control: t=0.33, p=0.75, 10 minutes: t=1.93, p=0.10, 60 minutes: t=2.20, p=0.06, 180 minutes: t=1.71, p=0.13).

GLM analysis of females alone revealed a significant effect of time at 10 minutes after stress (t=10.05, p<0.001, and 180 minutes after stress (t=-2.71, p<0.001), but no effect of prenatal treatment in either testosterone treated (t=0.54, p=0.59) or DEX treated (t=-0.33, p=0.74) females (Figure 16D; GLM: F_{5,65}=39.7, p<0.001).
**Figure 16: Plasma CORT levels following restraint stress.**

A: Regression plot comparing slopes of the CORT standard curve and pooled male and female plasma sample serial dilution. B: Plasma CORT in male and female mice. Females had elevated CORT compared to males. Females had altered CORT from baseline at 10 minutes and 180 minutes after stress, while males had altered CORT from baseline at 10 minutes and 60 minutes after stress. C: Plasma CORT in male mice following restraint stress. Testosterone treated males had reduced CORT levels compared to control males. D: Plasma CORT in female mice following restraint stress. + indicates a significant effect of sex, * indicates a significant effect of prenatal treatment, # indicates a significant effect of time (n=4-7 per group). Error bars ± SEM. *p<0.05, +++p<0.001. ###p<0.001, ##p<0.001.
3.15 Experiment 2: Adult testosterone levels

Serially diluted plasma samples yielded a displacement curve which was parallel to the CORT standard curve (Figure 11A). The volume of plasma determined to be the appropriate sample volume was 0.3125 µl, as this volume produced 48% binding. There was no significant effect of prenatal treatment on adult testosterone levels (Figure 17; ANOVA: F=0.31, p= 0.74).

Figure 17: Plasma testosterone levels in adult males (n=5-7/group). Error bars ± SEM.

3.16 Experiment 3: PD0 testosterone levels

While the data in Figure 9 seemed to support the hypothesis that the prenatal treatments altered post-natal testosterone secretion, the data were not unequivocal because levels of testosterone change rapidly in mice during the first 12h after birth (Motelica-Heino et al., 1988). Since the birth times were not recorded for the different treatment groups, it remained possible that if the time intervals between birth and
sacrifice where not the same for each treatment group, differences in mean testosterone levels could have been observed that were not in fact related to the prenatal treatment. To test this hypothesis, additional studies were performed, in which newborn male mice were sacrificed for blood collection at precisely timed intervals between 1 and 6h after birth.

As expected, testosterone levels changed significantly over time in the first 6 hours after birth (ANOVA: F=15.8, p<0.001). However, no significant differences were observed between prenatal testosterone treated, prenatal DEX treated, and SO control male mice (Figure 18: ANOVA: F=0.63, p=0.53). Furthermore, testosterone levels did not differ between treatment groups at 1 hour (WRST: H=0.58, df=2, p=0.75), 1.5 hours (WRST: H=2.57, df=2, P=0.28), 2 hours (WRST: H=1.39, df=2, p=0.50), 2.5 hours (WRST: H= 2.03, df=2, p=0.36), 4 hours (WRST: H=0.61, df=2, p=0.74), or 6 hours after birth (WRST: H=1.13, df=2, p=0.57).
Figure 18: Time-course of blood testosterone levels 1-6 hours after birth. (n=8-10 per group). Error bars ± SEM.

4 Discussion

4.1 Maternal testosterone levels

Analysis of maternal serum testosterone levels 24 hours after the last prenatal injection by RIA confirmed previous reports of testosterone levels measured in these samples by ELISA (Wasson, 2017). TP injected dams had serum testosterone levels which were 2.81 times that of SO injected dams when testosterone was measured by ELISA, and 2.86 times that of SO injected dams when testosterone was measured by
RIA. These comparisons increase confidence in the reliability of these assays for measuring testosterone levels in 3-4-month-old CD1 female mice.

In Wasson (2017), testosterone levels in maternal serum were also measured from samples taken on the day of birth, and no differences were detected in testosterone levels between dams injected with SO or TP. Thus, the prenatal TP injections produced a transient increase in maternal serum testosterone levels around the times of injection, and levels returned to baseline by the time of birth. Therefore, the aim to produce an early elevation in testosterone exposure in our TP injected litters was achieved.

4.2 Reproductive tissue weights, VO, and estrous cyclicity

The weights of the reproductive organs, timing of VO, and estrous cyclicity were measured to examine whether an early elevation in testosterone might disrupt sexual organ differentiation mechanisms and postnatal hormone profiles. We were unable to detect an effect of early prenatal testosterone on ovarian, uterine or testicular weight, nor were we able to detect a significant difference in VO or the day of first estrous between prenatal testosterone injected and prenatal SO injected mice. We were also unable to detect an effect of early prenatal testosterone on the average number of days per estrous cycle, or on the percentage of time mice were spending in each cycle stage. Together, these results suggest that treatment with low doses of prenatal testosterone did not disrupt sexual differentiation outcomes in CD1 mice.
Testes size is primarily determined by the number of Sertoli cells within the testes. In mice and rats, the final number of Sertoli cells in adulthood is partially influenced by proliferation during fetal and neonatal life (Sharpe, McKinnell, Kivlin, & Fisher, 2003). Indeed, prenatal androgen exposure has been demonstrated to influence Sertoli cell number in rams (Rojas-García et al., 2010). Additionally, mice who do not express functional ARs had reduced Sertoli cell number at birth, and this effect remained through adulthood (Johnston et al., 2004). However, AR expression in rats has not been detected in Sertoli cells until PD5, and thus the influence of androgens on Sertoli cells has been suggested to happen indirectly. Effects of androgens on Sertoli cells could occur through peritubular cells. Peritubular cells surround the seminiferous tubules in the testes, express AR during development, and secrete various substances including growth factors which influence testicular function (Maekawa, Kamimura, & Nagano, 2008). Influence of androgens on Sertoli cells could also occur by aromatization of estradiol, as estradiol is necessary for determining the end of Sertoli cell proliferation (Lucas et al., 2014; You & Sar, 1998).

