Role of Neonatal Testosterone in Shaping the Adult Male Hypothalamic-Pituitary-Adrenal Axis Response to Stress

by

Brenda Bingham

B.Sc., The University of British Columbia, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

February 2012

© Brenda Bingham, 2012
Abstract

Testosterone exposure during critical periods of development exerts major organizing effects on the hypothalamic-pituitary-adrenal (HPA) axis. The aim of this thesis was to determine how HPA axis activity is altered by neonatal testosterone, and where and how this might occur in the adult brain. In chapter two, I demonstrate that neonatal gonadectomy increased plasma corticosterone and Fos activation in the ACTH-regulating zone of the paraventricular nucleus of the hypothalamus under basal conditions and following restraint exposure. These responses were normalized with postnatal, but not adult testosterone replacement. Neonatal gonadectomized rats also had decreased numbers of AR (androgen receptors) and arginine vasopressin-positive cells in the bed nucleus of the stria terminalis and medial amygdala. This suggests that testosterone exposure during the neonatal period may prime adult HPA response to testosterone by altering AR levels and function within afferent mediators of HPA axis activity. Testosterone in the brain can be converted to estradiol by the aromatase enzyme, and estradiol usually impacts brain development directly. In chapter 3, I demonstrate that animals neonatally exposed to aromatase, or AR blockade fail to show a normal decline in corticosterone, or habituate, in response to repeat restraint exposure. By contrast, males castrated as adults show a significant reduction in corticosterone after repeated stress. These findings suggest an organizing influence of both ARs and estrogen conversion on HPA habituation, which occurs independently of activational effects of testosterone. The immediate early gene c-fos is rapidly induced in many brain regions following acute restraint stress and is an excellent tool for mapping functional differences in brain activation by stress. In chapter 4, we used c-fos mRNA as a tool to map changes in cellular activation in acutely stressed adult animals that received aromatase blockade.
neonatally. This treatment enhanced stress-induced c-fos expression in several limbic regions, including within the anterior cingulate and medial prefrontal cortex, lateral septum, anterior hypothalamic area, dorsal medial hypothalamus and medial amygdala, as well as at multiple levels of somatosensation. Based on these results, I propose estrogens exert effects during the neonatal period that result in systems-wide differences in adult neuroendocrine responses to homeostatic threat.
Preface

A version of Chapter 2 has been published: Bingham B, and Viau V. 2008. Neonatal gonadectomy and adult testosterone replacement suggest an involvement of limbic arginine vasopressin and ARs in the organization of the hypothalamic-pituitary-adrenal axis. Endocrinology, 149: 3581 – 91. I performed all the experiments and data analysis presented in this chapter. I wrote the manuscript that was subsequently revised and edited by Dr. Victor Viau.

A version of Chapter 3 has been published: Bingham B, Gray M, Sun T, Viau V (2011) Postnatal blockade of ARs and aromatase impair the expression of stress hypothalamic-pituitary-adrenal axis habituation in adult male rats. Psychoneuroendo. 36(2): 249-57. Megan Gray and Terri Sun assisted me with the animal experiments. I subsequently performed all of the assays and data analysis presented in Chapter 3. I wrote the manuscript that was subsequently revised and edited by Dr. Victor Viau.

A version of Chapter 4 has been published: Bingham B, Wang NX, Innala L, and Viau V (2012). Postnatal aromatase blockade increases $c$-$fos$ mRNA responses to acute restraint stress in adult male rats. Endocrinology (Feb 7. Epub ahead of print). I performed all of the animal experiments and assays. Nancy Wang assisted with performing the data analysis. I wrote the manuscript that was subsequently revised and edited by Dr. Victor Viau. Leyla Innala also assisted with manuscript revisions.

This work was approved by the UBC animal care committee (Application Number A07-0235).
Table of Contents

Abstract.................................................................................................................................... ii
Preface..................................................................................................................................... iv
Table of Contents .................................................................................................................... v
List of Tables ........................................................................................................................... x
List of Figures......................................................................................................................... xi
List Abbreviations................................................................................................................ xiii
Acknowledgements .............................................................................................................. xiv

Chapter 1: Introduction ........................................................................................................ 1

1.1 General Introduction .................................................................................................... 1
1.2 Literature Review ....................................................................................................... 4
1.3 Stress ............................................................................................................................ 4
1.4 Overview of the HPA Axis .......................................................................................... 7
  1.4.1 Neural regulation mpd PVN ............................................................................... 10
  1.4.2 Regulation of ACTH release ............................................................................. 12
  1.4.3 Regulation of glucocorticoid release ................................................................. 13
  1.4.4 Glucocorticoid negative feedback ...................................................................... 14
  1.4.5 HPA axis habituation ....................................................................................... 14
1.5 Sex Steroids ............................................................................................................... 16
  1.5.1 Determinants of health ....................................................................................... 16
  1.5.2 Sex differences in HPA axis activity ................................................................. 17
  1.5.3 Activational effects in males ............................................................................. 18
Chapter 1: Sex Steroid Hormone Receptors

1.6 Sex Steroid Hormone Receptors ................................................................. 20

1.6.1 Mechanism of action .............................................................................. 20

1.6.2 Role of sex steroid receptors in HPA axis regulation ......................... 23

1.7 The Perinatal Period .................................................................................. 24

1.7.1 Organizational-activational hypothesis ................................................ 25

1.7.2 Brain morphology and neurotransmitter systems ............................... 26

1.7.2.1 Androgen receptors ........................................................................ 27

1.7.2.2 Central vasopressin ........................................................................ 28

1.7.3 Perinatal testosterone and the HPA axis ............................................. 29

1.8 Overview and Objectives .......................................................................... 30

Chapter 2: Neonatal gonadectomy and adult testosterone replacement suggest an involvement of limbic arginine vasopressin and androgen receptors in the organization of the hypothalamic-pituitary-adrenal axis ......................................................... 32

2.1 Introduction .............................................................................................. 32

2.2 Methods .................................................................................................. 34

2.2.1 Postnatal gonadectomy and testosterone treatment .......................... 34

2.2.2 Adult testosterone replacement ............................................................. 36

2.2.3 Tissue and blood collection ................................................................ 37

2.2.4 Plasma hormones ............................................................................... 37

2.2.5 Fos immunohistochemistry ................................................................. 38

2.2.6 AR immunohistochemistry ................................................................. 39

2.2.7 Hybridization histochemistry ............................................................... 40

2.2.8 Statistics ............................................................................................. 43
Chapter 3: Postnatal blockade of androgen receptors or aromatase impair the expression of stress hypothalamic-pituitary-adrenal axis habituation in adult male rats
3.3.2 Efficacy of flutamide and ATD treatment ................................................................. 75
3.3.3 Plasma hormone responses ....................................................................................... 75
  3.3.3.1 Untreated and blank controls ............................................................................... 75
  3.3.3.2 Testosterone ....................................................................................................... 77
  3.3.3.3 Adrenocorticotropin ......................................................................................... 78
  3.3.3.4 Corticosterone ................................................................................................. 78
3.4 Discussion .................................................................................................................... 82
  3.4.1 Implications for stress disease research and future directions ............................. 86

Chapter 4: Postnatal aromatase blockade increases c-fos mRNA responses to acute restraint stress in adult male rats. ......................................................................................................................... 87
  4.1 Introduction ................................................................................................................ 87
  4.2 Methods ..................................................................................................................... 88
    4.2.1 Blood and tissue collection ................................................................................ 89
    4.2.2 Plasma hormones .............................................................................................. 89
    4.2.3 Hybridization histochemistry .......................................................................... 90
    4.2.4 Statistics .......................................................................................................... 91
  4.3 Results ....................................................................................................................... 91
    4.3.1 Hormone responses .......................................................................................... 91
    4.3.2 Extrahypothalamic AVP .................................................................................. 92
    4.3.3 Basal and stress c-fos mRNA expression ......................................................... 94
  4.4 Discussion ................................................................................................................. 97

Chapter 5: Discussion ....................................................................................................... 100
  5.1 Contributions to Original Knowledge .................................................................... 100
5.2 Methodological Considerations ................................................................. 102

5.2.1 Manipulating the neonatal hormone environment ............................. 102

5.2.2 Aromatase and flutamide antagonism .............................................. 103
  5.2.2.1 Drug delivery ............................................................................. 103
  5.2.2.2 Implant removal ..................................................................... 104

5.2.3 Immediate early genes and cellular activation .................................. 105

5.3 Recent Progress and Future Consideration .......................................... 106

5.3.1 AR expressing cells in the MeA and posterior BST .......................... 106

5.3.2 Arginine vasopressin in the posterior BST and MeA ....................... 108

5.3.3 Estrogen receptor-α and -β distribution during development .............. 110

5.3.4 Link to environment ....................................................................... 112

References ..................................................................................................... 115
List of Tables

Table 2.1 Correlations between AR and AVP cell numbers and basal and stress-HPA function. .................................................................................................................................................. 57

Table 3.1. Mean ± SEM duration (sec) of individual maternal behaviors in dams with experimental or control pups. .................................................................................................................. 75

Table 3.2. Mean ± SEM plasma testosterone concentrations (ng/ml) at 0 min of restraint under acute and repeat stress conditions in adults bearing blank, flutamide, or ATD implants at birth. ............................................................................................................................................ 77

Table 4.1. Mean ± SEM plasma testosterone and corticosterone concentrations (ng/ml) at 0 and 30 min of restraint stress exposure in adults bearing Blank or ATD implants at birth.... 92
List of Figures

Figure 1.1 Schematic illustrating the ACTH-regulating zone of the PVN ............................. 8
Figure 1.2 Diagram illustrating general regulation of the hypothalamic-pituitary-adrenal axis.
............................................................................................................................................... 9
Figure 1.3 Diagram illustrating the conversion of cholesterol to sex steroid hormones. .... 21
Figure 2.1 Mean plasma corticosterone concentrations and Fos-ir cell numbers in the mpd
region of the PVN under basal conditions. ............................................................................. 45
Figure 2.2 Mean plasma corticosterone concentrations and Fos-ir cell numbers in the mpd
region of the PVN under stress conditions. ........................................................................... 46
Figure 2.3 The AVP response to adult testosterone replacement in the posterior division of
the BST is reduced by neonatal GDX and restored with neonatal testosterone treatment. .... 49
Figure 2.4 Dark-field photomicrographs at 150- m intervals through the rostrocaudal extent
of the posterior division of the BST to show the relative distribution and strength of
hybridization signal for AVP mRNA. .................................................................................. 50
Figure 2.5 Neonatal testosterone treatment restores the number of AVP cells in the
anterodorsal part of the medial amygdala but not the capacity of individual cells to express
AVP mRNA. ....................................................................................................................... 51
Figure 2.6 Hybridization histochemical localization of AVP mRNA in the anterodorsal part
of the MeA in neo-sham, neo-GDX, and neo-GDX + T-treated rats. .................................... 52
Figure 2.7 Histochemical localization of AR-ir in the posterior division of the BST of neo-
sham, neo-GDX, and neo-GDX + T-treated rats ................................................................... 54
Figure 2.8 Histochemical localization of AR-ir in the MeA of neo-sham, neo-GDX, and neo-
GDX + T-treated rats. ........................................................................................................ 55
Figure 3.1 Volume of the sexually dimorphic nucleus of the hypothalamus in response to neonatal ATD and flutamide treatment and adult gonadectomy. ........................................ 76
Figure 3.2 Plasma ACTH and corticosterone on the first and last day of restraint under basal, stress and recovery conditions. ................................................................. 80
Figure 3.3 ACTH and corticosterone total hormone responses from baseline and on the last day of restraint as a percentage of the first day of restraint. ........................................ 81
Figure 4.1 AVP mRNA expression in the posterior division of the BST and MeA in response to neonatal ATD treatment................................................................. 93
Figure 4.2 Effects of neonatal ATD treatment on restraint-induced c-fos mRNA expression through forebrain regions in adult animals.................................................. 95
Figure 4.3 Relative optical density (OD) measures of basal and stress induced c-fos mRNA within limbic, motor and sensory regions.................................................. 96
**List Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>anterior cingulate</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropin hormone</td>
</tr>
<tr>
<td>AHA</td>
<td>anterior hypothalamic area</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ATD</td>
<td>1,4,6-androstatriene-3,17-dione</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>bfd</td>
<td>barrel field primary somatosensory</td>
</tr>
<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>CBG</td>
<td>cortisterone binding globulin</td>
</tr>
<tr>
<td>CA1</td>
<td>field CA1, cornu ammonis 1, hippocampus</td>
</tr>
<tr>
<td>CA3</td>
<td>field CA3, cornu ammonis 3, hippocampus</td>
</tr>
<tr>
<td>CeA</td>
<td>central nucleus of the amygdala</td>
</tr>
<tr>
<td>CP</td>
<td>caudoputamen</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial nucleus of the hypothalamus</td>
</tr>
<tr>
<td>EDC</td>
<td>endocrine disrupting chemical</td>
</tr>
<tr>
<td>Flu</td>
<td>flutamide</td>
</tr>
<tr>
<td>fx</td>
<td>columns of the fornix</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD 65</td>
<td>glutamic acid decarboxylase 65</td>
</tr>
<tr>
<td>GAD 67</td>
<td>glutamic acid decarboxylase 67</td>
</tr>
<tr>
<td>GDX</td>
<td>gonadectomy</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>HIP</td>
<td>hippocampus</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early-gene</td>
</tr>
<tr>
<td>int</td>
<td>internal capsule</td>
</tr>
<tr>
<td>LS</td>
<td>lateral septum</td>
</tr>
<tr>
<td>M1</td>
<td>primary motor area</td>
</tr>
<tr>
<td>MeA</td>
<td>medial nucleus of the amygdala</td>
</tr>
<tr>
<td>MPN</td>
<td>medial preoptic nucleus</td>
</tr>
<tr>
<td>Neo-GDX</td>
<td>neonatal gonadectomy</td>
</tr>
<tr>
<td>ot</td>
<td>optic tract</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>PVNmpd</td>
<td>paraventricular nucleus of the hypothalamus, medial parvocellular part, dorsal zone</td>
</tr>
<tr>
<td>PVT</td>
<td>paraventricular nucleus of the thalamus</td>
</tr>
<tr>
<td>sm</td>
<td>stria medularis</td>
</tr>
<tr>
<td>st</td>
<td>stria terminalis</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
</tr>
<tr>
<td>VPM</td>
<td>ventroposteromedial nucleus of the thalamus</td>
</tr>
<tr>
<td>V3</td>
<td>third ventricle</td>
</tr>
</tbody>
</table>
Acknowledgements

Firstly, I would like to thank Dr. Victor Viau, my PhD supervisor, for encouraging me to pursue a graduate degree. Your genuine enthusiasm for research and dedication to your work is contagious. I thank you for all the technical skills you have encouraged me to perfect, but mostly for the critical eye you helped me to develop. The quality of my work will always be better for your honest criticism and helpful suggestions.

My committee members, Dr. Liisa Galea, Dr. Timothy O’Connor and Dr. Joanne Weinberg, have been an incredible source of encouragement and knowledge throughout my graduate work. I am sincerely thankful!

I am grateful to all of the Viau lab members past and present. I am a better person, mentor and scientist because of all the amazing individuals I have had the opportunity to work with. I am particularly indebted to Patricia Lee, Megan Gray, Leyla Innala, Terri Sun, and Adam Anonuevo for all of the long hours spent in animal rooms, and to my long time lab mate Martin Williamson for a great friendship, and the help with all things ‘Figure’ related. My work would not be this pretty without your help.

I would also like to thank my friends and family for providing me with so much encouragement and support. Special thanks to my parents; you made me believe I could do anything if I worked hard and put my mind to it. To my ‘housemates’ Mike and Mary; I will be eternally grateful for your friendship and support, but mostly for all of the warm meals and many bottles of wine you supplied at the end of very long days. To my Oma; we miss you terribly but you will be happy to know someone else in the family will have the ‘Doctor title’.
I am grateful to the following sources of financial support throughout my graduate career: Michael Smith Foundation for Health Research Senior Trainee Award, the Canadian Institute for Health Research Doctoral Award and the Natural Sciences and Engineering Research Council for funding my project.

Finally, I would like to thank my husband Andy and daughter Makayla for helping me complete this chapter of my life. You are both a constant source of love, encouragement and motivation and I could not have done this without you.
Chapter 1: Introduction

1.1 General Introduction

The hypothalamic-pituitary-adrenal (HPA) axis provides a frontline of defense against threats to homeostasis by driving the release of glucocorticoids from the adrenal gland (cortisol in humans and corticosterone in rats). The release of glucocorticoids is centrally regulated by the paraventricular nucleus of the hypothalamus (PVN), which represents the final common pathway by which the brain ultimately regulates the HPA axis. Acute elevations in plasma glucocorticoids have several important adaptive functions, including metabolic, immune, and behavioral. Dysfunction of the HPA axis is associated with both psychosomatic and psychiatric disorders (see Chrousos et al., 1998; Raison and Miller, 2003; Tsigos and Chrousos, 2002 for reviews). For example, HPA hyperactivity is thought to contribute to health problems like cardiovascular disease (McEwen, 1998a), and several mood disorders, including major depression (Barden, 2004; Bjorntorp, 1996; Young et al., 2004). Mood disorders are common and according to the world health organization are among the leading causes of disability worldwide. Not only do these disorders greatly impact the quality of life of those affected, but also have a major economic impact through associated health care costs and lost work productivity (Kessler and Ustun, 2008).

Fortunately, under normal conditions there are mechanisms in place that dampen the secretion of glucocorticoids in the face of repetitive, non life-threatening stimuli. Animals repeatedly exposed to the same, or homotypic, stressor often show a decrease in the magnitude of the corticosterone response to stress (reviewed in Dallman, 2003; Grissom and Bhatnagar, 2009). In humans there is considerable variation in the ability of individuals to habituate to a given stressor. After repeated public speaking most people become habituated
and their cortisol secretion is reduced, however, approximately 10 percent of subjects continue to show elevations in cortisol secretion each time they speak in public (Kirschbaum et al., 1995). Understanding the mechanisms that create individual differences in the ability to habituate to stress will have significant implications for understanding individual differences in vulnerability to depression, addiction and other related mental illnesses.

The magnitude of the glucocorticoid response under stressful conditions is also subject to sex differences and sex steroid effects. Genuine differences in normal and abnormal HPA function between males and females have been implicated in sex-based differences in susceptibility to several forms of systemic, neurodegenerative and affective disorders, including depression (reviewed in Kudielka and Kirschbaum, 2005). Prevalence rates of several mood disorders, such as depression and anxiety, suggested a role for sex hormones in women (Rubinow and Schmidt, 2002; Shors, 2002). Studies demonstrating an association between depressive illness and hypogonadism in older men, also point to a potential role for testosterone in the predisposition and treatment of mood disorders related to HPA dysfunction (Schmidt et al., 2004; Shores et al., 2005). This provides every indication that sex steroids may play an important role in determining why some individuals are predisposed to stress-related neuropathology. Sex steroids, testosterone in males and estrogen in females, are considered integral in producing divergent HPA function in adult humans and rodents (Goldstein, 2002; Kajantie and Phillips, 2006; Kudielka and Kirschbaum, 2005). Indeed, testosterone exerts a strong inhibitory influence on stress-induced ACTH and corticosterone release in the adult male rat (Handa et al., 1994a and b, Viau and Meaney, 1996).
The relationship between the gonadal and adrenal axis appears to be established early on in development. The perinatal period is a critical time of brain growth, development and sexual differentiation. Male rats, unlike female rats, experience a prenatal and postnatal surge in testosterone, which have been shown to have a profound influence on brain morphology and behavior (Negri-Cesi et al., 2004; Wallen, 2005; Wilson and Davies, 2007). Evidence also suggests a role for neonatal testosterone in organizing HPA function in adulthood (McCormick et al., 1998; McCormick and Mahoney, 1999; Seale et al., 2005).

Male rats that are gonadectomized (castrated) during the neonatal period show elevated corticosterone levels as adults under basal (or no stress) conditions and in response to stress (McCormick et al., 1998). This elevated corticosterone response cannot be attenuated by testosterone replacement in adults, indicating that the neonatal testosterone exposure permanently alters how the adult HPA axis responds to testosterone in adulthood.

Although it is clearly evident that early life manipulation of gonadal steroids exerts potent effects on HPA function, it is unclear which regions of the adult brain are altered under acute stress conditions. Further, it is also unclear how neonatal sex hormone exposure in males might alter the capacity of adult animals to habituate to repeat stress conditions. Therefore, the aim of this thesis was to determine how HPA axis activity in adulthood is altered by neonatal testosterone, and where and how this might change the adult brain. In chapter 2 (Bingham and Viau, 2008), I will assess the impact of neonatal gonadectomy on androgen receptor (AR) and AVP mRNA expressing cells in PVN-regulating brain regions. This work provides insight into possible ways that the neonatal testosterone might permanently alter how the adult HPA axis responds to testosterone. Testosterone in the brain can be converted to estradiol by the aromatase enzyme. In many cases it is usually estradiol...
that directly impacts brain development. In chapter 3 (Bingham et al., 2011a), I will investigate the impact of neonatal aromatase or AR blockade on the HPA response to acute and repeated stress. In chapter 4, I will examine the impact of neonatal aromatase inhibition on stress-induced cellular activation in limbic, sensory and motor brain regions. The findings from these studies will be discussed in the context of the contribution of neonatal androgens and estrogens to changes in HPA axis activity in adult males and with respect to potential changes in neurotransmitter and neuromodulator systems in HPA-regulating brain regions. I hope this work will ultimately lead to the development of a better understanding of factors that contribute to individual differences in stress responsiveness.