Testicular weight was not affected by an early elevation in prenatal testosterone. Research examining the effect of prenatal testosterone injections on testicular weight in male offspring have shown inconsistent results. In rats exposed to a 3mg/day of testosterone from E16-19, testes size was shown to be reduced compared to controls in post-pubertal offspring (Tehrani et al., 2013), while no difference in testicular weight was seen in rats exposed to 1mg/kg/day of testosterone from E13-20 (Kita et al., 2016). Inconsistency in these studies could be a result of different dosages used in these
experiments. A dose of 1mg/kg/day translates to ~200-300\(\mu\)g/day, which is much lower than a dose of 3mg/day. Inconsistency could also be due to timing of androgen exposure. Early androgen exposure during the first trimester (E30-E58) in sheep reduced testes size in post-pubertal offspring (Scully et al., 2017). However, the same dose of testosterone given from E30-E90 has been demonstrated to increase testicular weight in ewes, suggesting the timing of androgen exposure is important for testis development (Bormann, Smith, Padmanabhan, & Lee, 2011). Therefore, the finding that testicular weight did not differ between prenatal testosterone treated and SO control offspring in experiment 1 was not completely unexpected.

No significant effect of early prenatal testosterone exposure on ovarian or uterine weight in female offspring was detected. This is in agreement with previous studies. Ovarian weight has generally not been observed to be influenced by exogenous prenatal testosterone exposure or endogenous fluctuations of hormones during the estrous cycle in previous experiments done on rats. Prenatal testosterone exposure ranging from 1.5mg/dose to 3mg/dose did not significantly influence ovary weight in post-pubertal offspring (Hotchkiss et al., 2007; Tehrani, Noroozzadeh, Zahediasl, Piryaei, Hashemi, et al., 2014). One experiment did find a significant reduction in ovary weight following prenatal testosterone treatment, however this was only at 10mg doses of TP (Wolf et al., 2002). Additionally, ovarian weights are not seen to fluctuate over the estrus cycle (Green, 1957). Uterine weight was also not significantly affected by 0.1mg-10 mg/dose prenatal testosterone treatment in previous experiments (Hotchkiss et al., 2007; Wolf et al., 2002). Thus, the lack of influence of prenatal testosterone on ovarian
and uterine weight in experiment 1 was consistent with previous experiments. Uterine weight is, however influenced by circulating estradiol levels (Y. Wang et al., 2008). As we have not yet determined plasma estradiol levels in prenatal testosterone and SO treated mice, the lack of change in uterine weight between these groups of mice suggests that prenatal testosterone treatment likely did not have a substantial influence on postnatal hormone levels in female mice.

Uterine weights were affected by surgical and hormone replacement conditions, where intact females and OVX-HR females replaced had larger uterine weights than OVX-VR females. Since uterine weight is influenced by circulating estradiol mice (Wang et al., 2008), these results also provide some evidence that gonadectomy surgeries were successful at reducing circulating estradiol, and that replacement with estradiol benzoate capsules restored circulating estradiol to a similar level to that of intact females.

The onset of VO occurs when the ovaries secrete sufficient amounts of E2 to stimulate VO (Rivest, 1991). Indeed, VO has been demonstrated to be initiated in mice by E2 injections (Rodriguez, Araki, Khatib, Martinou, & Vassalli, 1997). Thus, if our early prenatal testosterone injections influenced E2 secretion in prepubertal mice, VO could be affected. Previous research has demonstrated VO to be sensitive to high doses of prenatal testosterone treatment. Pregnant rats injected with a single 5mg dose of testosterone on either E16, 17, 18, 19 or 20 had female offspring with significantly delayed VO (Rhees et al., 1997). However, at low doses of prenatal testosterone treatment (0.1mg/dose and 0.5mg/dose), VO has been shown to remain unaffected.
(Wolf et al., 2002). We did not observe a significant effect of early testosterone treatment on VO, suggesting our testosterone treatment did not significantly affect E2 levels in prepubertal female mice. However, in Wasson (2017) the effect of prenatal testosterone treatment on VO was significant, thus, measurement of E2 levels are necessary to conclude whether TP injections influenced E2 levels in prepubertal female mice. The average day of VO in prenatal testosterone treated mice was 24.65 (outliers removed) or 23.84 (outliers included), and the average day of VO in SO treated mice was 24.52. When outliers were removed, we observed the same pattern as in Wasson (2017), where prenatal TP treated mice had VO ~0.5 days earlier than SO treated mice.

Observed VO in testosterone treated and SO control mice appear slightly earlier than previously recorded VO in CD1 mice, which ranges from 25.4 ± 2.69 to 31.3 ± 0.6 (Jefferson, Padilla-Banks, & Newbold, 2005; Thigpen et al., 2007). Additionally, Wasson (2017) observed VO to occur on PD29.56 in TP treated mice, and PD30.30 in SO treated mice. This discrepancy is likely due to multiple physiological and environmental factors which can also influence VO (Rivest, 1991). For example, factors such as light exposure, diet, and HPA axis activity have all been demonstrated to influence VO (Macfarland & Mann, 1977; Rivest, 1991; Thigpen et al., 2007). Thus, differences in handlers, season, or disturbances within the animal facility could underlie the observed differences in VO between our results and those of Wasson (2017).

Estrous cyclicity progresses following VO. For estrous cyclicity to occur, ovarian follicles must be developed and ready to release ova, and the pituitary must respond to a rise in E2 with secretion of luteinizing hormone and follicle stimulating hormone,
resulting in ovulation (Rivest, 1991). Therefore, if an early elevation in prenatal testosterone affected estradiol production in mice, or estrogen receptor expression in the pituitary, effects on estrous cyclicity could be expected. Estrous cyclicity has been previously demonstrated to be sensitive to androgen exposure during development. Female rats born to mothers exposed to 1.5 or 2.5mg/dose injections of TP during E14-18 had prolonged estrous cycles and a reduction in the percentage of rats cycling normally (Hotchkiss et al., 2007). Additionally, female rats born to mothers exposed to a single dose of 5mg of testosterone on E20 had prolonged and irregular estrous cycles (Tehrani, Noroozzadeh, Zahediasl, Piriaei, & Azizi, 2014). Estrous cyclicity was not affected in our testosterone treated female mice. There were no significant differences in the day of first estrous, the average length of the estrous cycle, or the average amount of time mice were spending in each cycle stage between testosterone treated and SO control female mice, further suggesting prenatal testosterone treatment did not significantly affect estradiol secretion or sexual differentiation in female mice.

Overall, these findings suggest that sexual differentiation outcomes were not altered by prenatal testosterone exposure in either male or female mice. As these findings may appear in opposition of previous studies which demonstrated that prenatal androgen exposure did affect sexual differentiation mechanisms, it must be noted that a large discrepancy exists between the doses of prenatal testosterone exposure in our experiments compared to those in the experiments described above. In our experiments, 10μg/dose of testosterone raised serum testosterone in dams only slightly, and testosterone levels remained within physiological range, while previous
experiments demonstrating a significant effect of prenatal testosterone on sexual differentiation were conducted using doses ranging from 1.5mg/dose to 10mg/dose. Thus, while previous work has demonstrated that elevated prenatal testosterone effected reproductive tissue weights, estrous cyclicity and hormone profiles in offspring, it is likely that the dose of testosterone we used was not sufficient to significantly disrupt sexual differentiation mechanisms in offspring.

4.3 Experiment 1: PD0 testosterone levels

We observed that exposure to testosterone or DEX on E12, 14 and 16 appeared to lower testosterone levels in males between 6-18 hours after birth compared to vehicle exposed mice. Testosterone levels from 6-18 hours after birth in prenatal SO treated males (0.55ng/mL) are consistent with testosterone levels shown in normal male mice, measured from 3-24 hours after birth (~0.58ng/mL; (Motelica-Heino et al., 1988)). Testosterone levels in prenatal testosterone treated males (mean: 0.39ng/mL) but not prenatal DEX treated males (mean: 0.25ng/mL) were significantly reduced compared to SO treated control males. The significance between testosterone treated males and control males was potentially revealed due to the large sample size in this group (n=29), as mean testosterone in DEX treated males (n=9), was lower than that of testosterone treated males.

Previous analyses of day of birth testosterone levels in mice have demonstrated that by approximately 6 hours after birth, testosterone levels in males were not significantly different than those in females (Corbier et al., 1992; Motelica-Heino et al., 1988). We demonstrated testosterone levels to be higher in males than females.
between 6 and 18 hours after birth, with many female samples falling below the limit of
detection of the assay. There was a significant interaction between the slope of the
testosterone standard curve and the female blood serial dilution, therefore conclusions
based on these results cannot be reliable.

These results provided preliminary evidence that hormone profiles might have
been disrupted in male mice after an early elevation in prenatal testosterone. However,
since the peak in testosterone levels in male mice happens two hours after birth, and is
complete by 6 hours, characterization of the testosterone surge was critical to more fully
understand how an early elevation in prenatal testosterone might have affected day of
birth testosterone levels (Motelica-Heino et al., 1988). Furthermore, since samples were
collected between 6 and 18 hours after birth and we did not know the precise time
interval in each litter, variation in sample collection time could have posed a significant
confounding influence on the results. Therefore, characterization of the day of birth
testosterone surge in male mice following early prenatal testosterone and prenatal DEX
exposure was carried out in experiment 3, discussed below.

4.4 Hair CORT levels

We measured CORT retained in male and female mouse’s hair to assess how an
early elevation in testosterone might affect stress levels in offspring over the first 6
weeks of life (Müller-Röver et al., 2001). The exact mechanism by which CORT gets
incorporated into hair is currently unknown, but it may be similar to that of other
lipophilic compounds, which are incorporated into the hair follicle during the active
growth phase via passive diffusion from blood vessels (Meyer & Novak, 2012). Support
for this mechanism comes from examining hair CORT levels in individuals that possess conditions which elevated plasma CORT, such as individuals with Cushing’s syndrome (Thomson et al., 2010).

We found that female CD1 mice had higher levels of CORT retained in their hair compared to males. This finding is consistent with previous literature showing that female mice and rats show elevated baseline plasma CORT compared to males (Weinstock et al., 1998). However, we must also consider possible differences in growth rate and handling between sexes, which could influence hair CORT levels. Since hair samples were obtained without previous shaving, they contained a combination of hairs which would have incorporated CORT throughout the time which hair was growing. Hair growth occurs in three stages: growth (anagen), regression (catagen) and rest (telogen) (Müller-Röver et al., 2001). In female C57BL/6 mice, initial hair growth occurs in the first 2 weeks of life, followed by catagen at 2-3 weeks, and telogen from 3-4 weeks (Müller-Röver et al., 2001). Anagen then occurs again from 4-6 weeks (Müller-Röver et al., 2001). Therefore, in hair samples collected from our mice on PD43, CORT might have been incorporated into hair during postnatal weeks 0-2 and 4-6. Prior to PD20, male and female offspring received the same handling, however, beginning daily on PD20, females were handled once per day during VO checks and lavages, while males were left unhandled. As this time period may overlap an anagen stage, it is possible that female CORT could be elevated compared to males partly due to a difference in handling. Additionally, if males and females have different times which they are in anagen, this could affect CORT levels within hair. However, hair growth varies by
mouse strain, sex, environmental conditions and nutrition, therefore, the exact times which CORT would be incorporated into hair are unknown in our CD1 mice (Müller-Röver et al., 2001). It is likely that these findings do reflect true difference in long-term baseline CORT levels between male and female mice, as baseline differences in plasma CORT have been previously reported and were also seen in restraint stress CORT analysis in experiment 2 (Figure 16; Weinstock et al., 1998).

No differences were seen in hair CORT levels between our testosterone treated and control mice. These results suggest that there were no differences in baseline CORT levels between these groups during the first few weeks of life. There were no environmental differences between the prenatal testosterone treated and prenatal SO offspring from birth to PD43 when hair was collected, thus, this result was likely unaffected by factors in the offspring’s environment. It is unknown whether an early elevation in prenatal testosterone could have affected hair growth in offspring, however, steroid hormones, including androgens, estrogens and CORT, can all affect hair growth (Paus & Cotsarelis, 1999). Androgens tend to influence hair follicle growth by shortening the anagen stage, while estrogens have the opposite effect (Paus & Cotsarelis, 1999). Since hair growth occurs postnatally in mice, exogenous prenatal testosterone treatment likely did not have direct effects on hair growth. Additionally, as testosterone levels were not altered postnatally in TP treated mice, it is unlikely that prenatal testosterone treatment effected hair growth in mice.