1.2 Literature Review

The following review of the literature will provide rationale for the experiments that contributed to this thesis. I will start by providing a description of the HPA axis activation, including the neural circuits and neuropeptides underlying its modulation. This will be followed by a description of the impact of sex differences on health and the HPA axis, with a focus on the role of gonadal hormones in HPA axis regulation in male rats. Finally, this chapter will conclude with a look at how testosterone exposure during critical periods of development might play a role in establishing the relationship between the adrenal and gonadal axis in adult males.

1.3 Stress

Mammals respond to stress with characteristic changes in behavior that coincide with changes in neuroendocrine, autonomic and immune systems aimed at reinstating the state of relative stability of core tissues, or homeostasis (Dallman, 2003; Day, 2005; Herman et al., 2003; McEwen and Wingfield, 2003). In the work described here, “stress” can be viewed as
a real or perceived threat that overwhelms selective homeostatic response mechanisms and
requires a multiple system redistribution of resources to meet the challenge at hand (Dallman,
2003; Day, 2005). The word “stress” should not be viewed as inherently negative, or
synonymous with dysfunction or disease (Day, 2005; McEwen, 1998b). It is when the
response to stress is abnormal or the response is sustained, as in chronic stress, that resultant
changes in brain and body may be deleterious to the organism (Dallman, 2003; McEwen,
1998b).

The hypothalamic-pituitary-adrenal axis (HPA) is a critical component of the stress
response system in vertebrates and is essential for adaptation and survival. Circadian- and
stress-induced increases in HPA activity culminate in the release of glucocorticoids from the
adrenal gland. Glucocorticoid hormones act at numerous levels to modulate physiology and
behavior, and help to coordinate the whole organism’s response to challenge (Kovacs et al.,
1986; Kovacs and Mezey, 1987; Swanson and Simmons, 1989). Maintaining normal daily
glucocorticoid rhythms and responses to stress are essential for health and survival of all
vertebrates (Dallman et al., 1987a). This includes the ability to decrease the secretion of
glucocorticoids, or habituate, in the face of repetitive, but non-life threatening stimuli. This
process of HPA habituation is adaptive, as it limits the deleterious consequences caused by
over activation of the HPA axis (Dallman, 2003).

The multifaceted arrays of stimuli that initiate the stress response are commonly
divided into two broad categories (Dayas et al., 2001; Herman et al., 2003; Pacak and
Palkovits, 2001; Sawchenko et al., 2000). “Systemic” stressors (also referred to as
homeostatic or physiological) are initiated by very real sensory stimuli. Examples of
“physiological” stressors include significant changes in cardiovascular tone, blood-borne
immune factors signaling infection, visceral and somatic pain, and inflammation. The other subtype of stressor can be categorized as “psychogenic”, “emotional”, or “neurogenic”. Responses to these types of stimuli are generated in anticipation of a challenge, rather than as a reaction to homeostatic disruption. Some “psychogenic” stressors are innate and species-specific (eg. open spaces or recognition of a predator), while others are learned through experience (Herman et al., 2003). Not only do these two classes of stressors differ on the basis of the sensory input that initiates the response, but they also differ in the pattern of activational responses within the brain and the circuitry that mediates adaptive responses to them (Reyes et al., 2003).

I have used the psychogenic stressor, restraint stress, in all of my studies. A single bout of restraint does not cause physical pain or gross metabolic disturbances, but does represent a stimulus of physically forced, novel confinement that is sufficient for eliciting widespread activation of brain stress response networks; including those regulating autonomic, behavioral, immune and neuroendocrine responses (Cullinan et al., 1995). Understanding mechanisms regulating this type of stress may be especially informative to understanding the neurobiology underlying stress-related disorders (Girotti et al., 2006). It also has several methodological advantages: it is easy to administer, reliably results in a robust increase in plasma concentrations of corticosterone, activation of the PVN and male rats repeatedly exposed to restraint stress reliably exhibit a marked reduction in HPA response (Bhatnagar et al., 2002; Cole et al., 2000; Girotti et al., 2006; Gray et al.; Viau and Meaney, 1996, 1991).
1.4 Overview of the HPA Axis

The daily functioning of the hypothalamic-pituitary-adrenal (HPA) axis controlling glucocorticoid secretion (cortisol in humans and corticosterone in the rat) is regulated by diurnal signals and activated by homeostatic disruptions, including stress (Dallman et al., 2002). Stress-induced elevations in circulating glucocorticoids coordinate the activity of a variety of systems, allowing the organism to meet the demands of the stressor. This response is essential for survival and may include temporary increases in glucose mobilization, cardiovascular and cognitive function, while simultaneously suppressing immune, reproductive and digestive function (Dallman, 2003; McEwen and Wingfield, 2003). Central regulation of the HPA axis is provided by a discrete set of hypophysiotrophic neurons in the medial dorsal parvocellular division of the paraventricular nucleus (mpd PVN) of the hypothalamus (Antoni, 1986; Swanson and Sawchenko, 1983, 1980; Whitnall, 1988) (See Figure 1.1). These neurons integrate excitatory and inhibitory signals from throughout the central nervous system to regulate HPA activity (Herman et al., 2002a; Herman et al., 2003). Under most conditions HPA activity is governed by two key peptides, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). In response to stress, these neurons are stimulated to release CRH/AVP into the pituitary-portal circulation, which stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH released into the general circulation stimulates the synthesis and release of adrenal glucocorticoids into the systemic circulation (see Figure 1.1). In addition to stress activation of the HPA axis, there is also a strong circadian rhythm of glucocorticoid release. The secretion of glucocorticoids peaks just before waking and are 40 to 50 fold higher than the low levels seen in the nadir (Dallman et al., 1987b).
Figure 1-1  A. Schematic depicting compartmentalization of the PVN at the level of the ACTH-regulating zone (mpd). Darkfield photomicrograph of the PVN showing the distribution of median eminence-projecting neurons retrogradely labeled after fluorogold injection into the jugular vein. Structures labeled for reference: dp, dorsal parvocellular; mpd, dorsal medial parvocellular; mpv, ventral medial parvocellular; pm, posterior magnocellular; pv, periventricular part of the PVN.
Figure 1-2. Diagram of the hypothalamic-pituitary-adrenal (HPA) axis. Threats to homeostasis (i.e., stress) activate many stress responsive brain regions, which in turn activate the medial dorsal parvocellular division of the PVN (mpd PVN) to release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These neuropeptides then stimulate the synthesis and release of adrenocorticotropic hormone (ACTH) from anterior pituitary corticotrophs, which then stimulates the adrenal cortex to synthesize and release glucocorticoid hormones.
1.4.1 Neural regulation mpd PVN

An impressive collection of cytoarchitectonic, connectional, phenotypic and functional studies suggest that the medial dorsal parvocellular PVN is regulated by multiple neuronal systems involving homeostasis, memory and emotionality (Herman et al., 2003; Sawchenko et al., 2000). This extensive collection of studies suggests the brain uses distinct neurocircuits to generate stress responses to specific stimuli. Inputs concerned with homeostasis emanate primarily from the brainstem, hypothalamus and basal forebrain and directly innervate the ACTH-regulating zone of the PVN (Dayas et al., 2001; Herman et al., 2003; Sawchenko et al., 2000). In stark contrast, limbic circuits activating the HPA axis in absence of physiological challenge are usually indirect (Herman et al., 2005). Thus, neurocircuitry that drives the response to predicted threats or danger associated with new environments (i.e. restraint stress) requires integration proximal to the PVN. Limbic system dysfunction has been implicated in contributing to numerous neuropsychiatric disease states (Drevets, 2000; Sheline, 1996). Given that these very structures are also implicated in stress control and psychosis, understanding their role in HPA axis regulation may prove informative for understanding stress-related neuropathology (Herman et al., 2005).

Specific limbic regions involved in regulation of the PVN include the lateral septum, medial amygdala, prefrontal cortex and hippocampus (Herman et al., 2005). Lesions of the medial amygdala increase restraint-induced activation of cells in the medial dorsal parvocellular PVN in response to restraint, suggesting a role for this region in HPA activation (Dayas et al., 1999). The lateral septum and hippocampus, on the other hand, are primarily implicated in HPA axis inhibition. Rats with lesions of the ventral hippocampus (or ventral subiculum) show a prolonged glucorticoid stress response following restraint or
open field, but not hypoxia (Bradbury et al., 1993; Herman et al., 1995; Herman et al., 1998), suggesting this region is important in terminating HPA axis responses to stress. These findings support the role of this structure in integration of HPA response to psychogenic stressors (Herman et al., 2005). Likewise, lesions to the lateral septum enhance plasma ACTH and corticosterone responses to forced swim stress (Singewald et al., 2011). Recent evidence suggests the prefrontal cortex regulates the HPA response to stress in a region specific manner. Radley et al (Radley et al., 2006) recently demonstrated that the prelimbic/anterior cingulate (dorsal) part inhibits stress-induced Fos protein and CRH mRNA expression, while the infralimbic part stimulates them in response to acute restraint stress.

Despite the clear involvement of these regions in HPA regulation, they do not appear to directly innervate the ACTH-regulating zone of the PVN. Rather, these regions make connections with first or second order relays to influence HPA output (Herman et al., 2005). The bed nucleus of the stria terminalis and other nuclei of the hypothalamus both receive and reciprocate information with the limbic system, and send substantial input to medial dorsal parvocellular PVN neurons (Dong and Swanson, 2004). The ventral hippocampus, prefrontal cortex, medial amygdala and lateral septum also send projections that terminate in the region immediately surrounding the PVN (Herman et al., 2002b; Risold and Swanson, 1997; Roland and Sawchenko, 1993; Swanson and Petrovich, 1998), which in turn sends interneuronal projections into the PVN. This places limbic structures in a key position to modulate HPA output through excitation or inhibition of interneuronal PVN projections (Herman et al., 2002b).
1.4.2 Regulation of ACTH release

Axons from neurons in the medial dorsal parvocellular part of the PVN project into the external lamina of the median eminence where they release CRH and AVP into the pituitary portal system. CRH and AVP then act on their respective G-protein coupled receptors, CRH receptor 1 and AVP receptor 1b, to stimulate corticotropes in the anterior pituitary to release ACTH (Jard et al., 1987; Zmijewski and Slominski). ACTH is synthesized as a part of the large precursor protein proopiomelanocortin (POMC), which is cleaved to produce ACTH (Eipper and Mains, 1980). CRH is a 41-amino acid peptide and is considered to be the primary stimulator of anterior corticotropes (reviewed in Bonfiglio et al., 2011). Seminal studies have shown that CRH strongly increases the synthesis of POMC and the secretion of ACTH from anterior pituitary corticotropes in culture (Vale et al., 1981; Vale et al., 1983). The 8-amino acid peptide AVP, on the other hand, is considered a weak ACTH secretagogue on its own (Yates et al., 1971). The majority of AVP in the hypothalamus is synthesized in the supraoptic nucleus and the posterior pituitary projecting magnocellular division of the PVN; however, 50% of CRH positive neurons in the medial dorsal parvocellular division of the PVN also express AVP.

AVP is generally thought to increase ACTH release through synergistic actions with CRH (Antoni, 1993; Gillies et al., 1982; Rivier and Vale, 1983). Recent evidence, however, supports a larger role for AVP in conditions of repeated (chronic) stress (reviewed in Roper et al., 2011). Studies have shown an increase in AVP expression in medial dorsal parvocellular PVN neurons (Ma et al., 1999), and an increase in pituitary AVP receptors following repeated stress (Aguilera, 1994). This shift from CRH- to AVP-mediated drive is
thought to maintain activation of the HPA axis during adaptation to repeated stress (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000; Harbuz and Lightman, 1992; Ma et al., 1997).

1.4.3 Regulation of glucocorticoid release

ACTH acts on melanocortin receptors present in the zona fasciculata of the adrenal cortex to stimulate rapid secretion of glucocorticoids into the peripheral blood supply (Baxter and Tyrell, 1987; Mountjoy et al., 1992). The type of glucocorticoid released is species specific. Cortisol is the major glucocorticoid found in humans, while corticosterone is the major glucocorticoid found in rodents. The presence of ACTH is considered essential for adrenocortical function (Baxter and Tyrell, 1987). Removal of the pituitary gland, or hypophysectomy, results in atrophy of the adrenal gland, and loss of the glucocorticoid response to stress (Baxter and Tyrell, 1987). Adrenal weight and glucocorticoid synthesis are restored with ACTH injection or infusion in hypophysectomized animals (Baxter and Tyrell, 1987).

Several ACTH-independent mechanisms have been shown to be important for appropriately fine-tuning and modulating the corticosterone response under different conditions (Bornstein et al., 2008). For example, activation of the preganglionic parasympathetic splanchnic nerve, which innervates the adrenal gland, leads to a prompt release of glucocorticoids. The splanchnic nerve is also thought to be essential for maintaining diurnal variations in glucocorticoids by altering the sensitivity of the adrenal gland to ACTH (Bornstein et al., 1990; Dallman et al., 1977; Engeland and Arnhold, 2005). Conditions of chronic stress (Ulrich-Lai et al., 2006), long-term voluntary exercise (Droste et al., 2007) and aging (Sonntag et al., 1987) have also been shown to alter adrenal secretions.
resulting in an apparent dissociation between ACTH and glucocorticoid responsiveness to stress.

1.4.4 Glucocorticoid negative feedback

Glucocorticoid release following stress has negative feedback actions on HPA activity that limit the magnitude and duration of the stress response (Dallman et al., 1987b). Glucocorticoids inhibit AVP and CRH expression at the level of the PVN, and POMC and ACTH secretion at the level of the pituitary (Kovacs et al., 1986). The activity of PVN CRH neurons can also be regulated indirectly by descending pathways from limbic (i.e. hippocampus, amygdala and septum) and brainstem regions. Transcriptional regulation by glucocorticoid hormones is mediated by two types of receptors: the type-1 high-affinity (Kd $[0^0 C:0.5-1.0 \times 10^{-9}]$) mineralocorticoid receptor (MR) and the type-2 low-affinity (Kd $[0^0 C:2.5-5.0 \times 10^{-9}]$) glucocorticoid receptor (GR) (Reul and de Kloet, 1985). In addition to feedback actions during stress, MR and GR are also essential for maintaining basal HPA activity (Antoni, 1986; de Kloet, 1991). MR is predominantly found in limbic regions, while GR is widely distributed in the brain with particularly high densities found in the PVN and pituitary corticotropes (reviewed in De Kloet et al., 1998). It should be noted that glucocorticoid feedback effects have also been shown to occur rapidly (within minutes) via non-genomic mechanisms (reviewed in de Kloet et al., 2008), suggesting the presence of a membrane receptor for corticosteroids.

1.4.5 HPA axis habituation

An important feature of many real-life stressors is that they can often reoccur and are persistent in nature. Under these circumstances, chronic elevation of glucocorticoids can increase risk to a number of pathologies including immune disorders, neurodegeneration and
major depression (Gold and Chrousos, 2002; McEwen and Stellar, 1993). Importantly, stress-induced elevations in ACTH and corticosterone release decline over repeated exposures to the same (homotypic) stimulus, protecting the organism from the potentially damaging effects of persistently high levels of circulating glucocorticoids (Dallman, 2007; Armario et al., 2004). Certain variables are known to enhance or diminish HPA axis habituation to a homotypic stressor, including frequency, intensity, predictability and type of stressor imposed (Grissom and Bhatnagar, 2009; Weinberg et al., 2007). HPA axis activity habituates to primarily psychological stressors and not to systemic stressors that involve an immediate physical threat, like water immersion and foot shock (Barnum et al., 2007; Retana-Marquez et al., 2003). In addition, HPA axis habituation does not prevent the glucocorticoid response to new challenges. Animals repeatedly exposed to the same stressor are still able to respond to a novel stressor and this is a distinct process termed stress facilitation (reviewed in Dallman et al., 1992; Grissom and Bhatnagar, 2009). The processes of habituation and facilitation act together to allow the organism to remain responsive to new challenges, while reducing the exposure to glucocorticoids in response to familiar challenges that hold no immediate threat (Bhatnagar and Dallman, 1998).

As several others have suggested, the decline in the magnitude of the ACTH and corticosterone responses to repeated aversive stimuli reflect the net influence of stimulatory drive and glucocorticoid mediated negative feedback regulation of the HPA axis (Dallman et al., 1987a). Several studies have shown that habituation to restraint stress is associated with a decrease in c-fos mRNA or Fos protein expression in many putative HPA-regulating regions, including the PVN, the ventrolateral septum, central and medial amygdala, and sensory regions involved with input from the vibrissae (whiskers) (Girotti et al., 2006; Melia
et al., 1994). However, decreased HPA axis activity appears not to be the result of a
generalized decrease in responsiveness of brain regions regulating the axis (Girotti et al.,
2006). As mentioned, habituation is very stimulus specific (Grissom and Bhatnagar, 2009)
and prior habituation to one stressor (homotypic stimulus) does not reduce the HPA response
to another stressor (heterotypic stimulus) (Akana and Dallman, 1997; Bhatnagar and
Dallman, 1998; Ma et al., 1999; Fernandes et al., 2002). With respect to negative feedback,
habituation to repeated stress can be blocked by antagonizing glucocorticoid and/or
mineralocorticoid receptors. Subcutaneous injection of a specific MR antagonist prior to the
last day of repeat restraint exposure blocked corticosterone-response habituation (Cole et al.,
2000; Jaferi et al., 2006). The paraventricular nucleus of the thalamus expresses MR and GR
receptors and is implicated in stress habituation (Bhatnagar et al., 2002). Daily injection of
specific MR and GR antagonists directly into the paraventricular thalamus on days one to
seven prior to restraint stress exposure blocked habituation on day 8 (Jaferi et al., 2006),
suggesting a role for MR and GR in this region in stress adaptation.

1.5 Sex Steroids

1.5.1 Determinants of health

Sex is an important determinant of health and differences exist in the prevalence rates
of many brain based disorders (Kudielka and Kirschbaum, 2005; Rubinow and Schmidt,
2002; Schmidt et al., 2004; Shors, 2002). For example, females are twice as likely as males
to be diagnosed with anxiety or depressive disorders (Kudielka and Kirschbaum, 2005). On
the other hand, men are more likely than women to commit suicide (Kessler and Ustun,
2008; Karch, 2011; Qin, 2011) or have drug dependance/abuse disorders (Lynch et al., 2002).
These differences in health status may be related, at least in part, to genuine sex differences
in sex steroid exposure during development and in adulthood (Glover et al., 2010; Kudielka and Kirschbaum, 2005; Mauri et al., 2003; Rubinow and Schmidt, 2002; Schmidt et al., 2004; Shors, 2002). In addition, sex steroid hormone fluctuations as a function of age, social status and reproductive experience appear to impact how an individual responds to stress (Bartke et al., 1973; Blanchard et al., 1993; Dallman et al., 2002; Seeman et al., 2001; Sencar-Cupovic and Milkovic, 1976; Viau, 2002). Sex differences in basal and stress-induced HPA activation are apparent across species (including rodents, humans and non-human primates) and in response to a variety of stressors (Halvorson et al., 1994; Handa et al., 1994b; Seeman et al., 1995; Seeman et al., 2001; Young et al., 2001). Given the link between sex steroids and stress control, it is important to determine the role sex steroids play in regulating HPA activity and individual differences in stress responsiveness (Williamson et al., 2005).

1.5.2 Sex differences in HPA axis activity

Different levels of circulating sex steroid hormones, including estrogens and androgens, are implicated in mediating the marked sex differences in HPA axis regulation (Chrousos, 1998; McEwen, 2000; Young et al., 2004). Sex differences in basal and stress-induced HPA activation have been found in a variety of species, including rodents, humans and primates (Kitay, 1961; Seeman et al., 1995; Seeman et al., 2001; Young, 1995). The following sections will discuss how sex hormones can regulate the HPA axis activity, focusing on the rodent. Male rats have lower levels of circulating ACTH and glucocorticoids under basal conditions and in response to stress compared to females (Kitay, 1961; Handa et al., 1994a). Corticosteroid-binding globulin (CBG) binds corticoisteroids and alters their bioavailability to target tissues (de Kloet et al., 1977; Koch et al., 1976). Female rats have
higher levels of circulating (CBG) than do males; however, the differences in CBG are not sufficient to negate sex differences in glucocorticoid secretion (McCormick et al., 1995).

The steroid hormone environment in adulthood acts to reversibly modulate, or activate, HPA axis activity. Estrogens, progestins and androgens have all been implicated in mediating HPA activity (Kudielka and Kirschbaum, 2005). Though most of this review will focus on males, it is important to mention that gonadal system is clearly capable of regulating HPA response in females. Ovariectomy decreases corticosterone secretion in female rats and this is attributed to the removal of ovarian hormones (Burgess and Handa, 1992). In addition, corticosterone responses to acute stress are pronounced during the proestrus phase of the estrous cycle, when estrogen levels are high (Viau and Meaney, 1991). Others have shown that estrogen treatment in female rats decreases ACTH, while increasing corticosterone in response to acute restraint stress (Figueiredo et al., 2007; Kitay et al., 1970). There is some evidence this effect may be at least partially mediated by estradiol-induced enhancement of adrenal sensitivity to ACTH (Figueiredo et al., 2007). These studies all highlight a stimulatory effect of ovarian steroids on HPA axis activity. Regulation of the HPA axis by ovarian steroids, however, maybe more complex than once thought. Not all studies have demonstrated enhanced HPA responsiveness in association with estrogens. Estradiol concentrations in the lower physiological range have been shown to inhibit, rather than stimulate, the HPA response to stress (Redei et al., 1994; Young, 1995).