This experiment is the first, to our knowledge, to extract CORT from mouse hair and measure extracted CORT using RIA. Previous measures of mouse hair CORT have
been done using ELISA and high-performance liquid chromatography mass spectrometry coupled with atmosphere pressure chemical ionization (LC-APIC-MS/MS). We have demonstrated that the optimum sample of hair to be used for RIA is much smaller (1mg) than that required for these methods. In previous analyses, optimum sample weights were 22mg and 31mg of mouse hair for ELISA (Erickson et al., 2017; Jarcho, Massner, Eggert, & Wichelt, 2016), and 20mg of hair for LC-APIC-MS/MS (Hohlbaum, Bert, Dietze, Palme, & Fink, 2017; Yu et al., 2015). Therefore, we have demonstrated that using RIA following mouse hair CORT extraction could be used in situations where it is difficult to obtain large hair samples.

The ability to measure CORT retained in hair provides a minimally invasive measure of HPA axis functioning over weeks to months. While CORT is most often measured in plasma or fecal samples, these measures provide data on GC levels from a few minutes to a few days (Heimbürge, Kanitz, & Otten, 2019). Future experiments utilizing the approach of measuring hair CORT should consider factors which influence hair growth (sex, hormone levels, differences in handling etc.) to improve data validity and reliability. To reduce potential confounds of hair growth timing differences between males and females and across species, histology can be done to determine periods of time when anagen occurs in both males and females (Azzi, El-Alfy, Martel, & Labrie, 2005). Hair can then be shaved prior to anagen, and then re-shaved at the end of anagen to determine CORT within this time period. Furthermore, analysis of hair CORT can be best used as part of an integrative study which might examine HPA axis activity by multiple means to heighten reliability in results.
4.5 Adult testosterone levels: Optimization and results

After unsuccessful attempts to measure testosterone levels with unextracted plasma, and diethyl ether extracted plasma, we determined that adult male mouse testosterone levels could be accurately measured using C18 SPE with RIA. Parallelism was observed between SPE serially diluted plasma samples and the SPE standard curve (Figure 11A). Additionally, mean testosterone levels in the male adult plasma pool were observed to be ~4ng/mL in 20μL to 1.25μL SPE sample volumes (Figure 11B). While there is a wide range of reported testosterone levels in intact adult mice, testosterone levels most consistently reported in CD1 mice have been demonstrated to be ~5-6ng/mL (Brouillette, Rivard, Lizotte, & Fiset, 2005; Machida et al., 1981). Taken together, these results suggested accurate measurements of testosterone in adult male mouse plasma samples could be achieved with RIA using sample volumes of 20μL to 1.25μL following SPE extraction.

We did not see any effect of prenatal treatment on testosterone levels in plasma samples collected on PD43, PD58 and PD77 in experiment 1, or in plasma samples collected in mice who did not receive restraint stress on PD77 in experiment 2. These results, in combination with our measurements of testicular weight and of testosterone levels on the day of birth, suggest that early prenatal testosterone treatment did not significantly affect offspring hormone profiles in male offspring. These results do not support our initial hypothesis that prenatal TP injections would affect sexual differentiation outcomes in male offspring. However, we intentionally used a very low dose of prenatal testosterone, which did not elevate maternal testosterone beyond the
normal physiological range, thus, the observation that prenatal testosterone treatment did not affect offspring sexual differentiation outcomes was not unexpected.

Testosterone measurements in GDX, GDX-VR, and GDX-HR male offspring were consistent with expected testosterone levels. Gonadectomy (PD58) was successful at reducing testosterone to very low levels (average testosterone: 0.1ng/mL ± 0.03ng/mL). Testosterone levels in mice that received vehicle replacement capsules also remained low (PD77 average testosterone: 0.25ng/mL ± 0.11ng/mL, two outliers removed). Hormone replacement capsules restored testosterone levels on average to 4.95ng/mL ± 0.90ng/mL (PD77), suggesting that our testosterone capsules were successful at restoring testosterone to average, physiological levels (Machida et al., 1981).

Testosterone measurements in intact males were overall consistent with expected testosterone levels. Average testosterone levels in experiment 1 were 1.67ng/mL ± 0.38ng/mL on PD43, 6.80 ± 2.19ng/mL on PD58, and 11.85 ± 2.58ng/mL on PD77, and average testosterone levels in experiment 2 were 5.70ng/mL ± 1.73ng/mL. Bell (2018) reported pubertal testosterone levels in male mice to peak on day 40 and then decline and level off by day 60, however the exact timing of puberty in mice can be affected by environmental and physiological factors (Kumar & Boehm, 2013). We speculate that puberty occurred slightly later in male mice in our study than those in Bell (2018), such that testosterone levels in PD43 mice were currently in the process of rising. Testosterone levels in male mice at PD58 and PD77 (experiment 2) were exactly what could be expected in adult male mice (Brouillette et al., 2005;
Machida et al., 1981). Testosterone levels in intact males at PD77 (experiment 1) were elevated somewhat compared to typical testosterone levels in adult male mice. We speculate this elevation was due to social isolation which occurred in male mice in experiment 1 from PD43-PD77. Prior to gonadectomy surgery in experiment 1 and for all of experiment 2, male mice were housed together, as male mice are generally tolerant toward familiar males. After gonadectomy or sham surgery, male mice were individually housed to reduce the risk of infection or complications. After recovery, males remained individually housed to ensure animal safety and well-being, as CD1 male mice are highly territorial and aggressive toward unfamiliar males (Van Loo, Van Zutphen, & Baumans, 2003). In group settings, male mice form hierarchies, with one male being dominant, and others becoming subordinate (Van Loo et al., 2003). Dominant males are observed to have elevated testosterone levels (mean=10.5 ± 2.5 ng/mL; Machida et al., 1981) compared to subordinate males (mean=2.2 ± 0.8 ng/mL; Machida et al., 1981), and this phenomena may account for some of the natural variability observed in male mouse testosterone levels (Machida et al., 1981). Socially isolated male rats have been observed to be more dominant than group-housed rats (Ward & Gerall, 1968). Furthermore, social isolation has been shown to increase testosterone levels in male mice (Sayegh, Kobar, Lajtha, & Vadasz, 1990). Thus, we speculate that the increased levels of testosterone in PD77 (experiment 1) intact male mice were observed as a result of social isolation in these animals.

Overall, our prediction that prenatal testosterone treatment would affect adult testosterone levels in male mice was not supported. Testosterone measurements did,
however, confirm gonadectomy and hormone replacement surgeries were successful at altering male testosterone profiles. These results demonstrate C18 SPE to be an effective means to clear interfering substances from adult male mice plasma samples prior to testosterone analysis using RIA.

4.6 Adult female estradiol levels

We have demonstrated SPE to improve validity in measuring estradiol in adult female mouse plasma samples. Estradiol in pooled adult female plasma following SPE was 3.93 ± 1.30 pg/mL in intact serum, and <0 pg/mL in GDX serum. Mean estradiol levels have been shown to range from <0.3pg/mL in estrus mice to ~8pg/mL in proestrus mice, thus the level of estradiol measured in our pooled intact serum sample was consistent with expected results (Nilsson et al., 2015).

SPE extraction prior to estradiol analysis by ELISA will be used in the future to obtain estradiol levels in mice from experiment 1, to assess potential effects of prenatal testosterone on adult hormone levels, and to confirm ovariectomy and hormone replacement were successful at altering female estradiol levels.

4.7 Prepulse inhibition of the startle response

PPI was attempted in experiment 2 to investigate the early prenatal testosterone mouse model as a potential model for behaviour typically associated with schizophrenia. We were unable to get meaningful data from this experiment, as most mice failed to exhibit a startle response. One possible explanation for a lack of startle responsivity is that CD1 mice can develop hereditary deafness (Shone, Raphael,
Miller, 1991). Thus, the mice would not exhibit startle responses to a loud tone if they could not hear it, or if it was not loud enough to be startling. Mice might also fail to startle if they were overly anxious from being in the startle chambers, as mice may experience anxiety when placed in a novel environment which they cannot escape (Misslin & Cigrang, 1986). Future directions for attempting to obtain startle responses in prenatal testosterone treated mice could include using a strain of mice which does not exhibit deafness, such as CBA/J mice (Shone et al., 1991), and considering habituating mice to the startle chamber prior to testing (Valsamis & Schmid, 2011).

4.8 HPA axis responsivity to restraint stress

A 30-minute restraint stress was used to provide an acute stressor to mice, and CORT was measured in plasma collected at 10 minutes, 1 hour and 3 hours after restraint to assess stress responsivity and recovery in offspring exposed to early prenatal testosterone or prenatal DEX. Acute restraint stress has been previously demonstrated to be a reliable method for measuring stress responsivity in mice (Zimprich et al., 2014). We observed SO control mice to demonstrate a typical HPA axis response and recovery to restraint stress, with CORT levels raising to comparable levels in previous experiments (Stewart et al., 2008). In addition, we observed that male mice who received early prenatal testosterone treatment had reduced stress responsivity overall compared to SO control and prenatal DEX treated mice. No significant changes in CORT were observed in DEX treated males or females, or testosterone treated females.
We have demonstrated that prenatal testosterone exposure on E12, 14 and 16 altered CORT responsivity in male offspring. The reduction in stress responsivity in early testosterone treated male offspring appears, at first sight, to contrast with previous data from our laboratories. Howes (2016), demonstrated that prenatal testosterone exposure selectively increased anxiety in male offspring. Anxiety behaviour is often associated with increased HPA axis activity, because CRH is anxiogenic (Chen, Zhou, Bai, Zhou, & Chen, 2015). Therefore, increased anxiety behaviour might be predicted to be associated with increased HPA stress responsiveness. Instead, we found the opposite.

We speculate that one potential explanation of these results is that the early prenatal testosterone injections affected anxiety behaviour and HPA axis responsivity through different mechanisms. Although some research has demonstrated a relationship between elevated anxiety and HPA axis hyperactivity, the findings that early prenatal testosterone decreased CORT responsivity to stress while increasing anxiety are not necessarily in opposition (Butler, Ariwodola, & Weiner, 2014). For example, although HPA axis functioning and anxiety appear strongly linked by CRH, different populations of CRH neurons are involved in these events. The CRH system regulating anxiety is located primarily within cells of the amygdala and the bed nucleus of the stria terminalis, while the CRH system regulating GC release is located primarily within the PVN. GCs can have different effects on these separate populations of CRH neurons (Schulkin, Gold, & McEwen, 1998). For example, while GC elevation has been demonstrated to downregulate CRH in the PVN, it was also shown to increase CRH in
the central nucleus of the amygdala (Schulkin et al., 1998). Reduced CRH in the PVN could then result in lower GC levels, while elevated CRH in the amygdala could result in upregulations of anxiety behaviour (Schulkin et al., 1998). Furthermore, if early prenatal testosterone selectively affected CRH expression in one of these regions, differential effects on HPA axis activity and anxiety could occur. CRH expression is detected in the developing mouse PVN by E13.5, presenting the possibility that early testosterone exposure could interact with the CRH system (Keegan et al., 1994).

We speculate that another potential explanation for effects of early prenatal testosterone on anxiety behaviour and stress responsivity could be dysregulated stress negative feedback. Reduced GC responses to stress could occur as a result of overactive feedback. For example, mice overexpressing GR demonstrate increased negative feedback, and reduced HPA axis activity and plasma CORT (Gass, Reichardt, Strekalova, Henn, & Tronche, 2001). Although anxiety behaviour has not been tested in GR overexpressing mice, mice with decreased GR expression show decreased anxiety behaviour (Montkowski et al., 1995). Therefore, we hypothesize that the opposite effect could occur in mice with elevated GR expression: upregulation of GR in the hippocampus, PVN or pituitary might lead to both decreased HPA activity and increased anxiety.

Beyond the HPA axis, numerous physiological systems are known to be involved in regulating anxiety, and the link between early prenatal testosterone and anxiety behaviour may be independent of HPA axis activity (Steimer, 2002). For example, anxiety behaviour can be influenced by gamma-aminobutyric acid (GABA) action in the
brain. KO mice heterozygous for the GABA receptor have been investigated as a model for anxiety behaviour (Crestani et al., 1999). Furthermore, testosterone, and its metabolites, DHT and 3α-diol have been shown to modulate GABA action in the brain and influence anxiety responses (Frye, Park, Tanaka, Rosellini, & Svare, 2001; Rupprecht et al., 2001). Therefore, investigation of early testosterone’s influence on other mechanisms involved in regulating anxiety should be done to further understand the link between these events.