1.5.3 Activational effects in males

Studies by Viau and Meaney (1996) demonstrate that individual differences in stress responsiveness in males vary as a function of basal (prestress) circulating testosterone levels. ACTH and corticosterone responses to acute restraint stress were shown to vary negatively
with circulating testosterone levels. Subsequent analysis of HPA activity following testosterone replacement at various physiological doses in gonadectomized males, demonstrated that stress-induced ACTH and corticosterone levels responded to testosterone in a dose-related manner. These results suggest that individual differences in HPA response to stress might be explained by naturally occurring variations in plasma testosterone (reviewed in Williamson et al., 2005).

Several other lines of evidence suggest that testosterone inhibits basal and stress-induced HPA axis activity in male rats. Under basal conditions, gonadectomized rats have higher plasma ACTH and cortisterone levels and increased corticosterone pulsatility (Seale et al., 2004). Gonadectomy also increases corticosterone and ACTH responses to numerous stressors, including restraint, lipopolysaccharide administration, noise, novel open field and footshock (Handa et al., 1994a; Handa et al., 1994b; Seale et al., 2004). At the level of the PVN, gonadectomy (castration) increases CRH and AVP mRNA expression under basal conditions (Bingaman et al., 1994; Viau and Meaney, 1996). In addition, gonadectomized male rats show increased cellular activation, and CRH and AVP heteronuclear RNA in response to restraint stress (Lund et al., 2004a; Viau et al., 2003). CRH and AVP heteronuclear RNA expression is increased in response to restraint, and this increase is believed to reflect a requirement to replenish peptide stores utilized during stress (Cole and Sawchenko, 2002; Herman et al., 1992; Herman et al., 1991). Taken together, these findings suggest that testosterone inhibits stress-induced cellular activation of medial dorsal parvocellular neurons and decreases their release of CRH and AVP. The stimulatory effect of gonadectomy on HPA axis activity in response to acute stress is reversed with testosterone or dihydrotosterone replacement, the reduced non-aromatizable form of testosterone
(Handa et al., 1994b; Viau and Meaney, 1996). These findings suggest that the inhibitory effects of androgens on HPA axis are most likely mediated via an AR dependent mechanism (reviewed in (Handa et al., 1994b; Williamson et al., 2005), but see (Handa et al., 2009). However, recent evidence suggests that dihydrotestosterone (DHT) can actually be converted to 3β-diol and may act through estrogen receptors to regulate the HPA axis (Lund et al., 2006; Weiser et al., 2009) (see Figure 1.3 for metabolic pathways for sex steroid synthesis).

1.6 Sex Steroid Hormone Receptors

1.6.1 Mechanism of action

Sex steroids, including estrogens and androgens, belong to a family of hormones that act through their cognate receptor to affect gene transcription in a variety of tissues. The receptors for estrogens and androgens are members of the nuclear receptor superfamily (Mangelsdorf et al., 1995). Nuclear receptors share a similar structure, with specialized domains for ligand binding, DNA binding and transcriptional activation (Jenster et al., 1991; Simental et al., 1991). Several protein types interact with sex steroid hormone receptors to regulate transcriptional activity. These include components of basal transcriptional machinery, specific transcription factors, and comodulatory proteins that act to increase (coactivators) or decrease (corepressors) transactivation of sex steroid hormone receptors (Tetel, 2009). Nuclear receptor comodulators (or coregulators) differ from general and specific transcription factors as they usually do not bind DNA, or affect the basal rate of transcription (Heinlein and Chang, 2001; Tetel, 2009). Rather, they influence transcription rates by modulating receptor folding to ensure stability and correct compartmentalization, or by acting at the target gene promoter region to facilitate DNA occupancy, chromatin
Figure 1-3. A diagram illustrating the conversion of cholesterol to sex steroid hormones, including androgens.
remodelling, and recruitment of general transcription factors associated with RNA polymerase II (nicely reviewed in (Heemers and Tindall, 2007). More than 200 nuclear receptor co-modulators have been identified, suggesting the action of sex steroid hormone receptors are regulated in an incredibly complex manner (Heemers and Tindall, 2007; Heinlein and Chang, 2001; Tetel, 2009; Wang et al., 2005). Interestingly, recent evidence suggests that androgens and estrogens may also act through respective membrane receptors to rapidly activate signal transduction pathways in a manner similar to neurotransmitters (review in Bennett et al., 2011; Pfaff et al., 2011).

Androgens released from the testis in male rats are responsible for sexual differentiation of the male phenotype during development, for male sexual maturation at puberty, and maintenance of male specific behaviour in adulthood (Simerly, 2002). In males, Leydig cells of the interstitial compartment of the testis convert cholesterol to testosterone through an enzymatic sequence of steps (Heinlein and Chang, 2002). Testosterone can be further metabolized to dihydrotestosterone by the enzyme 5α-reductase, or estradiol by the aromatase enzyme cytochrome p450, as well as other steroid end products (Keller et al., 1996; Lund et al., 2006) (see Figure 1.3). Testosterone and dihydrotestosterone both act via the ARs, while estradiol acts via two receptor subtypes: estrogen receptor-α and estrogen receptor-β (Heinlein and Chang, 2002; Pfaff et al., 2011). These receptors are distributed in high numbers throughout the central nervous system (Shughrue et al., 1997a; Shughrue et al., 1997b; Simerly et al., 1990), with modifications in expression patterns occurring during fetal and sexual development, and aging (Perez et al., 2003). Ultimately, several factors can contribute to the effect androgens and estrogens have on any given cell population, including
the presence of metabolic enzymes, steroid receptor subtypes present, and presence of comodulators.

1.6.2 Role of sex steroid receptors in HPA axis regulation

Connectivity data from our lab and others have demonstrated that ARs are not expressed by neurosecretory neurons in medial dorsal parvocellular division of the PVN (Bingham et al., 2006; Simerly et al., 1990; Stern and Zhang, 2003), nor are they expressed by anterior pituitary corticotropes (Morel et al., 1984). This suggests that testosterone acts upstream of the PVN to modulate HPA activity. However, several basal forebrain and hypothalamic structures believed to be responsible for regulating the HPA axis have been observed to contain a high number of cells that express sex steroid receptors (Simerly et al., 1990). Further, fluorogold retrograde and AR double-labeling studies have shown nuclei distributed throughout the brain both express ARs and project to the PVN (Williamson and Viau, 2007), including several important limbic relays mentioned previously. Notably, more than half the PVN projecting neurons in the posterior BST, and medial preoptic nucleus express ARs. Androgens have been shown to act at the level of the medial preoptic nucleus (Viau et al., 1996; Williamson et al., 2010; Williamson and Viau, 2008) and within the vicinity of the PVN region (Lund et al., 2006) to inhibit PVN cellular activation in response to acute stress. Thus, it is likely that the actions of testosterone in the brain are mediated through a variety of regions, and that the contribution of androgen regulation at any one site is stress and context specific (Williamson and Viau, 2007b).

Interestingly, recent work also implicates the estrogen receptor-β subtype in mediating the inhibitory effects of dihydrotestosterone ((Handa et al., 2009; Lund et al., 2006). As mentioned above, dihydrotestosterone can be converted to 3β-diol and act through
estrogen receptor-β to affect target tissues. Lund et al. demonstrated that 3β-diol and a selective estrogen receptor-β agonist suppressed pituitary-adrenal output and c-cf os mRNA responses to restraint stress in a manner similar to DHT. Like ARs, the estrogen receptor-β subtype is not distributed within PVN cells directed at the median eminance (Bingham et al., 2006). This remains consistent with the idea that circulating testosterone acts upstream of the PVN to regulate HPA axis activity. Ultimately, the actions of testosterone within any particular region depend on the presence of metabolic enzymes, and the relative levels of sex steroid receptors present in that region.

1.7 The Perinatal Period

The perinatal period is a critical time of brain growth, development and sexual differentiation (McCarthy, 2006). Due to the complexity of developmental processes and the requirement for precise cues and timing for the establishment of neural circuits, the fetus is extremely vulnerable to insults, and resultant changes in hormonal environment during this time. Manipulations that alter environmental variables, maternal-infant interactions and hormonal environment during this period have been shown to permanently modify the HPA axis (McCormick et al., 1998). Testosterone production by fetal Leydig cells in the developing testis is essential for masculinization of the Wolffian system, the external genitalia, and the brain (Negri-Cesi et al., 2004; Simerly, 2002). Male rats experience a surge in testosterone that occurs on days 18-19 of gestation (Weisz and Ward, 1980) and again during the first few hours following birth (Baum et al., 1988). These surges have been shown to exert remarkable changes in brain morphology, behavior, and HPA function (McCormick et al., 1998; Negri-Cesi et al., 2004; Wallen, 2005; Wilson and Davies, 2007). Several environment factors, including maternal stress and fetal alcohol exposure, have been
shown to influence both the timing and duration of testosterone exposure during the perinatal period and HPA function in adulthood (Ward et al., 2003) (McGivern et al., 1993). These findings underscore the importance of understanding how testosterone activity during critical periods may impact the development and maturation of the HPA axis.

1.7.1 Organizational-activational hypothesis

Phoenix, Goy, Gerall, and Young first proposed in 1959 that exposure to steroid hormones early in development masculinizes and defeminizes neural circuits, programming behavioral responses to hormones in adulthood (Phoenix et al., 1959). This led to the organizational-activational hypothesis, which suggests gonadal steroids influence sexually dimorphic behaviors in two fundamentally different ways. As described previously, they act to reversibly modulate (or activate) physiology and behavior in adulthood. On the other hand, during development they permanently alter (or organize) the brain and behavior. From this classic viewpoint chromosomal sex determines gonadal sex, which in turn determines sex specific brain and behaviour. The absence of high levels of gonadal hormones during the perinatal sensitive period results in feminization of the brain and is considered the default process. The promotion of male-typic brain and behavior and the suppression of female-typic brain and behaviour, or masculinization and defeminization, are considered to be separate processes driven by exposure to gonadal hormones. Though this thesis is focused on this hormonally driven process, it should be noted that recent work has led to a revision of the classic view of brain sexual differentiation. It is now clear that other factors, including genes and environment, act in parallel with sex hormones to cause sex differences in the brain (nicely reviewed in McCarthy and Arnold, 2011)
Ultimately, the majority of the organizing effects of steroid hormones are likely derived from the testicular androgen surge. However, androgens acting on ARs do not alone organize the male rat brain (Amateau et al., 2004; McCarthy, 2006; McCarthy et al., 2009). In addition to ARs (Lieberburg et al., 1980), the newborn male rat brain also has functional estrogen receptors (MacLusky et al., 1979; Perez et al., 2003) and high levels of the cytochrome P450 enzyme, aromatase (Shinoda et al., 1994; Zhao et al., 2007). In fact, the prevailing view is that estrogens are primarily responsible for masculinizing the rodent brain (Cooke et al., 1998; McCarthy et al., 2009; Simerly, 2002). The developing female brain is protected from the masculinizing effects of maternal estrogens by α-fetoprotein, which sequesters estradiol in the blood stream (Bakker, 2006; McEwen et al., 1975). Interestingly, several putative HPA-regulating brain regions, including the hippocampus, hypothalamus and preoptic area, exhibit higher aromatase activity during the perinatal period than at any other time in life (MacLusky et al., 1994).

1.7.2 Brain morphology and neurotransmitter systems

Sexual differentiation of the male brain results in structural, neurochemical and molecular changes. Hundreds of sex differences in neural structures have been found throughout the brain (De Vries, 2006). Structural sex differences ranging from microscopic changes in cell size, neurite morphology, dendritic length, or spine number to macroscopic changes in brain region area and volume, or projection density (De Vries, 2006; Johansen et al., 2004; Morris et al., 2004). The textbook example of a macroscopic change in morphology is provided by the sexually dimorphic nucleus of the hypothalamus (SDN-POA) (Gorski et al., 1978). Estradiol has been shown to prevent cell death in the SDN-POA of males during development (Arai et al., 1996; Davis et al., 1996). As such, the SDN-POA is
five times larger in males and contains more cells than in females (Dohler et al., 1986; Houtsmuller et al., 1994; Lund et al., 2000). Neurochemical and molecular changes are also numerous and distributed throughout the central nervous system. These include sex differences in neurotransmitters, enzymes, and hormone levels, epigenetic modifications, signaling cascade activation, and gene expression. There are a number of excellent reviews dedicated to sex differences in the central nervous system (De Vries, 2006; de Vries and Sodersten, 2009; McCarthy, 2006; McCarthy et al., 2009). However, I will focus on few examples that may be relevant to the study of sex differences in HPA axis activity.

1.7.2.1 Androgen receptors

The predominant view is that the inhibitory actions of testosterone on the HPA axis are mediated via actions through ARs (Handa et al., 1994b; Viau and Meaney, 1996 and reviewed in Williamson et al., 2005), but see (Handa et al., 2009). Sex differences in AR mRNA expression (Simerly et al., 1990) and AR immunoreactivity (Herbison, 1995; Roselli, 1991; Roselli et al., 1989) have been reported throughout the rat brain, with males generally expressing higher levels than females. The different levels of circulating sex hormones in males and females make it difficult to assess whether or not the difference is due to permanent changes that have occurred during the neonatal period. However, AR binding studies in adult gonadectomized animals have uncovered sex differences in several regions known to regulate the HPA axis; including the lateral septum, posterior BST, medial preoptic nucleus and medial amygdala (McGinnis and Katz, 1996). This opens the possibility that perinatal androgens may permanently alter AR expression in HPA-regulating regions.
1.7.2.2 Central vasopressin

Extrahypothalamic AVP circuits that originate in the bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA) are extremely sensitive to the influence of gonadal steroids and are prime candidates for relaying testosterone sensitive information that may ultimately influence HPA-output (Viau et al., 2005). In most vertebrates studied, males have more AVP in the BST and medial amygdala than females and denser projections than females (de Vries and Panzica, 2006). Han and De Vries (2003) demonstrated a 50% reduction in the number of AVP mRNA expressing cells in the BST in adult male rats that were gonadectomized as neonates as compared to controls. The effect of neonatal gonadectomy on AVP expression was restored with either estradiol or dihydrotestosterone on postnatal days 1, 3, and 5, suggesting a role for estrogens and androgens in sexual differentiation of AVP expression in the BST. Mice with a null mutation of the pro-cell death gene, BAX, had more cells in the BST in both sexes, but males still had 40% more vasopressin cells (de Vries et al., 2008). Therefore, cell death is unlikely to account for the sex difference in AVP cell number, suggesting differentiation of cell phenotype as the underlying mechanism (de Vries et al., 2008).

In adults, over 90% of AVP expressing cells in the BST and MeA colocalize with ARs (Zhou et al., 1994). Further, gonadectomy results in a dramatic decrease in detectable AVP mRNA in the BST and MeA, suggesting these AVP-expressing cells are directly responsive to testosterone in the adult male brain (de Vries and Miller, 1998; Miller et al., 1992). Further, the projection sites of the central AVP system show a close, if not overlapping relationship with many forebrain and hindbrain structures identified as AR-containing and PVN-projecting (Williamson and Viau, 2007b). These findings highlight the
sexually dimorphic AVP system as a candidate mediator of the inhibitory actions of testosterone on the PVN and HPA output (reviewed in Williamson et al., 2005).

1.7.3 Perinatal testosterone and the HPA axis

Levine and Mullins (1967) were one of the first to report effects of neonatal testosterone and estrogen treatment on the corticosterone response to stress in the adult rat. Since then, several studies have demonstrated that testosterone and its conversion to estrogen act during different periods of development to regulate multiple components of the HPA system. Males treated with the AR antagonist flutamide or the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) from prenatal day 13 to postnatal day 20, have elevated levels of CRH and AVP in the PVN (Seale et al., 2005). The mean level and amplitude of corticosterone release under basal conditions and in response to auditory stress and lipopolysaccharide treatment are also elevated following these treatments. Along parallel lines, testosterone replaced adult mice with the testicular feminization mutation (lack functional ARs) show elevated corticosterone responses under basal conditions and in response to exposure to open field with novel object. Interestingly, male rats gonadectomized within 12 hours of birth also have higher levels of plasma corticosterone and cfos activation in the PVN following 30 minutes of acute restraint stress (McCormick et al., 1998). This elevated adrenal response is attenuated with neonatal but not with adult testosterone replacement. This indicates that neonatal androgens determine how the HPA axis responds to testosterone later in life, suggesting possible changes in sex steroid receptor expression and/or regulation. Although it is clearly evident that early life manipulation of gonadal steroids exerts potent effects on HPA function, it is unclear which regions of the
adult brain are altered under acute stress conditions. Further, it is unclear how they influence the process of stress HPA activity under chronic stress conditions.

1.8 Overview and Objectives

The aim of this thesis was to determine how HPA axis activity is altered by neonatal testosterone, and where and how this might change the adult brain. The objectives of the present thesis are as follows:

Objective 1: To investigate the impact of neonatal gonadectomy on AR and AVP mRNA expressing cells in PVN-regulating brain regions. As mentioned, male rats that are neonatally gonadectomized do not respond to the inhibitory actions of testosterone on HPA axis activity as adults. Therefore, we examined how manipulation of neonatal testosterone exposure by gonadectomy alters the number of AR and AVP mRNA expressing cells in PVN-regulating brain regions. We chose to look at AVP mRNA in addition to AR because it is extremely sensitive to circulating testosterone and may also play a role in HPA regulation. In addition, hormonal and cellular markers of HPA and PVN function, respectively, were assessed under basal and stress conditions in adult rats. I hypothesized that the organizing effects of neonatal testosterone exposure could involve a shift in the capacity of neurons to express ARs and/or respond to adult levels of testosterone, particularly within brain nuclei projecting to and/or regulating the HPA axis.

Objective 2: To investigate the impact of neonatal aromatase or AR blockade on the HPA response to acute and repeated stress. Little is known about the organizational or activational effects of testosterone activity on HPA response to chronic stress in adulthood. Testosterone in the brain can be converted to estradiol by the aromatase enzyme, and it is actually estradiol that usually directly impacts brain development. In this study, we
examined the HPA response to acute and repeated restraint stress in adult male rats that received capsules containing an AR antagonist, or an aromatase inhibitor as neonates. I hypothesized that manipulating testosterone exposure in the postnatal period would alter HPA habituation to repeated stress.

**Objective 3: To investigate the impact of neonatal aromatase blockade on acute stress induced c-fos mRNA expression in limbic, sensory and motor regions.** The immediate early gene c-fos is rapidly induced in many brain regions following acute restraint stress. In this study, we used c-fos mRNA as a tool to map changes in cellular activation in response to acute restraint in adult animals that were exposed to neonatal aromatase blockade. Several sensory, motor and PVN-regulating limbic brain regions were examined.
Chapter 2: Neonatal gonadectomy and adult testosterone replacement suggest an involvement of limbic arginine vasopressin and androgen receptors in the organization of the hypothalamic-pituitary-adrenal axis

2.1 Introduction

Several processes influence the maturation and the response of the hypothalamic-pituitary-adrenal (HPA) axis. Manipulations that alter environmental variables, maternal-infant relationships, and sex steroid hormone levels during critical periods of development permanently modify the HPA axis. Alterations in androgen exposure and its active metabolites over the perinatal period in the male rat have been shown to exert remarkable changes in brain morphology and behavior as well as pituitary-adrenal function during adulthood (reviewed in McCormick et al., 1998; Negri-Cesi et al., 2004; Wallen, 2005; Wilson and Davies, 2007).

Levine and Mullins (1967) were one of the first to report effects of neonatal testosterone and estrogen treatment on the corticosterone response to stress in the adult rat. Since then, several studies have demonstrated that testosterone and its conversion to estrogen act during different periods of development to regulate multiple components of the HPA system. For example, male rats that are gonadectomized within 16 h of birth show increased corticosterone release as adults in response to restraint (McCormick et al., 1998). This elevated adrenal response is attenuated with neonatal but not with adult testosterone replacement.

---

1 A version of this chapter has been previously published: Bingham B, Viau V. Neonatal gonadectomy and adult testosterone replacement suggest an involvement of limbic arginine vasopressin and androgen receptors in the organization of the hypothalamic-pituitary-adrenal axis. Endocrinology, 2008; 149: 3581-91.
replacement, indicating that neonatal androgens determine how the HPA axis responds to testosterone later in life. Furthermore, adult male rats show higher levels of CRH and arginine vasopressin (AVP) mRNA in the paraventricular nucleus of the hypothalamus (PVN) when treated with the androgen receptor (AR) antagonist flutamide or the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) between d 13 of gestation and postnatal day 21 (Seale et al., 2005). These treatments also increase the mean level and amplitude of corticosterone release under basal conditions and the HPA response to auditory stress and lipopolysaccharide administration.

Despite the wealth of studies showing that the neonatal sex steroid hormone environment plays an important role in organizing the HPA axis, where this occurs in the brain remains poorly understood. Our connectional studies indicate that the AR and the estrogen receptor β-subtype are not distributed within PVN cells directed at the median eminence (Bingham et al., 2006). This raises the possibility that the central organizing influences of neonatal androgens are mediated outside the PVN. Notably, androgen and estrogen receptors are concentrated within several regions of the brain identified as regulating the HPA axis or projecting to the PVN region (Simerly et al., 1990; Williamson et al., 2005; Williamson and Viau, 2007b), including within the bed nuclei of the stria terminalis (BST) and the medial nucleus of the amygdala (MeA). Compared with their neuroendocrine counterparts in the PVN, AVP projections of the BST and MeA are extremely and uniquely sensitive to adult testosterone levels (de Vries and Panzica, 2006; Kalsbeek et al., 2002) and appear to exert an inhibitory influence on the neuroendocrine hypothalamus, including the PVN (reviewed in Williamson et al., 2005). Thus, the central inhibitory effects of testosterone on the HPA axis could involve some components of the
limbic-AVP system. All the more intriguing is that AVP expression in the posterior BST and MeA as well as its regulation by testosterone in adults depend on androgen exposure during the first week of life (de Vries and Panzica, 2006; del Abril et al., 1987; Han and De Vries, 2003; Wang, 1994).