Other findings from our experiments which could initially appear to be in opposition include the observations that early, elevated prenatal testosterone affected CORT responsivity to an acute stressor in male offspring, but not baseline CORT levels. We speculate that mechanisms behind these finding potentially involve changes in the GC receptors. Since MR has a higher affinity for CORT, it primarily binds CORT under baseline conditions. Indeed, Reul and de Kloet (1985) observed that MRs were bound by more than 80% endogenous ligands (CORT) under baseline conditions, compared to only ~10% binding in GRs. However, when GC concentrations were raised during times such as the circadian peak and during stress, GRs became increasingly occupied (Reul, & de Kloet, 1985). This has led us to hypothesize that prenatal testosterone may alter the expression of GR, but not MR in offspring. Indeed, MR expression is not observed in the mouse hippocampus until E15.5-E16.5, thus it is unlikely that an early elevation in testosterone would affect MR (Kretz et al., 2001; Noorlander, Graan, & Middeldorp, 2006). While it is unknown when GR is first expressed in the developing mouse brain, analysis of GR expression from whole mouse embryos determined that GR could be
detected as early as E9.5 (Cole et al., 1995). While, MR mRNA expression in the developing rat brain is not observed until E15.5, GR mRNA is expressed in a number of brain regions by E12.5-13.5 (Diaz et al., 1998). These findings suggest that GR expression may be present in the developing mouse brain during the time of our early prenatal testosterone treatment, and thus present the possibility the early prenatal testosterone might have affected GR expression in offspring. This hypothesis could explain the differential effects of early prenatal testosterone treatment on baseline CORT compared to CORT responsivity. Therefore, we speculate that early prenatal testosterone exposure selectively altered GR, but not MR development in the male fetal mouse brain.

Several sex differences emerged from measuring CORT in male and female mice. Overall, females had had higher levels of CORT compared to males. This result is consistent with previous research assessing restraint stress responsivity following restraint stress in rats and mice, as well as with the sex difference observed measuring CORT retained in mouse’s hair in experiment 1 (Aloisi et al., 1998; Goel & Bale, 2008). In addition, CORT levels in female mice returned to baseline levels by 60 minutes after stress, while CORT levels in male mice did not return to baseline until 180 minutes after stress, suggesting females were quicker to recover from stress. This is in agreement with previous research showing females to have quicker recovery to stress compared to males (Kitay, 1961; Lin et al., 2008).

Another observed sex difference was that the reduction in CORT observed in prenatal testosterone treated male offspring was not observed in female offspring. This
finding supports our prediction that males would be more affected by early prenatal testosterone exposure compared to females. It is also consistent with the findings from our lab’s previous studies, which observed that early prenatal testosterone treatment primarily affected behaviour in males (Howes, 2016; Wasson, 2017). As the mechanisms underlying the change in CORT in testosterone treated male offspring are not know, it is currently unclear why our treatment selectively affected males. This finding does, however, suggest that underlying molecular changes potentially induced by early testosterone treatment would likely have affected mechanisms which are typically sexually differentiated. Indeed, this prediction is consistent with our speculation that GR development was altered by early prenatal testosterone treatment in males. GR mRNA expression in the fetal hippocampus has been shown to have differential expression patterns in males and females. For example, in guinea pigs, female fetuses experience increased GR mRNA steady state levels in the hippocampus during late gestation compared to males (Owen & Matthews, 2003). Thus, we predict that differences in developmental GR expression patterns could have resulted in a scenario whereby an early elevation in prenatal testosterone could have selectively interfered with male GR development.

Prenatal DEX treatment has previously been demonstrated to affect stress responsivity in offspring, though the direction of change appears dependent on dose or species. Male rats exposed to a low dose of prenatal DEX (0.05mg/kg) on E17-19 exhibited increased CORT responsivity (Muneoka et al., 1997). Conversely, a high dose of prenatal DEX (1mg/kg) in guinea pigs on E40–41, E50–51, and E60–61
resulted in reduced CORT responsivity in male offspring (Lin et al., 2008). Therefore, the finding that prenatal DEX did not alter HPA axis responsivity in offspring in our experiment was somewhat unexpected, but it is likely that the dose of DEX used in experiment 2 was low enough to not significantly disrupt HPA axis development during the prenatal period. The dose of DEX was chosen to be similar to the dose of synthetic GCs which are often given to pregnant women at risk for preterm birth, as the long-term effects of prenatal GC exposure in humans are relatively unknown (American College of Obstetricians and Gynecologists, 2016). Thus, these results suggest that the risk associated with low doses of prenatal GC treatment during mid-late pregnancy are low in terms of long-term consequences on HPA axis responsivity in offspring. It is unknown, however, whether these results can be generalized to humans.

The finding that prenatal testosterone exposure and prenatal DEX did not influence offspring stress responsivity in the same way appears to be inconsistent with our initial hypothesis that prenatal GCs and prenatal testosterone might interact and have similar effects on the developing brain. As the dose of DEX used in our experiments was very low, we speculate that a higher dose of prenatal DEX might have had similar effects on stress responsivity as prenatal testosterone. It is also possible that prenatal stress, as opposed to prenatal DEX exposure, might have similar effects on development as low doses of prenatal testosterone. We designed experiment 2 using prenatal DEX instead of prenatal stress as we wanted to compare prenatal testosterone and prenatal GC exposure. Although prenatal stress raises GC levels, it can also activate multiple physiological systems, and thus effects of prenatal stress are
not necessarily attributed to heightened GCs alone (Harris & Seckl, 2011). Thus, it is likely that prenatal stress and prenatal injections of a synthetic GC have different effects on the developing brain. For example, although prenatal stress and prenatal DEX both raise GC levels, they have opposite effects on CRH. As prenatal stress stimulates the HPA axis, CRH increases in the brain prior to GC elevation. Following synthetic GC treatment, elevated GCs are maintained without an increase in CRH, and may stimulate negative feedback to the HPA axis, producing a downregulation in CRH release (Paragliola, Papi, Pontecorvi, & Corsello, 2017). Additionally, CORT and synthetic GCs have different binding affinities for GR and MR. While CORT has higher binding affinity for MR, DEX has higher binding affinity for GR (Spencer, Young, Choo, & McEwen, 1990). Thus, to further explore the hypothesis that prenatal testosterone and prenatal GC exposure may affect offspring development in similar ways, comparison of prenatal testosterone exposure and prenatal stress, should be done before completely discounting this hypothesis.

Overall, we have demonstrated that early prenatal testosterone treatment, but not prenatal DEX treatment, effected stress responsivity in offspring. Although the mechanisms behind this finding remains unknown, we speculate early prenatal testosterone might have impacted GR development in the male fetal mouse brain.

4.9 Experiment 3: PD0 testosterone levels

Testosterone levels were measured in male mice over the first 6 hours after birth to examine whether an early elevation in prenatal testosterone or exposure to prenatal DEX could alter the testosterone surge that occurs 2 hours after birth in male mice.
Contrary to preliminary evidence in experiment one (Figure 9), no effect of prenatal treatment was observed when measuring testosterone levels in male pups from 1 to 6 hours after birth. These results suggest that neither prenatal testosterone nor prenatal DEX exposure affected the testosterone spike following birth, further suggesting that these treatments did not alter sexual differentiation mechanisms in offspring.

While testosterone levels appeared to be lower in prenatal testosterone treated mice compared to control mice when testosterone was measured between 6 and 18 hours after birth, it is possible this effect was artifactual, as a result of differences in collection times. We did not know in this experiment the precise interval between birth and blood collection. Therefore, it was possible that the time interval between birth and collection could have been different in each of the treatment groups. Given the rapid rate of change of plasma testosterone levels on the day of birth, only a few hours difference in collection time could make a large difference in measured mean testosterone levels (Motelica-Heino et al., 1988). The time-course of changes in testosterone levels in the first 6 hours after birth can therefore be viewed as the more reliable measure of hormone profiles in male mice on the day of birth. Thus, we can conclude that testosterone levels on the day of birth were likely unaffected by treatment of early prenatal testosterone or prenatal DEX. However, it is still possible that small differences could exist between treatment groups but were not detected because of the large variation in individual sample measurements. Differences in mean levels of testosterone were evident at some timepoints after birth; but these were not statistically
significant because of the large range of individual testosterone levels. For example, mean testosterone in DEX treated mice at 1 hour after birth were 983.4 ± 313.34 pg/mL compared to 583 ± 137.99 pg/mL in control mice. Unfortunately, because of the rapid rate of change of circulating testosterone levels in newborn mouse pups, considerable animal to animal variability is to be expected. In future experiments, larger sample sizes could improve the power of the results amidst high variability (Biau, Kernéis, & Porcher, 2008).

4.10 Conclusions

We have demonstrated that an early increase in prenatal testosterone reduced stress responsivity in adult male offspring. As we used a low dose of testosterone during prenatal treatment, this finding may have implications for understanding mechanisms behind the etiology of stress implicated neuropsychiatric disorders, such as ASD and schizophrenia. Findings may also be extended to understanding mechanisms behind anxiety behaviour, and thus might have implications for psychological disorders where anxiety is a strong presenting factor, such as mood disorders and anxiety disorder (Mennin, Heimberg, Fresco, & Ritter, 2008). Furthermore, many non-significant results in this study may have positive implications in the context of human disorders, if findings are generalizable to humans.

The prenatal treatment used in this study was originally designed to investigate whether an early elevation in prenatal testosterone might produce physiological and behavioural changes similar to those observed in individuals with ASD. While some similarities have been demonstrated, overall findings have suggested that this mouse
model does not appear to strongly demonstrate an ASD-like phenotype. For example, ASD typically develops within the first few years of life and most behavioural changes which emerged following prenatal testosterone treatment did not occur until after adolescence (Howes, 2016; Shen & Piven, 2017; Wasson, 2017). Additionally, HPA axis activity is typically observed to be upregulated in individuals with ASD, while our results demonstrated early testosterone treatment decreased HPA axis activity in male mice (Figure 16; Spratt et al., 2012). However, while our early prenatal testosterone treated mice are not a “model” for ASD, they do provide a means to understand underlying mechanisms of etiological factors which might contribute to the development of male-biased neuropsychiatric disorders. For example, testosterone elevation and HPA axis downregulation have been observed in both schizophrenia and attention deficit/hyperactivity disorder (Abel et al., 2010; Bradley & Dinan, 2010; Ma, Chen, Chen, Liu, & Wang, 2011; L. J. Wang et al., 2017). Furthermore, these are complex, multifactorial disorders with numerous proposed risk factors, thus, drawing links between various risk factors will be necessary in determining precise etiology (Green, Naylor, & Davies, 2017; Karimi, Kamali, Mousavi, & Karahmadi, 2017; Sawa & Snyder, 2002). Therefore, our discovery that an early elevation in prenatal testosterone reduced HPA axis activity in male offspring may contribute to the overall understanding of how the male sex and stress dysregulation are connected in various neuropsychiatric disorders.

Although the experiments in this study presented mostly non-significant results, positive conclusions may be drawn from potential implications of these findings. We have shown that an early elevation in testosterone did not affect reproductive tissue...
weights, estrous cyclicity, or testosterone profiles in offspring. These findings suggest that exposure to slightly elevated testosterone during mid-late pregnancy did not have long-term effects on offspring sexual differentiation outcomes. The dose of testosterone given to dams in our study produced a slight elevation in maternal testosterone levels, raising testosterone levels to the upper end of physiological range (Figure 3). Elevated testosterone can occur naturally in women, especially in women with conditions that cause hormonal imbalances, such as polycystic ovarian syndrome (Lerchbaum, Schwetz, Rabe, Giuliani, & Obermayer-Pietsch, 2014). Therefore, the finding that exposure to higher physiological levels of testosterone during gestation did not affect sexual differentiation measures in male and female offspring could be viewed as a positive outcome. However, more work is needed to know if results from this study can be generalized to humans.