Based on these findings, we hypothesized that the organizing effects of neonatal testosterone exposure could involve a shift in the capacity of neurons to express ARs and/or respond to adult levels of testosterone, particularly within brain nuclei projecting to or regulating the HPA axis. To examine this possibility, we tested the effects of neonatal gonadectomy (GDX) with or without testosterone treatment during the first week of life on HPA function in adult animals bearing similar levels of testosterone replacement. Central AVP and AR expression levels were also assessed in the same animals to determine the impact of neonatal GDX on AR function as well as to reveal potential sites of testosterone’s upstream and organizational influences on the HPA axis.

2.2 Methods

2.2.1 Postnatal gonadectomy and testosterone treatment

Sprague Dawley rats were mated at the South Campus Animal Care Facility at the University of British Columbia, where they were housed in a controlled environment with a 12-h light, 12-h dark cycle, lights on at 0600 h, with food and water available ad libitum. Dams were preconditioned to pup removal by exposing them to a piece of latex glove laced with alcohol and Vetbond every day of gestation. On the day of birth, rat pups were subjected to sham GDX (neo-sham-GDX), GDX (neo-GDX), or GDX with testosterone treatment (neo-GDX + T). Two experimental male pups were used from each litter, one kept on a heating pad in a cage until surgery and the other pup anesthetized with isoflurane without
added heat supplementation. Incisions were made between the umbilicus and prepuce, and each testicle was brought out and clamped with a hemostat. Heated forceps were then used to cauterize the blood supply to the testicle, and the muscle layer was closed with 5-0 absorbable sutures, closing the skin with a drop of Vetbond tissue glue. Sham surgeries involved all the same procedures as the GDX surgeries, excluding testes removal. On postnatal d 1, 3, and 5, sham-operated pups received daily injections of 0.05 ml sesame oil (neo-sham-GDX), GDX rats received an injection of sesame oil either alone (neo-GDX) or with 500 µg testosterone propionate (neo-GDX + T), as previously described (Han and De Vries, 2003). Animals were weaned at 21 d of age and delivered to our animal housing facility, where they were housed two per cage under controlled temperature and lighting conditions (12-h light, 12-h dark cycle; lights on at 0600 h), with food and water available *ad libitum*. Rats were weighed every other day for 30 d before testing to monitor their health and to habituate the animals to the experimental conditions.

We are aware that pup litter environment plays an instrumental role in brain development. Unlike the majority of previous studies employing neonatal GDX ± T procedures, we adopted a more conservative approach to minimize litter disruption and to control for between-litter effects in maternal care (Liu et al., 2000) and nutritional load (Lesage et al., 2002). First, only two male rats per litter were experimentally manipulated. Second, total litter size in all cases was maintained at 12 pups per litter (two experimental, five male, and five female littermates). Third, litters remained intact and undisturbed until weaning at 21 d of age, and control, sham, and GDX ± T males were then pair housed for the remainder of the experiments. Finally, each study used a minimum of six litters per treatment and employed one experimental animal per litter in any given test as a single measure,
empirically controlling for litter effects (Abbey and Howard, 1973). To test the potential impact of handling and surgery on our results, we included in some of our studies animals from a subset of litters that were never handled. We found no significant differences in CRH mRNA expression levels in the PVN and no significant differences in restraint-induced plasma corticosterone levels between neo-sham-GDX and unhandled control animals. Pilot studies on maternal behavior, including temporal assessments of passive, arch-back and blanket nursing, licking and grooming, and maternal separation (time away from pups), indicated no obvious between-litter differences in maternal care.

2.2.2 Adult testosterone replacement

To unmask effects of neonatal androgen exposure on adult responses to testosterone, all rats received equivalent levels of testosterone replacement at 45 d of age. Testes from adult animals that were sham-GDX as neonates were removed under ketamine-xylazine-acepromazine anesthesia (administered sc, 77:1.5:1.5 mg/ml, respectively, 1 ml/kg). Each testis was delivered through the scrotal incision and exteriorized by severing the vas deferens and spermatic artery and ligated to maintain hemostasis. GDX was completed by closing the scrotal incision with 4-0 nonabsorbable sutures. As adults, neo-GDX and neo-GDX + T-treated animals were likewise subjected to ketamine-xylazine-acepromazine anesthesia but with sham GDX surgery. All animals received two sc SILASTIC brand (Dow Corning, Midland, MI) capsule implants (inner diameter 1.57 mm, outer diameter 3.18 mm, length 3.5 cm) packed with crystalline testosterone designed to deliver stable, adult levels of plasma testosterone (Viau and Meaney, 1996).
2.2.3 **Tissue and blood collection**

On day 20 of adult testosterone replacement, 65-day-old rats were anesthetized with a lethal dose of chloral hydrate (35% wt/vol, 1 ml per 100 g body weight, ip) for perfusion, either immediately after removal from the home cage or at the end of a single 30-min exposure to restraint. As verified by corneal, pedal, and tail-pinch reflexes, deep anesthesia was reliably achieved within 45–60 sec of chloral hydrate administration. Blood samples were obtained by tail nick immediately after the rat was placed in a restrainer (6.3 × 15 cm Plexiglas restrainer; Kent Scientific, Litchfield, CT), at 30 min of restraint exposure, and/or via the right auricle just before perfusate delivery. Blood samples collected in ice-chilled EDTA-treated Eppendorf tubes (3.75 mg EDTA/100 µl blood) were centrifuged at 3000 × g for 20 min and stored at −80 C until assayed.

Animals were perfused via the ascending aorta with ice-cold 0.9% saline (125 ml), followed by 500 ml ice-cold 4% paraformaldehyde (pH 9.5). The brains were postfixed for 4 h in a solution of the same fixative and cryoprotected in 15% sucrose in 0.1 m potassium PBS (KPBS, pH 7.4) overnight at 4 C. Five adjacent 1-in-5 series of 30-µm-thick frozen sections were collected and stored in cryoprotectant (30% ethylene glycol and 20% glycerol in 0.05 m KPBS buffer) at −20 C until processing. Adjacent series of tissues from each animal were used for *in situ* hybridization and immunohistochemical analyses. In all cases, one series was counterstained with thionin and alternately compared with dark- and bright-field illuminations for morphological and anatomical reference.

2.2.4 **Plasma hormones**

Plasma testosterone (25 µl) and corticosterone (5 µl) were measured using commercial RIA kits (MP Biomedicals, Costa Mesa, CA). For corticosterone, the plasma
samples were diluted 1:100 and 1:200 for prestress and poststress time intervals, respectively, to render hormone detection within the linear part of the standard curve. The intra- and interassay coefficients of variation for these assays ranged from 4–6 and 10–12%, respectively. $^{125}$I-labeled ligands were used as tracer in both cases. The testosterone antibody cross-reacts 100% with testosterone and slightly with $5\alpha$-dihydrotestosterone (3.40%), $5\alpha$-androstane-3$\beta,17\beta$-diol (2.2%), and 11-oxotestosterone (2%). The standard curve ED$_{50}$ for the testosterone RIA was 1.2 ng/ml, with a detection limit of 0.1 ng/ml. The corticosterone antibody cross-reacts 100% with corticosterone and slightly with desoxycorticosterone (0.34%), and testosterone and cortisol (0.10%). The standard curve ED$_{50}$ for the corticosterone RIA was 15 µg/dl, with a detection limit of 0.2 µg/dl. A freezer breakdown after these steroids were assayed prevented us from measuring plasma ACTH.

### 2.2.5 Fos immunohistochemistry

Cellular activation of the PVN under basal conditions and during restraint was determined by Fos-ir detection within the medial parvocellular part of the nucleus. Based on our previous time course studies, the 30 min poststress interval is optimal for detecting, within individual animals, both relative differences in stress-induced indices of HPA function and intervening levels of Fos in PVN attributable to differences in gonadal status (Viau et al., 2003). Fos-ir was detected using a standard avidin-biotin-immunoperoxidase procedure (Vectastain Elite ABC kit; Vector Laboratories, Burlington, CA) to localize a primary antiserum (1:40000) raised against amino acids 4–17 of human Fos protein (Oncogene Research Products, Boston, MA). As previously described (Viau et al., 2005), specific staining was abolished by preabsorbing primary antiserum with 50 µm synthetic Fos (Oncogene Research Products). An observer blind to treatment status took Fos-ir cell counts
in regularly spaced (150 µm) intervals through the extent of the PVN. Positive cells were identified as those expressing a black nuclear reaction product. Analyses of the number of cells recruited to express Fos protein in the medial parvocellular, dorsal part of the PVN was assisted by redirected sampling of an adjacent series of thionin stained tissue for anatomical reference. Cell number estimates were generated by counting bilaterally the number of Fos-positive cells through three levels of the medial parvocellular cell population located immediately adjacent to the laterally displaced posterior magnocellular cell group, averaged by dividing the total number of cell counts by slice number, corrected for double counting errors (Abercrombie, 1946; Guillery, 2002), and multiplying this product by a factor of five to account for slice frequency (one in five sections). Results represent estimates of the total number of Fos-positive cells through the medial parvocellular, dorsal part of the PVN.

### 2.2.6 AR immunohistochemistry

AR-immunoreactivity (AR-ir) was localized through several HPA-regulating brain regions, including the medial preoptic nucleus (MPN) and the CA1 region of the hippocampus, in addition to the posterior division of the BST and the posterodorsal part of the MeA. The CA1 region of the hippocampus was chosen as a negative control, because AR expression in this region appears to vary as a function of adult but not neonatal levels of testosterone (Xiao and Jordan, 2002).

Regions of interest were analyzed in tissues obtained under basal conditions and after acute restraint exposure so that correlations could be made between AR-ir cell number and HPA function. Because all of the animals were replaced with static levels of testosterone replacement as adults, we expected AR (as well as AVP in the BST and MeA) to change as a function of neonatal testosterone but not as a consequence of acute restraint exposure.
AR-ir was detected using a standard avidin-biotin-immunoperoxidase procedure to localize a primary antiserum (1:8000) raised against amino acids 1–20 of the rat AR (Santa Cruz Biotechnology, Santa Cruz, CA). Control experiments, in which the primary antiserum to AR was preadsorbed for 24 h at 4°C with 6.7 μm (10-fold excess) synthetic peptide immunogen, corresponding to N-terminal amino acids 1–21 or N-terminal amino acids 2–21 of the rat AR, failed to yield any evidence of specific AR staining (Bingham et al., 2006). Additional control experiments for antisera cross-reactivity, involving the omission of either primary or secondary antibody, yielded no specific labeling (Williamson and Viau, 2007b).

Light-level images of AR-positive neurons were counted bilaterally on an equal number of sections per region of interest per animal. The planes of the sections were standardized according to the atlas of Swanson (Swanson, 2004) and assisted by the morphological features provided by thionin staining of adjacent series of tissue. Areas to be measured were outlined using a standard frame for each of the following regions (Portillo et al., 2006): posterior division of the BST (0.67 mm²), posterodorsal part of the MeA (0.42 mm²), MPN (0.26 mm²), and CA1 region of the hippocampus (0.10 mm²). Final estimates of the total number of AR-positive cells were generated as described for Fos detection.

2.2.7 Hybridization histochemistry

cRNA probes were used to determine the relative expression levels of CRH and AVP mRNA in the PVN, AVP mRNA in the posterior BST and medial nucleus of the amygdala, and AR mRNA in the posterior BST, medial nucleus of the medial amygdala, and MPN. Hybridization histochemistry was carried out using [³³P]UTP-labeled (GE Healthcare Bio-Sciences, Baie d’Urfe, Canada) antisense cRNA exon-specific probes transcribed from a
680-bp fragment of the CRH gene, a 229-bp fragment of the rat vasopressin gene, and a 572-bp fragment of the AR.

Techniques for riboprobe synthesis, hybridization (Simmons et al., 1989), and the patterns of hybridization for these probes are described in greater detail elsewhere (Herman et al., 1992; Simerly et al., 1990; Viau and Sawchenko, 2002; Viau et al., 2001). Briefly, free-floating sections were first rinsed in KPBS to remove cryoprotectant and then mounted and vacuum dried on glass slides overnight. After postfixation with 10% formaldehyde for 30 min at room temperature, sections were digested in proteinase K (10 mg/ml, 37 C) for 30 min, acetylated for 10 min (2.5 mm acetic anhydride and 0.1 m triethanolamine, pH 8.0), rapidly dehydrated in ascending ethanol concentrations (50–100%), and then vacuum dried. Radionucleotide antisense cRNA probes were used at concentrations approximating $3 \times 10^7$ cpm/ml in a solution of 50% formamide, 0.3 m NaCl, 10 mm Tris (pH 8.0), 1 mm EDTA, 0.05% tRNA, and 10 mm dithiothreitol, 1× Denhardt’s solution, and 10% dextran sulfate and applied to individual slides. Slides were coverslipped and then incubated overnight at 57.5 C, after which the coverslips were removed and the sections washed three times in 4× standard saline citrate (SSC; 0.15 m NaCl and 15 mm citric acid, pH 7.0) at room temperature, treated with ribonuclease A (20 µg/ml) for 30 min at 37 C, desalted in descending SSC concentrations (2–0.1× SSC), washed in 0.1× SSC for 30 min at 60 C, and dehydrated in ascending ethanol concentrations. Hybridized sections were then exposed to x-ray film ($\beta$-max; GE Healthcare, Piscataway, NJ), defatted in xylene, coated with Kodak NTB2 liquid autoradiographic emulsion, and exposed at 4 C in the dark with desiccant. The duration on emulsion was determined by the strength of signal on x-ray film (6 d for AVP mRNA and 9 d for CRH mRNA in the PVN; 15 and 18 d for AVP mRNA in the posterior BST and MeA,
respectively; and 21 d for AR mRNA in the MPN, BST, and MeA). Slides were developed at 14 °C with Kodak D-19 for 3.5 min, briefly rinsed in distilled water for 15 sec, fixed in Kodak fixer for 6.5 min, and then washed in running water for 45 min at room temperature.

Based on the strength of the autoradiographic signal, exposure time to emulsion was optimized to ensure that mRNA levels detected were within the linear range of the assay and could be quantified by making relative comparisons in OD levels. OD readings, corrected by background subtraction, were taken at regularly spaced (150-µm) intervals through each region of interest. In the MPN, BST, and MeA, the planes of sections for in situ hybridization were analyzed using the same frame dimensions described above. Hybridized tissue series between animals were aligned along the rostrocaudal plane using white matter morphology as anatomical reference points in addition to the cytoarchitectonic features provided by an adjacent thionin stained tissue series. Analysis of the relative levels of CRH and AVP mRNA in the PVN was restricted to the medial parvocellular dorsal (mpd) region of the nucleus, likewise assisted by redirected sampling of adjacent thionin-stained material, as previously described (Viau and Sawchenko, 2002).

The dispersed nature by which AVP mRNA is expressed by neurons in the posterior BST and MeA allowed us to quantify the number of AVP-expressing cells in these nuclei. As previously described (Viau et al., 2001), any individual cell showing a developed silver grain density greater than five times background was deemed positive and counted. As discussed above with respect to AR, we expected changes in AVP mRNA levels in the BST and AVP to occur, in largest part, as a function of the organizing effects of neonatal testosterone but not as a consequence of acute restraint exposure. As shown in Results, the stability of AVP
mRNA (and AR cell numbers) across basal and stress conditions within each treatment group allowed us to correlate indices of HPA activity as a function of central AVP.

Assessing the number of grains per cell within individual neurons directly was impractical given the enormity of samples and numbers of AVP cells detected. Thus, to provide an indirect estimate of the capacity of individual neurons to express AVP, we divided the AVP OD values by the total number of AVP cells encountered through each region of interest.

Parceling of the rat brain followed the mapping of AVP expression and AR staining as defined by the morphological features provided by thionin staining of adjacent series of tissue, based on the terminology of Swanson (2004) to describe the posterior BST and Canteras et al. (1995) to describe the MeA. Light- and dark-level images were captured using a Retiga 1300 CCD digital camera (Q-imaging, Burnaby, British Columbia, Canada), analyzed using Macintosh OS X-driven, Open Lab Image Improvision version 3.0.9 (Quorum Technologies, Guelph, Ontario, Canada) and Image J version 1.3.5 software (National Institutes of Health, Bethesda, MD), and exported to Adobe Photoshop (version 7.0; San Jose, CA), where standard methods were used to adjust contrast and brightness and final assembly at a resolution of 300 dpi.

2.2.8 Statistics

Data are expressed as the mean ± sem and were analyzed by using one- and two-way ANOVA to detect neonatal treatment and neonatal treatment × stress effects, respectively. Post hoc analyses were performed when appropriate using Newman-Keuls test for multiple pairwise comparisons. Correlational analyses were performed using simple regressions to determine relations between plasma corticosterone and Fos-ir cell counts in the mpd PVN.
Likewise, linear regressions were also performed between basal and stress-induced parameters of HPA function and the number of cells expressing AVP mRNA and AR-ir. Immunohistochemical, in situ hybridization-histochemical, and statistical comparisons were made by observer-blind by assigning coded designations to the tissue and data sets in advance.

2.3 Results

2.3.1 Adult testosterone replacement and corticosterone levels

Testosterone replacement levels were comparable between neonatal treatment groups. Plasma testosterone concentrations = 3.4 ± 0.4, 3.8 ± 0.5, and 3.6 ± 0.5 ng/ml in neo-sham-GDX, neo-GDX, and neo-GDX + T-treated animals, respectively. Analysis of plasma corticosterone concentrations revealed significant main effects of neonatal treatment \( [F_{(2,30)} = 6.70; P = 0.003] \) and stress \( [F_{(1,30)} = 338.00; P < 0.0001] \), and a significant neonatal treatment × stress interaction \( [F_{(2,30)} = 3.59; P = 0.04] \). This interaction was attributed to significantly higher levels of corticosterone in adult testosterone-replaced, neo-GDX rats under basal conditions (Figure 2.1) and in response to restraint stress exposure (Figure 2.2).

2.3.2 Fos-ir, CRH, and AVP mRNA in mpd PVN

Quantitative assessment of the number of Fos-responding neurons in the HPA-regulating, mpd part of the PVN revealed significant main effects of neonatal treatment \( [F_{(2,30)} = 8.61; P = 0.001] \) and stress \( [F_{(1,30)} = 413.37; P < 0.0001] \) but no significant neonatal treatment × stress interaction \( [F_{(2,30)} = 2.01; P = 0.15] \). Compared with neo-sham-GDX and neo-GDX + T-treated rats, neo-GDX rats showed higher numbers of Fos-ir cells under basal conditions (Figure 2.1) as well as higher numbers of mpd cells recruited to express Fos-ir during restraint (Figure 2.2). Correlational analyses revealed reliable positive relationships
Figure 2-1. A and B, Mean ± sem plasma corticosterone concentrations (A) and Fos-ir cell numbers in the mpd region of the PVN (B) under basal conditions in adult male rats bearing similar levels of testosterone replacement (see Results, testosterone replacement levels). As neonates, these animals were either neo-sham), neo-GDX, or neo-GDX + T during the first week of life. *, P < 0.05 vs. neo-sham and neo-GDX + T animals (n = 6 per group). C, Scattergram shows a strong positive correlation between the numbers of Fos cells in the mpd region of the PVN and plasma corticosterone concentrations in unstressed animals (n = 18).
Figure 2-2. A and B, Mean ± sem plasma corticosterone concentrations (A) and Fos-ir cell numbers in the mpd region of the PVN (B) under stress conditions in adult male rats bearing similar levels of testosterone replacement. *, P < 0.05 vs. neo-sham and neo-GDX + T animals (n = 6 per group). C, Scattergram shows a strong positive correlation between the numbers of mpd PVN cells recruited to express Fos protein and plasma corticosterone concentrations in stressed animals (n = 18).
between plasma corticosterone concentrations and Fos cell counts under basal [F(1,16) = 7.65; P = 0.014] and stress [F(1,16) = 11.4; P = 0.001] conditions (Figures 2.1 and 2.2, respectively).

Densitometric measurements within the mpd region of the PVN revealed no effect of neonatal treatment on CRH mRNA under basal conditions [F(2,15) = 0.77; P = 0.92] or at 30 min of restraint exposure [F(2,15) = 1.52; P = 0.25]. Likewise, there was no effect of neonatal treatment on AVP mRNA levels under basal conditions [F(2,15) = 1.66; P = 0.22] or after restraint [F(2,15) = 1.12; P = 0.35].