The non-significant effects of prenatal DEX exposure on offspring testosterone profiles and stress responsivity can also be viewed as a positive outcome. When mothers are at risk for preterm birth, the current recommendation by the American College of Obstetricians and Gynecologists to treat these women with a low dose of synthetic GCs to promote fetal maturation before delivery (American College of Obstetricians and Gynecologists, 2017). The dose of DEX (0.1mg/kg) in our experiments was chosen to be similar to the dose of synthetic GCs which are often given to pregnant women at risk for preterm birth. The current recommended treatment for antenatal corticosteroid therapy is two 12mg injections of betamethasone (0.2mg/kg for a 60kg woman) or four 6mg injections of dexamethasone (0.1mg/kg for a 60kg
woman) (American College of Obstetricians and Gynecologists, 2016). As long-term consequences of clinical prenatal GC exposure in humans are relatively unknown, our study suggested that low doses of synthetic GCs did not have long-term implications on HPA axis responsivity to acute stress or on testosterone profiles in offspring, and, thus, offers support for the continued use of these treatments. However, as previously stated, more work is required to know if these findings can be generalized to humans.

In summary, an early elevation in prenatal testosterone reduced stress responsivity in male offspring, while having no significant effect on female offspring. It is currently unknown, however, whether the reduction in HPA axis responsivity in TP treated males might provide a physiological advantage or disadvantage. Testosterone treatment did not affect reproductive tissue weights in males or females, estrous cyclicity in females, or testosterone levels in males. Additionally, prenatal DEX exposure did not affect stress responsivity in male or female offspring and did not affect testosterone levels in males. In conclusion, the physiologically relevant doses of testosterone and DEX used in this study enable our findings to have potential implications for conditions in which testosterone or DEX is elevated during pregnancy in humans.

4.11 Limitations

The longitudinal nature of this study resulted in different litters of mice being subject to variations in research staff, season, and testing rooms. Handling can be a potential source of variation in animal studies, thus, changes in research staff overtime who have differing handling techniques and skills could potentially effect physiological
responses in mice (Balcombe, Barnard, & Sandusky, 2004). Seasonality has been demonstrated to effect animals even when in controlled lab environments and may be another unexplained source of variation (Rivest, 1991). Additionally, slight variations in environment from different testing rooms, including noise, light, and scent, could affect study results (Castelhano-Carlos & Baumans, 2009).

Another limitation comes from the potential confounding effect of light stress on the animals. As mice were on reverse light-dark cycles, daytime testing would be conducted under red light as to minimize light stress. While mice are less sensitive to red light than white light, they are still responsive to red light, and thus, exposure to red light during the dark cycle potentially posed as a confounding stressor for the animals (Peirson, Brown, Pothecary, Benson, & Fisk, 2018). This issue could have particularly affected mice in experiment 3, as pregnant dams were monitors constantly from E19 until birth under red light.

Another potential source of variation within stress responsivity measures comes from variation in the time of day of sample collection. Plasma samples could only be collected from one mouse at a time, such that slight temporal separations were necessary for restraint stress and sample collection. Since basal CORT varies over the course of the day, variations in plasma collection time could have potentially influenced stress responsivity results. However, this is not likely to have a large effect on results, as all collections occurred in the afternoon (13:00-17:00h), and the circadian peak occurs at the beginning of the active period (~20:00h in our mice) (Romero, 2002).
Findings from this study may be limited from small sample sizes. Large variations in individual sample were observed within some of the data, and particularly in the day of birth testosterone experiment (n=8-10). Small sample sizes can increase the possibility of type II error and may have masked significant effects. Thus, increasing sample sizes in the future may provide a means to instill confidence in results.

4.12 Future directions

Tissue samples which remain from all three experiments will be used to examine how an early elevation in prenatal testosterone might result in structural and functional changes in brain regions involved in sexual differentiation and stress. This will allow us to better understand molecular mechanisms underlying changes in HPA axis responsivity in prenatal TP exposed male offspring. Furthermore, we will continue with the preliminary attempts to measure estradiol levels in plasma samples from our adult female offspring, to determine whether early prenatal testosterone influenced estradiol levels, and to verify whether ovariectomy and hormone replacement were successful at altering estradiol profiles.

To further explore the effects of early prenatal testosterone exposure on the dynamics of the HPA axis, brains harvested from experiment 1 will be used to examine potential changes in GR and MR in brain regions involved in stress negative feedback, such as the hippocampus and the PVN. Brains were flash frozen at the time of sacrifice and are therefore available for examination of receptor quantities at both the mRNA and the protein levels. As brains from intact, GDX, and hormone replaced animals exist, we
can also characterize both organizational and activational effects of steroid hormones on the expression of GR and MR.

To investigate the effects of steroid hormone exposure on hippocampal morphology, we will conduct Golgi-cox analysis on brains from animals in experiment 2. Following restraint stress and sacrifice, brains from each animal were prepared for Golgi-Cox dendritic morphology analysis. Coronal brain sections will be used to examine changes in hippocampal dendritic morphology including changes in dendritic branching and spine density. The effects of environmental manipulations on the hippocampus are often studied by examining the morphology of hippocampal CA1 and CA3 pyramidal neurons because they are highly plastic, sexually differentiated and often structurally remodel in response to factors such as stress, estrogens and androgens (Atwi, McMahon, Scharfman, & MacLusky, 2016; Gould, Woolley, Frankfurt, & McEwen, 1990; Juraska, Fitch, & Washburne, 1989; Leuner & Gould, 2010; Bruce S. McEwen, Nasca, & Gray, 2016). Thus, we can investigate the influence of sex, prenatal steroid treatment, and acute restraint stress on hippocampal morphology in male and female offspring using Golgi-cox prepared slides.

To further investigate how early prenatal testosterone and prenatal DEX treatment might affect neonatal brain development, we will examine expression of genes involved in sexual differentiation mechanisms and HPA axis functioning in pups on the day of birth. From experiment 3, brains from pups were flash frozen at each timepoint of sacrifice. Brains from pups at 2 hours after birth, which is the time at which testosterone peaks in male mice (Figure 2; Motelica-Heino et al., 1988), will be deep-
sequenced, to determine steady state levels of mRNA in the brains of newborn pups. Relevant changes will be followed up by quantitative polymerase chain reaction analysis, at multiple time points after birth, to determine whether prenatal steroid exposure influenced neonatal genetic profiles.
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