2.3.3 AVP mRNA responses to neonatal GDX and adult testosterone replacement

ANOVA indicated no significant effect of stress or a significant stress × neonatal interaction on AVP mRNA levels in the BST and MeA (P ≥ 0.52 in all cases). Thus, the relative levels of the transcript through these regions were assessed in basal and stress tissue combined and analyzed, a priori, as a function of neonatal treatment. As revealed by densitometric analyses, there was a significant main effect of neonatal treatment on AVP mRNA in the BST [F(2,33) = 12.95; P < 0.0001], credited to significantly lower levels of AVP mRNA in neo-GDX animals compared with neo-sham-GDX and neo-GDX + T-treated animals (Figure 2.3, top panel). There was a main effect of neonatal treatment on AVP cell numbers in the BST [F(2,33) = 43.06; P < 0.0001]. This main effect was likewise credited to the neo-GDX group, because they showed significantly lower numbers of AVP-expressing cells compared with neo-sham-GDX and neo-GDX + T-treated animals (Figure 2.3, middle panel). Taken as an index of the capacity of individual cells to express AVP (see Figure 2.3, bottom panel), there was no neonatal treatment effect on the AVP OD to cell number ratio [F(2,33) = 0.27; P = 0.76]. As illustrated in Figure 2.4, the bulk of AVP mRNA in the posterior
BST was found to be most concentrated within the vicinity of the transverse and intrafascicular nuclei, although scattered and far less intense clusters of hybridized cells were routinely found within the principal nucleus. Furthermore, qualitative assessment of the spatial pattern of cells hybridized for AVP indicated that the effects of neo-GDX ± T were most apparent within the transverse and intrafascicular nuclei but far less reliable within the principal nucleus (Figure 2.4). As we optimized the time on emulsion to capture the majority of AVP mRNA-expressing cells in the posterior division of the BST, our inability to detect reliable differences in the principal nucleus may be a matter of detection given the scarcity and relatively weaker labeling of this AVP population.

In the MeA, there was a significant main effect of neonatal treatment on the density of AVP mRNA \(F_{(2,33)} = 8.33; P = 0.0012\) within the anterodorsal region of the nucleus (Figure 2.5). The neonatal treatment effect was credited to both the neo-GDX and the neo-GDX + T treatment group, showing significantly lower levels of AVP mRNA compared with neo-sham-GDX animals (see Figure 2.5, top panel). There was a main effect of neonatal treatment on AVP cell numbers in the MeA \(F_{(2,33)} = 5.71; P < 0.0074\) attributed to neo-GDX but not to neo-GDX + T-treated rats. Thus, neo-GDX animals showed significantly lower numbers of AVP mRNA-expressing cells compared with neo-sham-GDX and neo-GDX + T-treated rats (Figure 2.5, middle panel). Finally, there was a main effect of neonatal treatment on the capacity of individual cells to express AVP \(F_{(2,33)} = 5.64; P = 0.0078\). This main effect was credited to significantly lower AVP OD: cell number ratios in neo-GDX and neo-GDX + T-treated animals compared with neo-sham-GDX rats (see Figure 2.5, bottom panel). AVP mRNA cells were detected through the longitudinal extent of the MeA, represented by concentrated clusters in the anterodorsal part and by a vastly smaller and
Figure 2-3. The AVP response to adult testosterone replacement in the posterior division of the BST is reduced by neonatal GDX and restored with neonatal testosterone treatment. Mean ± sem relative levels of AVP mRNA (A), AVP mRNA-expressing cell numbers (B), and AVP mRNA to AVP cell number ratios (C) in neo-sham and neo-GDX ± T rats bearing similar levels of adult testosterone replacement. *, P < 0.05 vs. neo-sham and neo-GDX + T animals (n = 12 per group).
Figure 2-4. A–D, Dark-field photomicrographs at 150-μm intervals through the rostrocaudal extent of the posterior division of the BST to show the relative distribution and strength of hybridization signal for AVP mRNA in control animals. Scale bar, 500 μm (A–D).

E, Mean ± sem relative levels of AVP mRNA in neo-sham and neo-GDX ± T-treated rats through the rostrocaudal extent of the posterior division of the BST. Intervals A–D shown on the x-axis correspond to the slice intervals illustrated in the photomicrographs. Group differences in AVP mRNA were detected through the rostral to mid-extent of the posterior BST (slice levels B and C), most apparent within cells hybridized for AVP in the vicinity of the intrafascicular and transverse nuclei. *, P < 0.05 vs. neo-sham and neo-GDX + T animals (n = 12 per group).
Figure 2-5. Neonatal testosterone treatment restores the number of AVP cells in the anterodorsal part of the medial amygdala but not the capacity of individual cells to express AVP mRNA. A–C, Mean ± sem relative levels of AVP mRNA (A), AVP mRNA-expressing cell numbers (B), and AVP mRNA to AVP cell number ratios (C) in neo-sham and neo-GDX ± T rats bearing similar levels of adult testosterone replacement. *, P < 0.05 vs. neo-sham (A and C); *, P < 0.05 vs. neo-sham and neo-GDX + T (C) (n = 12 per group).
Figure 2-6. Hybridization histochemical localization of AVP mRNA in the anterodorsal part of the MeA. A–C, Dark-field photomicrographs of coronal sections through a comparable level of the BST to show the distribution and relative strength of hybridization signal in neo-sham (A), neo-GDX (B), and neo-GDX + T-treated rats (C) bearing similar adult testosterone replacement levels. Note, as described in Fig. 5, that although neo-GDX + T restored the number of AVP-expressing cells in the MeA, this treatment did not reverse the inhibitory effect of Neo-GDX on the AVP signal within individual cells (compare B and C). For anatomical reference, the optic tract is illuminated in the upper left corners. Scale bar, 100 μm (A–C).
weakly hybridized contingent in the posterodorsal part. As illustrated in Figure 2.6, neonatal GDX ± T treatment effects on AVP were clearly pronounced within the anterodorsal part.

2.3.4 AR-ir responses to neonatal GDX and adult testosterone replacement

ANOVA indicated main effects of neonatal treatment \( F(2,30) = 22.78; P < 0.0001 \) and region \( F(3,90) = 167.97; P < 0.0001 \) but no significant effect of stress \( F(1,30) = 0.16; P = 0.69 \). Thus, the relative levels of AR staining were assessed in basal and stress tissue combined. There was a significant main effect of neonatal treatment on AR cell numbers in the posterior BST \( F(2,33) = 16.11; P < 0.0001 \) and in the posterodorsal part of the MeA \( F(2,33) = 7.62; P = 0.0019 \) but not in the CA-1 region of the hippocampus \( F(2,33) = 0.44; P = 0.90 \), although a trend toward significance was found in the MPN \( F(2,33) = 3.39; P = 0.084 \). Treatment effects in both the posterior BST and MeA were attributed to the neo-GDX group, showing significantly lower numbers of AR-positive cells than neo-sham-GDX and neo-GDX + T-treated rats (Figure 2.7 and 2.8, respectively). No significant effect of neonatal treatment was found on the relative levels of AR mRNA in the MPN, BST, and MeA \( P = 0.65 \). Relative to neo-sham-GDX and neo-GDX + T-treated animals, neo-GDX rats showed a substantial reduction in the number of AR-ir cell numbers throughout the posterior BST but without any apparent bias between the principal, transverse, and intrafascicular nuclei (Figure 2.7). Group differences in AR staining (lowest in neo-GDX animals) were noted throughout the region of the MeA, most conspicuously in the posterodorsal part of the nucleus (Figure 2.8).
Figure 2-7. Histochemical localization of AR-ir in the posterior division of the BST. A–C, Bright-field photomicrographs of coronal sections through a comparable level of the BST to show the distribution and relative strength in AR staining in neo-sham (A), neo-GDX (B), and neo-GDX + T-treated rats (C) bearing similar adult testosterone replacement levels. Scale bar, 250 μm (A–C). At the level depicted, neo-GDX resulted in a decrease in AR staining within the vicinity of the principal nucleus, located just lateral to the stria medullaris (sm) and fornix (fx). A decrease in staining was also apparent within the vicinity of the intrafascicular and transverse nuclei, located medial to the ventral tips of the stria terminalis (st) and the internal capsule (int), respectively. Neo-GDX + T restored AR staining in both regions (compare B and C). D, Mean ± sem total estimates of AR-ir cell numbers in neo-sham and neo-GDX ± T-treated rats bearing similar adult testosterone replacement levels. *, P < 0.05 vs. neo-sham and neo-GDX + T animals (n = 12 per group).
Figure 2-8. Histochemical localization of AR-ir in the posterodorsal part of the MeA. A–C, Bright-field photomicrographs of coronal sections through a comparable level of the MeA to show the distribution and relative strength in AR staining in neo-sham (A), neo-GDX (B), and neo-GDX + T-treated rats (C) bearing similar adult testosterone replacement levels. Scale bar, 250 μm (A–C). Neo-GDX resulted in a decrease in AR staining throughout the entire posterodorsal part, reversed with neonatal testosterone treatment. Structures are labeled for reference: ot, optic tract; st, stria terminalis. D, Mean ± sem total estimates of AR-ir cell numbers in neo-sham and neo-GDX ± T-treated rats bearing similar adult testosterone replacement levels. *, P < 0.05 vs. neo-sham and neo-GDX + T animals (n = 12 per group).
2.3.5 Correlations between AVP and AR-ir cells and HPA function

Several of our findings implied relations between the capacity of animals to express AVP and AR-ir and to respond to adult testosterone replacement. The stability of AR-ir and AVP mRNA across basal and stress conditions within each treatment group allowed us to perform linear regressions between these variables and basal and stress indices of HPA across animals (see Table 2.1). Thus, under nonstressed conditions, a strong negative correlation was found between the number of AR-ir cells in the MeA and resting-state levels of Fos in the mpd part of the PVN. A strong negative correlation was also found between AVP cells in the posterior BST and basal levels of Fos-ir in the PVN. Basal corticosterone levels tended to vary as a function of AVP in the BST and AR cell numbers in the MeA (P = 0.07 for both). In animals exposed to 30 min of restraint, stress-induced levels of Fos in the PVN and corticosterone in plasma varied strongly and negatively with AR and AVP in the posterior BST.
Table 2.1 Correlations between AR and AVP cell numbers and basal and stress-HPA function.

<table>
<thead>
<tr>
<th>Variable/Region</th>
<th>PVN Fos-ir</th>
<th>Plasma corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>r value</td>
</tr>
<tr>
<td><strong>AR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>posterior BST</td>
<td>0.1489</td>
<td>0.35</td>
</tr>
<tr>
<td>medial amygdala</td>
<td>0.0216</td>
<td>- 0.54</td>
</tr>
<tr>
<td><strong>AVP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>posterior BST</td>
<td>0.0051</td>
<td>- 0.63</td>
</tr>
<tr>
<td>medial amygdala</td>
<td>0.7102</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable/Region</th>
<th>PVN Fos-ir</th>
<th>Plasma corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>r value</td>
</tr>
<tr>
<td><strong>AR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>posterior BST</td>
<td>0.0452</td>
<td>- 0.48</td>
</tr>
<tr>
<td>medial amygdala</td>
<td>0.6748</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>AVP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>posterior BST</td>
<td>0.0088</td>
<td>- 0.59</td>
</tr>
<tr>
<td>medial amygdala</td>
<td>0.2171</td>
<td>0.31</td>
</tr>
</tbody>
</table>

1. Correlations were determined by simple regression across all animals (n = 18 for each condition).
2. Fos-ir cell counts determined in the medial parvocellular, dorsal part of the PVN.
2.4 Discussion

Although it is becoming increasingly clear that the sex steroid hormone environment during the perinatal period plays an important role in organizing the HPA axis, where this occurs in the brain remains poorly understood. The present findings suggest that the organizing effects of testosterone might be mediated upstream from the PVN, particularly within brain regions previously acknowledged as regulating the HPA axis and concentrating ARs. Thus, we now have a testable framework for examining in future studies whether the critical effects of neonatal androgens occur within AR- and/or AVP-expressing cells that communicate with the HPA axis.

Our findings support previous reports showing a stimulatory effect of neonatal gonadectomy on both basal and stress-induced corticosterone release in adult male rats (McCormick et al., 1998). The effects of neo-GDX on HPA function that we observed were restored with neonatal testosterone treatment but not with adult testosterone replacement. Based on our previous GDX and dose-replacement experiments (Viau and Meaney, 1996), the adult testosterone replacement levels achieved in the present study were more than sufficient to reverse the stimulatory effects of GDX on HPA function in adults. Importantly, it has been previously demonstrated that neo-GDX animals show similar elevations in HPA responses to stress regardless of testosterone (Libertun and Lau, 1972; McCormick et al., 1998). Thus, because we did not study adult animals without testosterone replacement, we probably underestimated the capacity of neo-GDX animals to show reduced HPA responses to testosterone. Nonetheless, relative to neo-sham and neo-GDX + T-treated animals, HPA activity was significantly higher in neo-GDX animals despite similar levels of adult testosterone replacement.
CRH and AVP expression in the PVN did not vary as a function of neonatal treatment. However, basal and stress-induced levels of corticosterone varied strongly and positively with the number of Fos-positive cells in the mpd part of the PVN. These findings provide a strong indication that neonatal testosterone can organize the cellular activation of PVN neurons governing ACTH release under basal and stress conditions. Seale et al., (2005) recently demonstrated elevated CRH and AVP mRNA levels in animals that received either the AR antagonist flutamide or the aromatase inhibitor ATD during the perinatal period. This discrepancy with our findings may be explained by normal androgenic and estrogenic influences exerted before our postnatal manipulation (Baum et al., 1988; Weisz and Ward, 1980). Thus, there are likely several critical periods over which testosterone and its metabolites are capable of organizing and operating on different aspects of the HPA system, including ACTH secretagogue synthesis and release and, as our current findings suggest, afferents supplying androgen-sensitive input to the PVN.

2.4.1 Mechanisms to explain changes in AR and AVP

Neuronal cell numbers in the posterior BST and medial amygdala are greater in adult male rodents than in females, and testosterone contributes to this sex difference. Neonatal GDX results in a decrease in neuronal soma size and cell numbers in the posterior BST of adult males, and this testosterone-reversible effect is most conspicuous in the vicinity of the principal nucleus (reviewed in de Vries and Panzica, 2006, and see Hines et al., 1992). The sex difference in neuronal cell numbers in the MeA is most apparent with the posterodorsal part of the nucleus and likewise appears to be organized by testosterone, at least before adulthood (Morris et al., 2008). As we observed, neonatal GDX ± T altered AVP expression within the vicinity of the transverse and intrafascicular nuclei of the posterior BST (Fig 2.4)
and in the anterodorsal part of the MeA (Fig. 2.6), regions least associated with testosterone- and gender-dependent differences in neuronal volume and cell number. Effects on AR-ir cell numbers were also localized, but not limited to, these same regions of the BST and MeA (Figs. 2.7 and 2.8). Taken together, the neurotrophic effects of testosterone that occur during the neonatal period remain an important consideration; however, the group differences in AVP expression and AR staining are unlikely to be explained solely by developmental influences on neuronal cell number.

Our findings could also reflect a change in the ability of existing neurons to express the AR. A shift in the capacity of these neurons to register and respond to circulating levels of testosterone is also possible, considering that androgens often regulate the synthesis of their own receptors (Xiao and Jordan, 2002). Because we detected no differences in AR mRNA, regional differences in AR-ir detection could be explained by site- and cell-specific differences in testosterone metabolism and, ultimately, AR and mRNA stability (see Lu et al., 1999). In addition, several AR coregulators have been identified as candidate molecules capable of influencing the stability of the AR ligand complex and nuclear translocation (reviewed in Heinlein and Chang, 2002). Thus, local mechanisms regulating the capacity of cells to register circulating levels of testosterone might explain why neo-GDX animals displayed less AR staining in the cell nucleus and functionally lower HPA and central AVP responses to adult testosterone replacement.

2.4.2 Mechanisms to explain changes in the adult HPA response to testosterone

The strong associations observed between AR-ir cell numbers, plasma corticosterone, and intervening levels of Fos in the PVN (see Table 1.1) suggest that the posterior BST and MeA are important candidate mediators of the developmental effects of testosterone on HPA
function in adulthood. The posterodorsal part of the MeA and the posterior division of the BST, including the principle, intrafascicular, and transverse subnuclei, are among several forebrain regions with the highest densities of neurons that express androgen and estrogen receptors (Shughrue et al., 1997; Simerly et al., 1990). The posterior BST sends robust projections to the PVN region, toward the medial parvocellular part of the nucleus (Dong and Swanson, 2004), and to the PVN surround, targeting several cell groups that are in a position to integrate, albeit indirectly, input from the limbic forebrain, including the prefrontal cortex, lateral septum, MeA, and ventral subiculum (reviewed in Williamson and Viau, 2007).

Lesions in the vicinity of the posterior division of the BST and the dorsal aspect of the MeA indicate that both of these structures are capable of providing inhibitory inputs to the PVN (Choi et al., 2007; Dayas et al., 1999). Superimposing our current findings onto this functional and connectional data, we propose that neonatal testosterone primes the adult HPA response to testosterone by defining the number of ARs within the posterior BST and MeA. Although our previous connectional studies show a high containment of ARs within PVN projecting nuclei in the posterior BST, and a small contingent in the MeA that projects to the perinuclear region of the PVN (Williamson and Viau, 2007), the extent to which neonatal testosterone alters ARs within these PVN afferents remains to be confirmed.

The group differences in the number of AVP-expressing cells in the BST and MeA confirm several previous reports showing that testosterone stimulation of AVP expression within these regions depends on the level of testosterone exposure during the neonatal period (reviewed in de Vries and Panzica, 2006). AVP mRNA expression and synthesis in the BST and MeA, as well as their projections, are extremely dependent on testosterone levels in adult males (reviewed in de Vries and Panzica, 2006 and Kalsbeek et al., 2002). Despite similar
adult testosterone replacement levels, however, neo-GDX rats expressed lower levels of AVP mRNA in both the BST and MeA (Figs. 2.3 and 2.6, respectively). This could reflect a loss in testosterone responsiveness within existing AVP neurons, at least in the MeA. In addition to the differences in the number of cells that express AVP, neo-GDX animals showed less labeling per cell in the MeA (Figs. 2.6 and 2.7). In the BST, our analyses suggest that alterations in AVP mRNA expression are produced by changes in the number of neurons expressing the transcript and not by changes in the amount of AVP mRNA expressed per cell. The dense packing of AVP-expressing neurons in the posterior BST, particularly within the vicinity of the intrafascicular and transverse nuclei, could have impacted our ability to detect neonatal-dependent changes in the capacity of individual neurons to express AVP. Otherwise, the testosterone-dependent organization of AVP neurons in the BST and MeA may be mediated by distinct mechanisms.

In line with this possibility, neo-GDX + T-treated animals showed region-specific differences in their cellular responsiveness to adult testosterone replacement. In the BST, neonatal testosterone treatment restored the number of neurons expressing the AVP transcript. In the MeA, neonatal testosterone treatment likewise restored the number of AVP cells, but not the capacity of individual cells to express AVP mRNA (Fig. 6). Previous labeling studies in gonadal-intact, adult male rats indicate that virtually all AVP neurons in the BST and MeA colocalize with androgen and estrogen receptors in the BST and MeA (reviewed in de Vries and Panzica, 2006). Based on our current findings, however, we are compelled to conclude that in contrast to the MeA, the AVP response to adult testosterone in the BST may be more evenly coupled to changes in ARs and/or similar periodic influences of testosterone. What should be emphasized at this point is that the animals used in the current
study were robbed of testosterone exposure up to the age of 45 d. During this time in male rodents, major age-related increases in AVP mRNA, AVP-ir, and AR cell numbers in the BST and MeA occur, attributed to an increase in plasma testosterone (Gomez et al., 2004; Romeo et al., 2000). Furthermore, a recent report suggests that puberty is also met by a testosterone-dependent increase in neurogenesis in the MeA (Ahmed et al., 2008). Thus, the differential AVP cellular responses in the MeA may have been shaped by the absence of testosterone exposure over the course of pubertal development.

Notwithstanding these caveats, we found several correlations in support of our hypothesis that the limbic system may be responsible for organizing the adult HPA response to testosterone and that AVP may be a key contributor to this response, at least within the posterior BST (see Table 2.1). We are not the first to make this association between central AVP and HPA function. Gomez et al. (2004) identified strong negative relationships between the number of AVP-positive cells in the BST and the capacity of mpc neurons in the PVN to express CRH- or AVP-ir. Although these studies were performed in the context of puberty, steady-state and stress-induced levels of AVP in the BST were also shown to vary as a function of resting and stress-induced levels of testosterone between pre- and postpubertal animals. Taken together with our findings, changes in AVP expression and its regulation by testosterone in the BST could signify a means by which the central and organizing influences of testosterone are transmitted to the PVN. Compared with the BST, the MeA is functionally at least one or several steps removed from the PVN, which might explain why we were unable to detect significant correlations between AVP cell numbers in this region and basal and stress-induced indices of HPA function.
2.4.3 Conclusion and future considerations

We have shown that neonatal testosterone exerts profound effects on the HPA response to testosterone during adulthood. The extent to which this is executed by changes in AVP and AR within the same cells and/or represented within unique projections to the HPA axis requires further clarification. As discussed earlier, neonatal gonadectomy exerts remarkable changes in adult brain morphology, including effects on neuronal soma size and cell numbers within subregions of the posterior BST and the MeA. This could have an important bearing on the extended circuitries of the PVN, including its functional connectivity with the limbic system. Using an anterograde tract-tracing tracer approach, Gu et al. (2003) showed that neonatal gonadectomy can decrease the projection densities of the principal nucleus of the posterior BST, at least within the anteroventral periventricular nucleus and the ventral premammillary nucleus of the hypothalamus. Thus, future studies employing a retrograde tracer injection approach in the PVN could answer whether neonatal androgens are capable of altering the number of neurons targeting the PVN directly as well as their containment of ARs and AVP. Because the group differences in the number of AR cells were several orders of magnitude higher than the number of AVP cells, we anticipate that there are likely several androgen-sensitive, candidate neurotransmitters driving the organizational actions of testosterone. Nonetheless, the group difference in AVP responsiveness to adult testosterone replacement provides some indication that AR function may be altered by neonatal testosterone exposure.

A clear majority of studies have shown that the organizing effects of testosterone are mediated, in large part, by its conversion to estrogen (reviewed in Baum, 2003). Furthermore, testosterone and estrogen interact on the central regulation of AVP during adulthood (Brot et
al., 1993, De Vries et al., 1994) as well as during the neonatal period (Kuhnemann et al., 1995). Thus, in addition to ARs, the organization of the adult HPA response to testosterone probably involves important changes in estrogen receptor expression and function as well as cellular and region-specific differences in aromatase activity (Balthazart, 1997). Based on the strength of our current findings, we now have an anatomical and functional framework for exploring these possibilities.
Chapter 3: Postnatal blockade of androgen receptors or aromatase impair the expression of stress hypothalamic-pituitary-adrenal axis habituation in adult male rats

3.1 Introduction

The perinatal period is a critical time of brain growth, development and sexual differentiation. Due to the complexity of developmental processes and the requirement for precise cues and timing for the establishment of neural circuits, the fetus is extremely vulnerable to insults and concomitant changes in hormonal environment during this time. Manipulations that alter environmental variables, maternal–infant interactions, and the glucocorticoid and/or sex steroid hormone environment during the neonatal period permanently alter the magnitude of the hypothalamic-pituitary-adrenal (HPA) axis response to acute stress (Arai and Gorski, 1968; Levine and Mullins, 1967; McCormick et al., 1998; Raisman and Field, 1971). Male rats experience a surge in testosterone that occurs on days 18–19 of gestation (Weisz and Ward, 1980) and again during the first few hours of birth (Baum et al., 1988). During both of these periods, testosterone has been shown to exert remarkable changes in brain morphology, behavior and HPA function. Several environment factors, including maternal stress and fetal alcohol exposure, have also been shown to influence both the onset and duration of testosterone exposure during the perinatal period and to permanently alter the HPA axis (McGivern et al., 1993; Ward et al., 2003). These findings

---

2 This chapter has been previously published:
underscore the importance of understanding how testosterone activity during critical periods may affect the development and maturation of the HPA axis.

Circadian- and stress-induced increases in HPA activity involve the sequential release of different classes of hormones from the hypothalamus to the adrenals. Activation of HPA effector neurons in the paraventricular nucleus (PVN) of the hypothalamus triggers the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from their terminal stores in the media eminence. At the level of the anterior pituitary corticotroph, these neuropeptides stimulate the release of ACTH, which in circulation stimulates the de novo synthesis and release of glucocorticoid steroid hormones from the adrenals (cortisol in humans, corticosterone in the rat).

The HPA axis provides a frontline of defense against threats to homeostasis (i.e. stress). Acute elevations in glucocorticoids are adaptive, as they meet the metabolic demands of stress critical for survival. Under normal conditions there are mechanisms in place that dampen the secretion of glucocorticoids in the face of repetitive, but non-life-threatening stimuli (McEwen, 1998b). Stress-induced elevations in ACTH and corticosterone release decline over repeated exposures to the same stimulus. The process of HPA habituation is adaptive as it limits the detrimental effects of chronic elevations in circulating glucocorticoids, while allowing the system to remain fully responsive to new challenges (Dallman, 2007).

During development rapid increases in sex steroid hormones permanently alter, or organize, the brain and behavior, while sex steroids in adulthood act to reversibly modulate, or activate, physiology and behavior (Ward et al., 2003). In a fully developed male nervous system the magnitude of the HPA response to acute stressors varies strongly and negatively
with individual differences in plasma testosterone concentrations. Manipulations of the gonadal axis by castration and testosterone replacement in adult male rodents indicate that the main effect of testosterone is to decrease the magnitude of the ACTH and corticosterone release, at least under acute stress conditions (reviewed in Williamson et al., 2005). Likewise, testosterone exposure during critical periods of development influences both the magnitude of the HPA response to stress and the capacity of the HPA axis to respond to testosterone during adulthood (McCormick et al., 1998). Male rats that are castrated within 16 h of birth show higher numbers of PVN neurons recruited to express Fos protein and corticosterone responses to stress as adults (Bingham and Viau, 2008). Although these effects are reversible with neonatal testosterone replacement, they are not reversed with adult testosterone replacement (Bingham and Viau, 2008; McCormick et al., 1998).

The organizing influence of testosterone on the adult HPA axis may be dependent on both androgen receptors (ARs) and estrogen receptors. In addition to ARs (Lieberburg et al., 1980), the newborn male rat brain has functional estrogen receptors (MacLusky et al., 1979) and high levels of the cytochrome P450 enzyme, aromatase (Shinoda et al., 1994). Male rats treated with either the AR antagonist flutamide or the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) from prenatal day 13 to postnatal day 20 show elevations in the mean level and amplitude of corticosterone release under basal conditions, as well as in response to acute auditory and immune challenges (Seale et al., 2005). While it is clear that early exposure to sex steroid hormones influences the development and sexual differentiation of the HPA axis under acute stress conditions, how they influence the process of stress HPA habituation in response to repeat, intermittent stress has yet to be considered.
We hypothesized that manipulating testosterone exposure in the postnatal period would alter HPA habituation to repeated stress. Thus, in the current study we examined HPA responses to repeated restraint stress in adult male rats that received subcutaneous capsules containing the antiandrogen, flutamide, or the aromatase inhibitor, ATD, introduced within 12 h of birth and removed on day 21 of weaning. A group of adult, gonadectomized male rats was also included to distinguish between the organizational and activational effects of testosterone.

3.2 Methods

3.2.1 Postnatal flutamide and ATD treatment

Sprague–Dawley rats were mated at the South Campus Animal Care Facility at the University of British Columbia, where they were housed in a controlled environment with a 12:12 light/dark cycle, lights on at 06:00 h, with food and water available ad libitum. Dams were pre-conditioned for pup removal by exposing them to a piece of latex glove laced with alcohol and Vetbond every day of gestation. Within 12 h of birth, 3 male Sprague–Dawley pups per litter were subcutaneously implanted with Silastic capsules (i.d. 1.5 mm, o.d. 2.1 mm, length 5 mm) that were either blank (Blank), packed with crystalline flutamide to block ARs, or crystalline 1,4,6-androstatriene-3,17-dione (ATD) to inhibit the conversion of testosterone to estrogen. A single animal from each litter that received no surgery served as an additional control (untreated). Using a microchip trocar, implants were inserted into the subcutaneous space along the midline just caudal to the shoulder blades, and the skin was closed using a drop of tissue glue. The capsules were removed on day 21 of weaning, as puberty also represents a critical period for the sexual differentiation of the brain (Schulz et al., 2009a), including the HPA axis (Gomez et al., 2004) and (McCormick and Mathews,
2007). Animals were also weaned at 21 days of age and delivered to our animal housing facility, where they were housed two per cage under controlled temperature and lighting conditions (12-h light, 12-h dark cycle; lights on at 06:00 h), with food and water available 

*ad libitum.* All experimental protocols were approved by the University of British Columbia Animal Care Committee.

To reveal an activational influence of the testes on stress-HPA habituation, a subset of control male rats were bilaterally gonadectomized (adult-GDX) at 60 days of age. Testes were removed via a scrotal incision under ketamine–xylazine–acepromazine anesthesia (77:1.5:1.5 mg/ml, respectively; 1 ml/kg sc). Each testis was delivered separately through the scrotal incision and exteriorized by severing the vas deferens and spermatic artery, which was ligated to maintain homeostasis. Gonadectomy was completed by closing the scrotal incision with 4–0 non-absorbable sutures. All other groups (untreated, Blank, flutamide and ATD) received sham gonadectomy at 60 days of age, which was achieved by clipping and suturing the scrotum under ketamine-xylazine-acepromazine anesthesia. Rats were adapted to handling and daily weighing (08:00 h) for 7 days prior to stress testing.

### 3.2.2 Blood collection

Starting at 65 days of age, rats were subjected to a repeated form of psychogenic stress, which involved 10 single exposures to 30 min of restraint, repeated daily. Employing a repeated measure design, individual rats were tail bled at 0, 30 and 60 min from the onset of restraint on the first day, and then sampled once again on the tenth day of restraint. Blood samples were obtained by tail nick immediately after the rat was placed in a restrainer (6.3 cm × 15 cm Plexiglas restrainer, Kent Scientific, Litchfield, CT), at 30 min of restraint exposure, and 30 min after return to the home cage. Blood samples (300 µl) were collected in
chilled EDTA (3.75 mg/100 µl of blood) and Aprotinin (100 KIU/100 µl of blood) treated Eppendorf tubes, centrifuged at 3000 × g for 20 min and stored at −80 °C until assayed.

### 3.2.3 Tissue collection and microscopy

Twenty four hours after the last restraint session, rats were anesthetized for perfusion using a lethal dose of chloral hydrate (700 mg/kg, intraperitoneal), and sequentially perfused via the ascending aorta with 0.9% saline and then 4% paraformaldehyde (pH 9.5) at 4 °C, delivered over 5 and 20 min, respectively, at a flow rate of 20–25 ml/min. Brains were post-fixed 4 h and cryoprotected overnight with 10% sucrose in 0.1 M potassium phosphate buffered saline (KPBS) pH 7.3 prior to slicing. Regularly spaced series of 100 µm-thick sections through the rostrocaudal extent of the hypothalamus were collected and stained with thionin to validate treatment effects on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA).

Bright-field images were captured using a Retiga 1300 CCD digital camera (Q-imaging, Burnaby, BC), and analyzed using Macintosh OS X-driven, Open Lab Image Improvision v. 3.0.9 (Quorum Technologies, Guelph, ON) and Image J v. 1.3.5 software (NIH, Bethesda, MD). An investigator blind to each animals treatment group traced cross-sectional areas within individual serial sections using a 5× objective, compiled to reconstruct the total volume of the SDN-POA using Open Lab software.

### 3.2.4 Maternal behavior

Variations in maternal care during the early postnatal period have been shown to impact neuroendocrine responses to stress in adult male offspring (Meaney and Szyf, 2005a, b). To validate that our surgical manipulations were not causing a shift in maternal care, we compared the maternal behavior of cohorts of lactating female rats with litters that contained
experimentally manipulated pups to those bearing unhandled control animals. The behavior of each dam was observed for three 10 min daily observation periods in undisturbed litters from days 3 to 6 postpartum. Observations occurred at regular times each day, with three periods during the light (08:00, 11:00, and 14:00 h) phase of the light/dark cycle. Within each 10 min observation period, the behavior of each mother was scored every 5 s, equaling 120 observations per mother each day to determine the total durations for each of the following behaviors (Champagne et al., 2001; Pawluski et al., 2006): licking/grooming (body licking and genital licking with the dam off the pups); licking/grooming and nursing; arched-back nursing; “blanket” nursing; passive nursing; and time off pups. All licking behaviors (licking/grooming, licking/grooming and nursing) were summed to create a single licking/grooming variable as previously described (Liu et al., 2000).

A concerted effort was also made to minimize litter disruption and to control for between-litter effects in maternal care and nutritional load (Lesage et al., 2002). First, only three male rats per litter were experimentally manipulated. Second, total litter size in all cases was maintained at 12 pups per litter. Third, litters remained intact and undisturbed until weaning at 21 days of age. A subset of unhandled, non-experimental animals served as a control for surgery and handling effects. After weaning, animals from each treatment group were pair housed for the remainder of the experiments. Finally, each study used a minimum of six litters per treatment and employed 1 experimental animal per litter for any given test as a single measure, empirically controlling for litter effects (Abbey and Howard, 1973).

3.2.5 Plasma hormones

Plasma testosterone (25 µl), corticosterone (5 µl, diluted 1:200 as per kit instructions), and ACTH (50 µl) concentrations were measured using commercial RIA kits (MP
Biomedical, Solon, OH). For corticosterone, the plasma samples were diluted 1:100 and 1:200 for prestress and poststress time intervals, respectively, to render hormone detection within the linear part of the standard curve. The intra- and interassay coefficients of variation for all of the assays typically ranged from 3 to 6 and 10 to 12%, respectively, and I-125 labeled ligands were used as tracer in all cases. The testosterone antibody cross-reacts 100% with testosterone and slightly with 5α-dihydrotestosterone (3.40%), 5α-androstane-3β,17β-diol (2.2%), and 11-oxotestosterone (2%). The standard curve ED50 for the testosterone RIA was 1.2 ng/ml, with a detection limit of 0.1 ng/ml. The corticosterone antibody cross-reacts 100% with corticosterone and slightly with desoxycorticosterone (0.34%), testosterone, and cortisol (0.10%), but does not react with the progestins or estrogens (<0.01%). The standard curve ED50 for the corticosterone RIA was 17 µg/dl, with a detection limit of 0.625 µg/dl. The ACTH antibody cross-reacts 100% with ACTH1–39 and ACTH1–24, but not with β-endorphin, α- and β-melanocyte-stimulating hormone, and α- and β-lipotropin (all <0.8%). The standard curve ED50 for the ACTH RIA was 82 pg/ml, with a detection limit of 20 pg/ml.

3.2.6 Statistics

A three-way mixed (between treatment, within subject) design ANOVA was used to analyze hormone responses using restraint and time as repeated measures. When interactions were found to be significant ($P < 0.05$) or approached significance ($P < 0.1$), further comparisons were made using a two-way mixed design ANOVA using time as the repeated measure to analyze hormone responses on the first and last day of restraint. To gauge treatment effects on HPA habituation, total hormone responses from baseline were determined with the trapezoidal rule (Pruessner et al., 2003), and the data expressed as area
under the curve were analyzed using a two-way mixed design ANOVA, using day of restraint as the repeated measure. The size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in adult males is determined by sex hormone exposure early in development (Jacobson et al., 1981), and is reduced in size by neonatal treatment with flutamide (Lund et al., 2000) and ATD (Houtsmuller et al., 1994). Therefore, we compared the size of the SDN-POA in the same animals used for the repeated stress study to independently assess the efficacy of flutamide and ATD treatment, analyzed by one-way ANOVA. When appropriate, post hoc pairwise comparisons in all cases were made using Newman-Keuls. Maternal behaviors between dams with and without manipulated pups were compared using unpaired t-tests.

3.3 Results

3.3.1 Maternal behavior

Between dams with experimentally manipulated and unhandled controls pups, there was no significant differences in the duration of any of the maternal behaviors scored (see Table 3.1), including arched-back nursing \([F_{(1,6)} = 0.110, P = 0.75]\), blanket nursing \([F_{(1,6)} = 0.72, P = 0.80]\), passive nursing \([F_{(1,6)} = 0.054, P = 0.82]\), time off pups \([F_{(1,6)} = 0.144, P = 0.72]\), and licking and grooming behaviors combined \([F_{(1,6)} = 0.125, P = 0.74]\).
Table 3.1. Mean ± SEM duration (sec) of individual maternal behaviors in dams with experimental or control pups.

<table>
<thead>
<tr>
<th>Maternal Group</th>
<th>Experimental</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arched-back Nursing</td>
<td>956 ± 377</td>
<td>813 ± 209</td>
</tr>
<tr>
<td>Blanket Nursing</td>
<td>1746 ± 199</td>
<td>1812 ± 148</td>
</tr>
<tr>
<td>Passive Nursing</td>
<td>718 ± 370</td>
<td>594 ± 378</td>
</tr>
<tr>
<td>Licking/Grooming</td>
<td>733 ± 135</td>
<td>829 ± 237</td>
</tr>
<tr>
<td>Time off pups</td>
<td>671 ± 156</td>
<td>764 ± 189</td>
</tr>
</tbody>
</table>

3.3.2 Efficacy of flutamide and ATD treatment

A significant effect of treatment \([F_{(3, 20)} = 33.70, P < 0.0001]\) was revealed for the volume of the SDN-POA. Mean volumes estimates of the SDN-POA for each treatment group are presented in Figure 3.1. Post hoc analysis confirmed that adult rats treated with flutamide and ATD at birth had SDN-POA volumes that were significantly smaller than control and adult-GDX animals.

3.3.3 Plasma hormone responses

3.3.3.1 Untreated and blank controls

To validate that animals bearing blank implants could serve as appropriate controls, we first compared adult plasma hormone (testosterone, ACTH, and corticosterone) responses under acute and repeat stress conditions between sham-surgical, untreated animals and those receiving blank Silastic implants at birth. For testosterone, ACTH, and corticosterone, there were no significant three-way (stress × treatment × time), or two-way interactions between blank and untreated control animals \((P > 0.5 \text{ in all cases})\). For both ACTH and corticosterone, there was a significant interaction between day of restraint and time \((P < 0.0001)\). Post hoc analyses confirmed, as expected, that the magnitude of the
Figure 3-1. The volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) decreases in response to neonatal treatment with 1,4,6-androstatriene-3,17-dione (ATD) and flutamide (FLU), but not with adult gonadectomy (adult-GDX). Brightfield photomicrograph illustrating the size and location of the SDN within the preoptic area (left). Structures labeled for reference: ac, anterior commissure; ot, optic tract. Volume of the SDN-POA mean ± SEM in blank, FLU, ATD and adult-GDX animals (right). **P < 0.01 vs. blank and adult-GDX animals (n = 12/group).
ACTH and corticosterone responses were significantly lower on the last day than on the first day of restraint exposure ($P < 0.01$) for both untreated and blank controls. Taken together, as there were no significant differences between untreated animals and those bearing blank implants for all hormones of interest, only neonatal pups that received blank implants were used as controls for subsequent comparisons of flutamide and ATD treatment effects.

### 3.3.3.2 Testosterone

The magnitude of the HPA response to acute stress varies strongly and negatively as a function of prestress levels of testosterone in circulation (Viau and Meaney, 1996). Therefore, basal testosterone levels (time 0) were measured on the first and tenth day of repeated restraint (see Table 3.2). There were no significant effects of treatment [$F(2, 15) = 1.51; P = 0.25$] and stress [$F(1, 2) = 0.50; P = 0.49$], and no significant interaction between treatment and stress [$F(2, 15) = 0.11; P > 0.05$].

**Table 3.2.** Mean ± SEM plasma testosterone concentrations (ng/ml) at 0 min of restraint under acute and repeat stress conditions in adults bearing blank, flutamide, or ATD implants at birth.

<table>
<thead>
<tr>
<th>Stress Group</th>
<th>Acute</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>1.00 ± 0.22</td>
<td>1.03 ± 0.11</td>
</tr>
<tr>
<td>Flutamide</td>
<td>2.10 ± 0.46</td>
<td>1.48 ± 0.23</td>
</tr>
<tr>
<td>ATD</td>
<td>1.02 ± 0.58</td>
<td>1.12 ± 0.23</td>
</tr>
</tbody>
</table>
3.3.3.3 Adrenocorticotropicin

A three-way ANOVA revealed significant interactions between treatment and time \([F(6, 40) = 6.40; P < 0.0001]\), restraint and time \([F(2, 6) = 35.75; P < 0.0001]\), and a marginal interaction between restraint and treatment \([F(3, 20) = 2.39; P = 0.09]\). Two-way ANOVAs revealed significant interactions between treatment and time on the first \([F(6, 40) = 4.56; P = 0.001]\) and last day \([F(6, 40) = 5.14; P = 0.0005]\) of restraint exposure. On both the first and last day of restraint, neonatally ATD treated and adult-GDX rats had larger ACTH responses (time 30) than control rats and those treated with flutamide (Figure 3.2, top).

To determine the basis for the restraint and treatment interaction, the total area under the curve (AUC) for the ACTH responses at 30 and 60 min (different from 0 time) were calculated for the first and last day of restraint exposure. As illustrated in Figure 3.3 (panel A), the absolute decline in ACTH responses was significant for all groups \((P \leq 0.007\) in all cases). However, as a percentage of the total response on the first day of restraint (Figure 3.3, panel B), on the last day of restraint adult-GDX, ATD, and flutamide treated rats displayed smaller declines in ACTH responses \((60.9 \pm 6.8, 63.3 \pm 2.9, 62.9 \pm 7.2\%\), respectively) than control animals \((85.2 \pm 2.6\%\).

3.3.3.4 Corticosterone

A three-way ANOVA revealed significant interactions between treatment and time \([F(6, 40) = 5.51; P = 0.003]\), restraint and time \([F(2, 6) = 15.04; P < 0.0001]\), restraint and treatment \([F(3, 20) = 5.22; P = 0.09]\), and the three-way interaction between main effects was marginally significant \([F(6, 40) = 2.29; P = 0.0537]\). Two-way ANOVAs revealed significant interactions between treatment and time on both the first \([F(6, 40) = 3.27; P = 0.01]\) and last day \([F(6, 40) = 5.84; P = 0.0002]\) of restraint exposure. On the first day of restraint (Figure 3.2,
bottom), ATD treated and adult-GDX rats had larger corticosterone responses (time 30) than control rats and those treated with flutamide. On the last day of restraint, moreover, adult-GDX rats and those treated with ATD and flutamide had larger corticosterone responses (time 30) than control animals (Figure 3.2, bottom).

Comparisons of the total area under the curve (AUC) for the corticosterone responses at 30 and 60 min (different from 0 time) for the first and last day of restraint exposure revealed a significant interaction between treatment and day of restraint ($P = 0.01$). As indicated in Figure 3.3 (panel C), the absolute decline in corticosterone responses was significant for adult-GDX ($P < 0.001$) and control rats ($P = 0.002$), but not for those treated with ATD ($P = 0.237$) and flutamide ($P = 0.141$). As a percentage of the total response on the first day of restraint (Figure 3.3, panel D), on the last day of restraint ATD and flutamide treated rats displayed smaller declines in corticosterone responses (13.8 ± 12 and 16.4 ± 8.9%, respectively) than control and adult-GDX rats (57.5 ± 9.2 and 47.5 ± 5.2%, respectively).
Figure 3-2. Plasma ACTH (top) and corticosterone (bottom) on the first (acute) and last day of restraint (repeat) under basal (0 min), stress (30 min) and recovery conditions (60 min) to show main effects of treatment on the magnitude of responses (mean ± SEM). *P < 0.05 vs. blank at 30 min (n = 6/group).
Figure 3-3. ACTH (A) and corticosterone (C) total hormone responses from baseline (mean ± SEM area under the curve) to show a main effect of repeated restraint. *P < 0.05 vs. acute response (n = 6/group). Total hormone responses on the last day of restraint as a percentage of the first day of restraint to show main effects of treatment on ACTH (B) and corticosterone (D) decline. *P < 0.05 vs. blank (n = 6/group).
3.4 Discussion

Studies examining early sex steroid hormone influences on the development of the HPA axis have focused primarily on glucocorticoid responses to acute or single challenges only (Bingham and Viau, 2008; McCormick et al., 1998; Patchev et al., 1999; Seale et al., 2005). While the magnitude of the glucocorticoid hormone response certainly contributes to pathology, how the organism adapts to stress may ultimately determine its fate. We used a model of repeated psychogenic stress to determine the organizational and activational impact of testosterone on HPA habituation in adulthood. The results of the current study suggest that both the activation of ARs and the conversion of testosterone to estrogens during the neonatal period in males are essential for the expression of stress HPA habituation to occur during adulthood. In contrast, neonatal estrogens appear to be solely responsible for the majority of the organizing effects of testosterone on the acute stress response.

By comparing HPA responses within individual animals under acute and repeat restraint conditions, we found that neonatal flutamide and ATD treatment reduced the decline in the magnitude of the ACTH response to repeated restraint, and completely prevented the normal decline in corticosterone. Similar to flutamide and ATD treatment, animals castrated (GDX) as adults showed a reduced capacity for the ACTH response to habituate. However, unlike those treated with flutamide and ATD, the repeated stress induced decline in plasma corticosterone responses in adult GDX animals was comparable to those receiving blank implants at birth. Taken together, neonatal flutamide and ATD altered the normal declines in ACTH and corticosterone responses to repeated restraint, whereas the main and largest effect of adult GDX was to increase the magnitude of the ACTH and corticosterone responses under acute and repeat stress conditions.
From the preceding, it is important to consider the nature by which the organizing influences of testosterone on HPA habituation occur, if at all, independently of its activational effects. In adult males with testes, the magnitude of the HPA response to stress is negatively related to plasma testosterone concentrations (Viau et al., 1996). Conversely, adult GDX causes a testosterone-reversible increase in ACTH and corticosterone responses to stress (Handa et al., 1994b; Viau and Meaney, 1996). Based on our current findings in adult GDX animals, we suggest that variations in testosterone in adulthood do not affect the process of HPA habituation, but nevertheless operate on the stress-induced drive to the HPA axis to regulate the amplitude of ACTH and corticosterone responses (Viau et al., 2003). Thus, in so far as castration represents the removal of testosterone's activational effects, the current findings reflect a permanent requirement for neonatal, but not adult testosterone to alter the process of stress habituation. In other words, the capacity of animals to express HPA habituation is determined prior to adulthood, independently of testosterone's activational effects on the HPA axis.

In addition to failing to habituate to repeated restraint, ATD treated animals also had higher ACTH and corticosterone responses to acute restraint exposure. This confirms previous work demonstrating an important influence of perinatal aromatase to organize the magnitude of the HPA response to acute stress challenges (Seale et al., 2005). However, unlike previous studies of adult offspring treated with flutamide from prenatal day 13 to postnatal day 20 (McCormick and Mahoney, 1999), we found no enduring effect of postnatal flutamide treatment on ACTH and corticosterone responses under acute stress conditions. These results remain consistent with several previous studies demonstrating that estrogens are primarily responsible for masculinizing the rodent brain (reviewed by Cooke et al., 1998).
Volume estimates of the SDN-POA, determined in the same animals subjected to repeated restraint, confirmed demasculinizing effects for both flutamide and ATD treatment (Houtsmuller et al., 1994; Lund et al., 2000). Further, our implant approach effectively avoided disruptions to maternal behavior and care. Taken together with our current findings, we propose that the masculinization of the HPA axis, at least with respect to acute stress, is affected by testosterone and ARs prior to parturition, and by estrogens throughout the perinatal period.

Nevertheless, our findings remain in keeping with an overlapping influence of testosterone and aromatase to permanently alter HPA habituation. The nature of this influence might be represented within converging cellular pathways. Aromatase is found within many sexually differentiated regions of the neonate brain, and both AR activation and testosterone are positive regulators of aromatase (Zhao et al., 2007). Further, pharmacological studies have shown that antagonism of ARs decreases the accumulation of testosterone within target cells (McGinnis and Mirth, 1986), reducing the substrate available for aromatase and subsequent formation of estrogens. Therefore, the impact of neonatal flutamide to alter the process of stress HPA habituation in adults likely includes effects on estrogen exposure, in addition to processes specific to AR activation.

As several others have suggested, the decline in the magnitude of the ACTH and corticosterone responses to repeated aversive stimuli reflect the net influence of stimulatory drive and glucocorticoid mediated negative feedback regulation of the HPA axis (Dallman et al., 1987b). Current evidence would suggest that the latter represents a candidate mechanism for testosterone's organizing influence on HPA habituation. Perinatal flutamide and ATD treatment, as well as postnatal GDX in males, decrease the expression and binding capacity
of glucocorticoid (GR) and mineralocorticoid receptors (MR) in the pituitary, PVN, and hippocampus (McCormick et al., 1998; Patchev et al., 1999; Seale et al., 2005). Each of these brain regions have been shown to mediate glucocorticoid negative feedback regulation of the HPA axis under basal and stress conditions (Herman and Cullinan, 1997). Interestingly, the effectiveness of a MR antagonist to increase corticosteroid responses is greater in animals exposed to repeated restraint than those exposed for the first time (Cole et al., 2000). Further, blockade of both MR and GR in the paraventricular nucleus of the thalamus on successive days of restraint exposure reduces the level of ACTH habituation (Jaferi et al., 2006). Thus, we predict that both neonatal testosterone and its estrogen metabolites are required for the development of the male's dependency on MR and GR receptors to regulate the expression of HPA habituation.

Another possible mechanism is revealed by the dissociations seen between treatment effects on stress-induced ACTH and corticosterone release. While neonatal flutamide and ATD treatment slightly, but reliably attenuated the normal decline in ACTH responses to repeated restraint, even greater departures were observed for corticosterone. Strikingly, there was no significant decline in corticosterone responses between the first and last day of restraint in flutamide and ATD treated animals. In fact, relative to the first day of restraint, half of the animals within each treatment group showed an absolute increase in corticosterone secretion during the last day of restraint. The basis for the dissociation between ACTH and corticosterone may rest within central autonomic pathways that serve to alter adrenal sensitivity to ACTH or to regulate adrenocortical steroidogenesis independently of ACTH (Bornstein et al., 2008). In line with the latter possibility, corticosterone production is altered in adrenal slices or homogenates obtained from hypophysectomized, pubertal male
rats given testosterone (Kitay et al., 1966). Moreover, there is evidence to suggest a stimulatory influence of estrogen, at least in female rats, to promote the synthesis and/or release of corticosterone independently of ACTH (Figueiredo et al., 2007). Although there was no effect of neonatal flutamide and ATD on adult testosterone levels, we cannot rule out possible treatment effects on the metabolism of testosterone to estrogen and subsequent estrogenic effects on the adrenal regulation.

3.4.1 Implications for stress disease research and future directions

In summary, the data suggest that both the activation of ARs and the conversion of testosterone to estrogens during the neonatal period are essential for normal habituation of the HPA axis to repeated psychogenic stress. Whether this occurs by alterations in peripheral or central mechanisms to regulate the HPA axis, as discussed above, has yet to be determined. The neonatal hormone environment is extremely sensitive to environmental perturbations, and this appears to be a critical period for the development of individual differences in stress responsiveness (Meaney and Szyf, 2005a, b). There is a great deal of animal literature emphasizing the need for human work on alterations in glucocorticoid exposure in development on child and adult outcomes (Weinstock, 2008, 2005). Our current data suggest that the lasting effects of neonatal stress and normal fetal programming may ultimately depend on interactions between testosterone and glucocorticoid steroid hormones, and highlight the need for further investigation in this area (see Bergman et al., 2010).
Chapter 4: Postnatal aromatase blockade increases \(c\text{-}fos\) mRNA responses to acute restraint stress in adult male rats.³

4.1 Introduction

The paraventricular nucleus (PVN) of the hypothalamus receives multimodal information from afferent mediators of homeostasis and represents the final common pathway by which the brain regulates hypothalamic-pituitary-adrenal (HPA) axis output. The magnitude of the HPA axis response is subject to activational and organizational effects of testosterone. In adult male rats, gonadectomy causes an increase in pituitary ACTH and adrenal glucocorticoid steroid hormone responses to stress, reversible with testosterone replacement (Williamson et al., 2005). Adult animals that are gonadectomized at birth show increased corticosterone responses to acute restraint exposure (McCormick et al., 1998), in addition to enhanced Fos induction within the neurosecretory region of the PVN (Bingham and Viau, 2008). Both of these effects are reversible with neonatal, but not adult testosterone replacement.

In addition to androgens, estrogens have been shown to induce remarkable changes in brain morphology, behavior, and HPA function (McCarthy et al., 2009; Seale et al., 2005; de Vries and Panzica, 2006). The prenatal and neonatal male rat brain has high levels of estrogen receptors (MacLusky et al., 1979) and cytochrome P450 enzyme aromatase (Shinoda et al., 1994), which converts testosterone to estrogen. Perinatal or neonatal treatment with an aromatase inhibitor increases HPA output in adult males (Bingham et al.,

³ A version of this chapter has been previously published: Bingham B, Wang NX, Innala L, and Viau V (2012) Endocrinology (Feb 7. Epub).
2011 and Seale, 2005), indicating that the conversion of testosterone to estrogens is an important mechanism for the organization of the HPA axis.

Although current findings suggest a role for neonatal estrogens in altering PVN neuroendocrine output during adulthood, substantially less attention has been paid to other HPA regulating nuclei. Guided by patterns of c-fos mRNA expression induced by acute restraint, here we describe the effects of postnatal aromatase blockade on the cellular activation of the PVN and its extended circuitries in adult male rats. Limbic regions analyzed were chosen on their capacity to regulate PVN and neuroendocrine responses (reviewed in Ulrich-Lai and Herman, 2009). As per Girotti et al. (Girotti et al., 2006), we examined areas of the sensory thalamus and primary somatosensory cortex harboring tactile information from the nose and mouth region (facial) and vibrissae (barrel field). We also assessed stress-induced activation of the primary motor cortex and caudoputamen, likely engaged to drive such stress specific motor responses as struggling behavior (Grissom et al., 2008).

4.2 Methods

Sprague-Dawley rats were mated at the South Campus Animal Care Facility at the University of British Columbia and housed in a controlled environment with 12:12 light/dark cycle, lights on at 0600 h, with food and water available ad libitum. Within 12 hours of birth, 2 pups from each litter received subcutaneous Silastic implants (total length = 5 mm, inner diameter = 1.5 mm, outer diameter = 2.1 mm) that were blank, or filled with the aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (Bingham et al., 2011). A total of 6 animals were included for each treatment group. Since puberty represents another critical period for sexual differentiation of the brain and HPA axis, implants were removed on postnatal day 21.
(Gomez et al., 2004; McCormick and Mathews, 2007). After transfer to our colony room, animals from the same treatment group were housed 2 per cage, and then handled daily (0800h) for 1 week before stress testing at age 65 days. All experimental protocols were approved by the University of British Columbia Animal Care Committee.

4.2.1 Blood and tissue collection

Blood samples (300 ul) were obtained via the tail vein following removal from the home cage, or immediately following 30 minutes of restraint stress exposure using flat bottom restrainers (8.5 x 21.5 cm; Kent Scientific, Litchfield, CT). Animals were then anesthetized for perfusion under basal conditions or 30 min after termination of restraint exposure using chloral hydrate (350 mg/kg, ip). Animals were perfused (20 ml/min) with ice-cold 0.9% saline (5 min) and then 4% paraformaldehyde (20 min). Brains were post-fixed for 4 hours and stored overnight with cryoprotectant prior to slicing. Five series of frozen 30 µm-thick coronal sections collected through the length of the brain were then stored in cryoprotectant at -20°C.

4.2.2 Plasma hormones

Plasma corticosterone and testosterone concentrations were measured using RIA kits containing I-125 labeled ligands (MP Biomedical, Solon, OH) to confirm the stimulatory effect of neonatal ATD treatment on adult HPA output (Bingham et al., 2011), as well as to discount possible gonadal and adrenal axis interactions (Viau et al., 1999). The intra- and interassay coefficients of variation for both assays ranged from 3 to 6 and 10 to 12%, respectively. The standard curve ED50 for the testosterone and corticosterone RIA were 1.2 ng/ml with a detection limit of 0.1 ng/ml and 17 µg/dl with a detection limit of 0.625 µg/dl, respectively.
4.2.3 *Hybridization histochemistry.*

Described elsewhere (Simmons et al., 1989; Williamson and Viau, 2007), in situ hybridization was performed using [33P]UTP-labeled antisense cRNA probes encoding the rat c-fos gene [2.1 kb; Dr. T. Curran, The Roche Institute (Curran and Morgan, 1985), and the 3’ end of exon C encoding the rat arginine vasopressin (AVP) gene (Dr. D. Richter, University of Hamberg, Germany).

Semiquantitative densitometric analysis of relative levels of c-fos mRNA was performed on X-ray film using ImageJ software (version 1.38, NIH, Bethesda, MD). The 30 min post-stress interval for c-fos mRNA detection was chosen on the basis of previous studies showing this time point optimal for recording restraint-induced increments in c-fos expression (Cullinan et al., 1995). The number of AVP expressing cells in extrahypothalamic regions depends on estrogen exposure during the neonatal period (de Vries and Panzica, 2006). To validate ATD delivery, estimates of AVP positive cell numbers in medial amygdala and posterior bed nuclei were performed on emulsion-coated slides by counting the number of nuclei that were overlain by silver grains in excess of 5 times background, as previously described (Viau et al., 2001).

Guided by the atlas of Swanson (Dong and Swanson, 2004), standard frames of reference were used to determine AVP cell counts and to generate average optical density (OD) values for c-fos expression, determined bilaterally on regularly spaced (150 um) intervals through each nucleus of interest. The number of sections analyzed depended on the size of each area of interest: 3 sections for PVN, anterior hypothalamic nucleus, dorsomedial
nucleus of the hypothalamus, ventral medial nucleus of the thalamus, and medial amygdala; 4 sections for prefrontal cortex, and sensory areas for nose and mouth and vibrissae; 6 sections for the paraventricular nucleus of the thalamus, piriform cortex, and hippocampus; and 8 sections for the primary motor region and striatum.

4.2.4 Statistics

Hormone and in situ hybridization comparisons were made observer-blind by assigning coded designations to data sets in advance. Two-way ANOVAs were used to compare hormone responses. A Two-way mixed ANOVAs was used to examine AVP mRNA (within region and between treatment). A three-way mixed ANOVA was initially used to examine c-fos mRNA using brain region as the within group factor, and treatment and stress as between factors. When interactions were found to be significant, additional comparisons were made within basal and restraint conditions using two-way ANOVAs (within region, between treatment). Newman–Keuls post hoc tests were used, when appropriate, to assess statistical significance of treatment (Aabel 3.0.4).

4.3 Results

4.3.1 Hormone responses

Analysis of corticosterone indicated significant effects of treatment \( F_{(1,20)} = 4.37; P = 0.05 \) and stress \( F_{(1,20)} = 266.11; P < 0.001 \), and a significant interaction between treatment and stress \( F_{(1,20)} = 4.39; P = 0.049 \). Consistent with our previous findings, subsequent post hoc analysis confirmed that corticosterone levels were higher in ATD treated animals compared to control animals under stress conditions (Table 4.1). For testosterone, there were no significant effects of treatment \( F_{(1,20)} = 0.35; P > 0.5 \) and stress \( F_{(1,20)} = 2.70; P = 0.12 \), and no significant interaction between treatment and stress \( F_{(1,20)} = 0.29; P > 0.5 \).
Table 4.1. Mean ± SEM plasma testosterone and corticosterone concentrations (ng/ml) at 0 and 30 min of restraint stress exposure in adults bearing Blank or ATD implants at birth.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>1.1 ± 0.2</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>ATD</td>
<td>1.4 ± 0.2</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td><strong>Corticosterone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>12.75 ± 3.4</td>
<td>312.2 ± 21.6**</td>
</tr>
<tr>
<td>ATD</td>
<td>12.64 ± 2.4</td>
<td>400.4 ± 35.9**</td>
</tr>
</tbody>
</table>

**, P < 0.001 vs. Basal; †, P < 0.05 vs. Blank (n = 6 per group).

4.3.2 Extrahypothalamic AVP

Analysis of AVP cell numbers indicated a significant effect of treatment \([F_{(1,10)} = 20.45; P = 0.0011]\). For both the posterior BST and medial amygdala, post hoc analysis confirmed lower numbers of AVP-expressing cells in ATD treated animals compared those with blank implants (Figure 4.1). Based on the masculinizing effect of neonatal estrogen treatment to increase extrahypothalamic AVP in adulthood (de Vries and Panzica, 2006), the present findings indicate that the ATD implants effectively reduced estrogen exposure.
Figure 4-1. Localization of AVP mRNA through the mid-extent of the posterior division of the BST to show the relative distribution and strength of hybridization signal in animals bearing blank and ATD implants at birth (A and B, respectively). Scale bar = 250 μm. C, Mean ± SEM number of AVP mRNA positive cells within medial amygdala and posterior bed nuclei in control and ATD treated animals. **, *; P < 0.01 and P < 0.05, respectively, vs. blank (n = 6 per group).
4.3.3 Basal and stress c-fos mRNA expression

A significant three-way interaction was revealed between treatment, stress and region \( [F_{(14,280)} = 4.58; P < 0.0001] \). In unstressed animals, there was a significant effect of region \( [F_{(14,140)} = 12.27; P < 0.0001] \), but no significant effect of treatment \( [F_{(1,10)} = 0.31; P = 0.59] \) and no significant interaction between treatment and region \( [F_{(14,140)} = 0.27; P = 0.996] \). In animals exposed to restraint, there were significant effects of region \( [F_{(14,140)} = 57.79; P < 0.0001] \) and treatment \( [F_{(1,10)} = 20.63; P = 0.0011] \), and a significant interaction between region and treatment \( [F_{(14,140)} = 5.113; P < 0.0001] \). Subsequent analyses of stress-induced c-fos mRNA revealed significant effects of treatment (P’s ≤ 0.003) in almost all regions analyzed (Figures 4.2 and 4.3), credited in these cases to higher responses in ATD animals. There were no significant effects of treatment for the caudoputamen primary, motor cortex, anterior hypothalamic area, hippocampus and medial amygdala \( (P \geq 0.06) \). However, a priori we expected ATD treated animals to show increased activation in the medial amygdala and hippocampus due to their connectivity with the HPA axis and sensitivity to neonatal sex steroids. Fisher’s LSD tests revealed that ATD treated animals had higher stress-induced c-fos expression in the medial amygdala \( (P = 0.03) \), but not for hippocampus \( (P = 0.375) \), compared to those with blank implants.
Figure 4-2. Effects of neonatal ATD treatment on restraint-induced increases in c-fos mRNA through forebrain regions sampled in adult animals. Key areas (left column) are shaded in black (see Figure 3 for abbreviations). Remaining columns (from left to right) are representative autoradiographs taken from unstressed animals (Basal) and those treated at birth with Blank or ATD implants under acute restraint conditions.
Figure 4-3. In animals bearing Blank or ATD implants at birth, mean ± SEM relative optical density (OD) measures of c-fos mRNA in the paraventricular nucleus of the hypothalamus and limbic-related regions under basal (A) and restraint-stress (C) conditions; and within motor and sensory regions, likewise under basal (B) and stress (D) conditions. *P = 0.03, ** P = 0.003 and ***P< 0.0001 vs. blank (n= 6 per group). Regions abbreviated along rostrocaudal plane (A, C): AC, anterior cingulate gyrus; PFC, prefrontal cortex; LS, lateral septal nucleus; AHA, anterior hypothalamic area; PVN, paraventricular nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; HIP, hippocampus; MeA, medial amygdala. Regions abbreviated (B, D): CP, caudoputamen; M1, primary motor cortex; Face, representing somatosensory areas for nose and mouth; bfd, barrel field; PIF, piriform cortex; VPM, ventroposteromedial nucleus of the thalamus.
4.4 Discussion

We examined the effect of neonatal aromatase inhibition on c-fos mRNA responses in adult males within forebrain regions previously identified as showing marked activation in response to restraint exposure. With the exception of motor regions analyzed, the basic effect of ATD was to enhance c-fos mRNA responses within neuroendocrine neurons of the PVN, somatosensory and limbic nuclei. Taken together with elevated corticosterone responses in ATD treated animals, our results suggest that neonatal estrogens may organize the HPA axis by altering neuronal circuits related to somatosensory processing, as well as those mediating emotional and behavioural responses to stress.

Our results are consistent with previous work demonstrating an organizational role for perinatal sex hormone exposure on stress-induced HPA axis activity (Seale et al., 2005). Perinatal aromatase inhibition or androgen receptor (AR) blockade increases corticosterone responses to auditory and immune challenges in adult males. Postnatal AR blockade can also increase the magnitude of the corticosterone response to restraint (McCormick and Mahoney, 1999), but see (Bingham et al., 2011). Thus, in addition to estrogens, the organization of the HPA axis also involves androgenic mechanisms. Given the general stimulatory effect of ATD on adult HPA output, we expect neonatal estrogens to enhance cellular activation to a diverse array of stressful stimuli (immune, auditory and psychogenic).

Adult females often show higher glucocorticoid responses to stress than males, attributed to the stimulatory and inhibitory effects of estrogen and testosterone, respectively. Moreover, HPA output and PVN Fos responses in males varies strongly and negatively as a function of testosterone concentrations in circulation (Viau and Meaney, 1996; Viau et al., 2003). In the present study, ATD treated animals showed no differences in plasma
testosterone to explain their enhanced *c-fos* and corticosterone responses to restraint. Studies examining sex differences in the restraint-induced activation of the PVN and forebrain nuclei consistently indicate lower, or equivalent levels of induction in males compared to females (Figueiredo et al., 2002). Thus, increased *c-fos* activation in ATD treated animals suggests that estrogen may act on processes related to the masculinization of afferents driving the HPA axis, rather than defeminization.

A potential mechanism for the pervasive stimulatory effect of ATD in the current study may be related to the enhanced activation of sensory pathways. As previously proposed, somatosensory pathways may be preferentially involved in initiating widespread activational responses commonly observed in stress-naïve animals (Girotti et al., 2006; Li and Sawchenko, 1998). Thereafter, various cortical regions and subcortical relays might then be recruited to direct autonomic, behavioural and neurosecretory responses. This possibility is reminiscent of a substantial body of studies implicating aromatase and estrogen in the differentiation and maturation of several different sensory systems, including olfaction, vision and pain processing (Forger, 2006). The extent to which neonatal ATD alters cellular activation within sensory regions in response to innocuous stimuli remains to be examined.

Neonatal aromatase inhibition also lowers the capacity for adult males to express HPA axis habituation to repeated restraint exposure (Bingham et al., 2011). Several regions identified in the present study as showing enhanced *c-fos* responses may be more readily engaged under chronic stress conditions involving anticipatory stress and emotional appraisal (see Herman et al., 2003) including: medial prefrontal and anterior cingulate cortices, lateral septum, and medial amygdala. We recently reported that blocking central AVP receptors increases the magnitude of the corticosterone response under repeated, but not under acute
restraint stress conditions (Gray et al., 2010). Coupled to the inhibitory effect of ATD on extrahypothalamic AVP, we propose that limbic-related circuits showing enhanced \textit{c-fos} responses and in receipt of central AVP projections could comprise targets for the organizational influence of estrogen on neuroendocrine habituation. The paraventricular nucleus of the thalamus (PVT) adheres to this scheme, as well as represents a critical neural substrate for the process of stress adaptation (Bhatnagar et al., 2002).

The contribution of neonatal sex hormones to the development of the HPA axis is often overlooked. For example, prenatal stress and prenatal ethanol exposure, can also influence the onset and duration of the perinatal testosterone exposure, in addition to adult HPA responses (McGivern et al., 1993; Ward et al., 2003; Ward and Weisz, 1980). We predict environmental events that impact testosterone release and its conversion to estrogen have the potential of redirecting any number of central stress response networks, including those mediating behavioral, neuroendocrine and immune responses. Keeping in mind the impact of neonatal sex steroid hormones on these networks promises to increase our understanding of early influences on normal responses to homeostatic threat, as well as predisposition to stress-related disease.
Chapter 5: Discussion

5.1 Contributions to Original Knowledge

The studies presented in this thesis contribute to understanding the role of the neonatal testosterone in shaping the adult HPA response to stress. In particular, I manipulated the sex hormone environment in neonates to determine how HPA axis activity is altered by neonatal testosterone, and where and how this might change the adult brain.

1. Testosterone does not have an inhibitory influence on the HPA response to stress in animals that are neonatally gonadectomized, suggesting neonatal testosterone exposure could alter the capacity of neurons to respond to testosterone by altering AR expression. These experiments are the first to show that neonatal gonadectomy dramatically decreases AR numbers in two HPA-regulating brain regions: the posterior BST and medial amygdala. In addition, there was a negative correlation between the number of receptors, and corticosterone concentrations in plasma and Fos levels in the medial parovocellular PVN under basal and stress conditions. The ability of ARs in these regions to regulate the HPA response to stress still requires further investigation.

2. Along similar lines, I found a negative correlation between the number of arginine vasopressin (AVP)-expressing neurons in both of these nuclei and HPA activity in the same animals. Taken together, these findings suggest that testosterone exposure during the neonatal period may increase the sensitivity of the HPA axis to testosterone by increasing AR number and/or androgen sensitive neurochemistry in HPA-regulating regions.

3. Although I was able to confirm that the neonatal testosterone surge organized the HPA response to acute stress, the contribution of aromatase on the conversion of testosterone to estrogens in HPA axis development had not been addressed previously. The work
presented here demonstrates that neonatal aromatase inhibition, but not AR blockade, increased plasma ACTH and corticosterone levels in adult males in response to acute restraint stress. This would suggest that neonatal estrogens are responsible for the majority of the organizing effects of testosterone on the acute stress response.

4. Given the inhibitory role of testosterone in stress regulation under acute stress conditions, we suspected that testosterone might play a role in organizing habituation to repeat stress exposure. The normal decline in corticosterone failed to occur in flutamide- and ATD-treated animals in response to daily repeat restraint exposure. By contrast, males that were castrated as adults showed a significant reduction in corticosterone after repeated stress. Taken together, these findings suggest an organizing influence of both ARs and estrogen conversion on HPA habituation to repeated psychogenic stress that appears to occur independently of the activational effects of testosterone.

5. In hopes of identifying a few select HPA-regulating regions that might be impacted by neonatal testosterone, I used c-fos mRNA as a tool to map changes in cellular activation in response to acute restraint in adult animals that were exposed to neonatal aromatase blockade. I found higher c-fos expression levels in the paraventricular nucleus (PVN) of the hypothalamus and elevated corticosterone responses to acute restraint stress in ATD treated animals. ATD also enhanced stress-induced c-fos expression in several limbic regions, including within the anterior cingulate and medial prefrontal cortex, lateral septum, anterior hypothalamic area, dorsal medial hypothalamus and medial amygdala, as well as at multiple levels of somatosensation. Due to the widespread nature of our findings and the highly interconnected nature of the brain regions examined, it is not possible to ascertain which of the regions are directly targeted by estrogens during development. However, it is
clear that the conversion of testosterone to estrogen during the neonatal period exerts effects that result in a systems-wide increase in brain activation in response to stress.

5.2 Methodological Considerations

5.2.1 Manipulating the neonatal hormone environment

The most difficult decision in designing the studies for this thesis was the choice of how to manipulate the perinatal hormone environment to best study the effects of sex hormone exposure in males on HPA function. Recent studies have implicated genetic factors in contributing to the sex differences in brain morphology and neurochemistry in male and female rodents (Arnold, 2009; Arnold et al., 2009; McCarthy and Arnold, 2011). Thus, the testosterone surge is not the only factor that creates differences between the male and female brain. For this reason we preferred to manipulate the sex hormone environment in male rat pups, rather than use the more common approach of giving sex steroid hormone injections to masculinize female pups.

This left two choices; either manipulate hormones perinatally by manipulating the testosterone surge that occurs on prenatal day 18 and postnatal day 1, or manipulate only the postnatal testosterone surge. Manipulating the perinatal hormone environment typically involves injecting the dam daily with agonists or antagonists from prenatal day 13 until parturition (usually 10 days). Daily injections that manipulate the steroid hormone environment are stressful and likely alter the hormone environment of the dam, which in turn could impact HPA axis development in the fetus. For these reasons, we felt a neonatal manipulation was the best approach for studying the role of testosterone in organizing the HPA response to stress.
Our choice of neonatal manipulation, however, was not without potential pitfalls. The HPA axis is extremely plastic during the neonatal period, with manipulations such as neonatal malnutrition (Lesage et al., 2002), maternal separation (Levine, 1994), maternal behavior (Liu et al., 2000) and neonatal handling (Smythe et al., 1996) permanently altering HPA axis activity in adulthood. Our paradigm requires that pups undergo anesthesia on the first day of life and are periodically handled to ensure good health and that surgical sites have not become infected. To assess the impact of handling and surgery on my results, my initial studies (Chapters 3) included animals from each litter that have not been handled. In addition, I compared maternal behavior between dams with pups that had received surgery and those with pups that had not been disturbed (Chapter 3). There were no detectable differences in the occurrence of any of the behaviors between groups of dams, ruling out an effect of group differences in maternal behavior on my results. In addition, all litters were culled to twelve pups to equalize the nutritional load on the dams and only one pup from each litter was used in any group to maintain genetic diversity in my studies (Abbey and Howard, 1973). All of these measures have allowed me to draw conservative conclusions from my data.

5.2.2 Aromatase and flutamide antagonism

5.2.2.1 Drug delivery

There is an extensive literature that demonstrates that handling rat pups neonatally can significantly impact HPA axis activity in adulthood (for reviews see Gutman and Nemeroff, 2002; Meaney et al., 1991; Korosi and Baram, 2010). I preferred a drug implant approach rather than a chronic drug injection approach because it limited handling of pups. The size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in adult males is
determined by sex hormone exposure early in development (Jacobson et al., 1981), and is reduced in size by neonatal treatment with flutamide (Lund et al., 2000), and ATD (Houtsmuller et al., 1994). Therefore, I compared the size of the SDN-POA in the same animals used for the repeated stress study to independently assess the efficacy of flutamide and ATD treatment. I found that both flutamide and ATD treatment significantly reduced the volume of the SDN-POA, suggesting enough drug is released from the silastic implants to effectively prevent sexual differentiation of neural structures.

5.2.2.2 Implant removal

An added benefit of the implant approach over the neonatal gonadectomy approach is implants could be removed prior to puberty. The peripubertal period also represents a critical period for the sexual differentiation of the brain (Schulz et al., 2009b), including the HPA axis. Another surge in testosterone occurs during adolescence/puberty and recent evidence suggests this surge may also be responsible for organizing the HPA axis (Evuarherhe et al., 2009; Gomez et al., 2004; McCormick and Mathews, 2007; Romeo, 2010). Lightman’s group (2009) demonstrated that testosterone treatment decreased the HPA-response to noise stress and LPS injection in adult males that were gonadectomized as adults, but not in those that were gonadectomized peripubertally. Similar to the effects of neonatal gonadectomy, this suggested that peripubertally castrated rats are resistant to the inhibitory influence of testosterone on HPA axis activity. In contrast to neonatally castrated rats, however, peripubertally castrated rats do not show an increase in HPA output following stress. They suggest that the androgen mediated organizational mechanisms taking place during puberty are different from those taking place perinatally and this certainly warrants future investigation.
5.2.3 Immediate early genes and cellular activation

The experiments done in Chapter 2 and Chapter 4 relied on the expression of the immediate early gene Fos as a marker of cellular activation. The induction of c-Fos has been widely used as a marker of neuronal activation in the brain in response to a variety of psychogenic stressors; including restraint, swim stress and novelty (Chan et al., 1993; Chan and Sawchenko, 1994; Chen and Herbert, 1995; Cullinan et al., 1995). I chose to use c-fos mRNA as a marker of cellular activation because it is generally expressed at extremely low levels under basal conditions, and is rapidly and reliably induced in response to restraint stress (for reviews see (Chaudhuri, 1997; Hoffman and Lyo, 2002; Kovács, 1998)). Further, immediate early gene mapping is one of the only techniques available in the rat that allows for analysis of large areas of the brain while maintaining cellular-level resolution (Kovács, 2008).

It is important to keep in mind that not all neurons that are activated (metabolically or electrophysiologically) by a stimulus will respond with the induction of c-fos mRNA or protein; therefore, no conclusions can be drawn from a lack of induction (Kovács, 2008; Ziolkowska and Przewlocki, 2002). For example, Fos is not induced in cells with a net inhibitory synaptic or transcriptional drive (Kovács, 2008; Ziolkowska and Przewlocki, 2002). With respect to the results of Chapter 4, this means I can say that neonatal aromatase inhibition affected the response to restraint stress of neurons in a large number of brain regions in some way. However, regions that did not show c-fos mRNA induction may have also been impacted and should not be ruled out as potential sites that may be influenced by neonatal testosterone exposure. Several other immediate early genes have been used to map stress responsive circuits in the rat brain and their specific time course of induction and
expression patterns differ (Girotti et al., 2006; Girotti et al., 2007; Weinberg et al., 2007; Ziolkowska and Przewlocki, 2002). Future studies aimed at comparisons of expression patterns using multiple immediate early genes would provide further insight into stress regulating brain regions that are altered by neonatal testosterone.

5.3 Recent Progress and Future Consideration

5.3.1 AR expressing cells in the MeA and posterior BST

From my early work in neonatally gonadectomized males, it is clear that the neonatal testosterone surge is required for the HPA axis to be sensitive to testosterone under acute stress conditions in adults. I was able to demonstrate that this could be mediated through neonatal testosterone induced changes in AR expression, or AVP expression at the level of the posterior BST and medial amygdala. The extent to which ARs and/or AVP expressing cells in these regions are actually capable of regulating the HPA axis under acute stress conditions has been suggested many times (Williamson et al., 2005; Williamson and Viau, 2007), but until recently had not been directly tested.

I have recently started to examine the role of ARs in the posterior BST in regulating the acute HPA response to stress. Neonatal gonadectomy decreased ARs in all three major nuclei of the posterior BST: principal, interfascicular, and transverse (Chapter 2). Our most recent findings suggest that a subset of AR-expressing cells within these nuclei may actually be responsible for increasing the biosynthetic capacity and stress-induced drive of the PVN (Bingham et al., 2011b). To test a role for ARs in the posterior BST to inhibit the PVN, I compared the effects of the non-aromatizable androgen dihydrotestosterone (DHT), the AR antagonist hydroxyflutamide (HF), or a combination of both drugs implanted unilaterally within the posterior BST of adult male rats. PVN AVP mRNA and stress-induced Fos were
augmented in response to AR agonist and attenuated in response to antagonist on the side of the implant. This was opposite to our expectations of an inhibitory influence of ARs in the posterior BST on biosynthesis and cellular activation in the PVN, making ARs in this region an unlikely target for the organizing influence of the neonatal testosterone surge on the HPA axis. This finding should not diminish the potential importance of the ability of neonatal testosterone to increase AR expression in the posterior BST. In addition to having the capacity to directly or indirectly regulate the PVN, the nuclei in the posterior BST are also implicated in the control of sexually dimorphic defensive and reproductive behaviors (Dong and Swanson, 2004; Simerly, 2002), providing potential alternative functions for increased AR expression in the posterior BST. Future studies examining the role of ARs in the medial amygdala are also required before their role in HPA axis regulation is certain.

Further, recent evidence also suggests that future analysis should be expanded to include estrogen receptor-β. The predominant view at the time the experiments in Chapter 2 were undertaken was that testosterone acted through the AR to inhibit the HPA axis. Early studies had shown that the effects of gonadectomy are reversed with replacement of the testosterone metabolite dihydrotestosterone (Handa et al., 1994b). Unlike testosterone, dihydrotestosterone is not converted by the aromatize enzyme and was thought to be a specific AR agonist (Handa et al., 2009). As mentioned in the introduction, recent evidence suggests that DHT can in fact be converted to 3β-diol by enzymes other than aromatase (shown in Figure 1.3) and act through estrogen receptor-β to regulate the PVN (Lund et al., 2006; Lund et al., 2004b). Therefore, the neonatal testosterone surge may organize the HPA-response to testosterone through changes in estrogen receptor-β rather than, or in addition to, changes in ARs.
As discussed in chapter 2, neonatal gonadectomy exerts remarkable changes in adult brain morphology, including effects on neuronal soma size and cell numbers within subregions of the posterior BST and the MeA. This could have an important bearing on the extended circuitries of the PVN, including its functional connectivity with the limbic system. Using an anterograde tract-tracing approach, Gu et al. (2003) showed that neonatal gonadectomy decreases the projection densities of the principal nucleus of the posterior BST to many regions, including the anteroventral periventricular nucleus of the hypothalamus (AVPV) and medial amygdala. Thus, future studies employing a retrograde tracer injection approach in the PVN could answer whether neonatal androgens are capable of altering the number of neurons targeting the PVN directly as well as their containment of ARs and estrogen receptor-β.

5.3.2 Arginine vasopressin in the posterior BST and MeA

With respect to AVP, recent work in our lab suggests this peptide plays a role in regulating HPA responses to repeated, but not acute, stimuli in adult male rats. We previously examined relations between stress HPA habituation and changes in arginine vasopressin (AVP) mRNA expression within limbic regions (Gray et al., 2010b). Animals exposed to five daily episodes of 3 hour restraint or ten days of 30 minute restraint, showed significant reductions in ACTH and corticosterone responses, as well as an increase in AVP mRNA levels in the posterior bed nucleus of the stria terminalis and medial amygdala. Megan Gray (Gray et al., 2010a) in our lab also recently measured plasma ACTH and corticosterone responses to repeated episodes of 3 hour restraint in animals bearing continuous infusion of the AVP V1a receptor antagonist into the lateral ventricle. Relative to
vehicle-infused control groups, V1a antagonism increased the ACTH and corticosterone responses to restraint on the last day, but not on the first day of restraint exposure.

As demonstrated in Chapter 3, adult animals bearing ATD and Flu implants at birth also express a lower capacity for expressing HPA axis habituation to repeated restraint (Bingham et al., 2011a). In addition to the evidence shown in Chapter 2 and 4, others have shown that normal expression of AVP cells in the pBST and MeA is dependant on both androgen and estrogen exposure in the developing male rat (Han and De Vries, 2003). Taken together with the role of AVP in HPA axis habituation in adult animals, the evidence strongly suggests extrahypothalamic AVP cells could comprise targets for estrogen and/or androgen mediated influences on neuroendocrine habituation, but may not be directly involved in acute HPA responses. AVP synthesized in the posterior BST and medial amygdala is transported to axon terminals, which project to many putative HPA regulating regions (de Vries and Miller, 1998). Studies aimed at selective disruption of AVP circuits originating from the posterior BST and/or medial amygdala during development and during adulthood could provide further support to this hypothesis.

In addition to AVP, sex steroids have been shown to interact with several neurotransmitter systems both organizationally and activationally that are capable of regulating the HPA axis (De Vries, 2006). Another possible neurotransmitter that may be targeted by neonatal testosterone to alter the sensitivity of the HPA axis to sex steroid hormones in adulthood is GABA. Stressed induced changes in GABA neurotransmission within PVN-projecting populations have been proposed to provide a mechanism for regulating HPA axis responses (Herman et al., 2005). PVN neurons are richly innervated by GABAergic cell populations from several HPA-regulating regions that are sensitive to
testosterone, including the peri-PVN region, posterior BST and medial preoptic nucleus (Herman et al., 2002a; Polston et al., 2004). Further, GABA synthesis and turnover in the brain is remarkably sensitive to alterations in circulating testosterone concentrations (Grattan and Selmanoff, 1993). Researchers have shown that the GABA system is also an endpoint of sexual differentiation (McCarthy and Arnold, 2011), resulting in sex differences in somatostatin secretion (Murray et al., 1999) and GABAergic projection profiles from the posterior BST (Gu et al., 2003). Thus, it remains possible that the organizing influence of testosterone on HPA axis activity also involves changes in other neurotransmitter or peptidergic systems that regulate the PVN.

5.3.3 Estrogen receptor-α and -β distribution during development

The widespread nature of the enhanced c-fos mRNA found in males treated with an aromatase inhibitor neonatally suggests estrogens may act at one or more brain regions upstream of the PVN to alter how HPA axis-regulating neurocircuits response to stress. This is further complicated by the fact that almost all of the regions identified express sex hormone receptors during development and in adulthood (McGinnis and Katz, 1996; Perez et al., 2003; Shughrue et al., 1997a; Simerly et al., 1990). Uncovering the brain regions that are specifically altered by neonatal estrogen under acute stress conditions is essential to further our understanding of how the neonatal testosterone surge alters afferent mediators of HPA function. In addition mapping changes in sex-steroid hormone expressing PVN projecting nuclei in adult animals as described above, and unique expression patterns of sex steroid hormone receptors during development, may also provide insight into which brain regions are altered by the neonatal testosterone surge.
The current studies reveal that the conversion of testosterone to estrogen by the aromatase enzyme is essential for the organizing effects of testosterone on the HPA axis response to acute psychogenic stress. Estrogen may act at either estrogen receptor-α and/or -β to alter developing neurocircuitry dedicated to regulating the HPA axis (Kudwa et al., 2006). Estrogen receptor-α and -β are expressed at high levels in many regions of the developing rat brain (Perez et al., 2003), including those regions examined in Chapter 4 that showed an enhanced c-fos mRNA to acute restraint in ATD treated animals. In addition, regions that did not show a c-fos mRNA response to acute restraint (Chapter 4), but are recognized as contributing to testosterone’s inhibitory influence on HPA axis regulation in adults, also express ERs postnatally, including the peri-PVN region, and the medial preoptic nucleus. These receptors have overlapping, but distinct patterns of expression in the neonatal rat brain (Perez et al., 2003). Uncovering the individual contributions of estrogen receptor α or -β to organizational influences on the HPA axis using specific receptor antagonists would help to narrow down which regions involved in the stress response are directly transformed by neonatal estradiol.

Perhaps of particular interest, the expression of estrogen receptor-α peaks in the developing neocortex in the rat brain on postnatal day three and then steadily declines, while estrogen receptor-β does not appear until postnatal day 14 (Perez et al., 2003; Yokosuka et al., 1995). Estrogen receptor-α is also selectively distributed in thalamic nuclei, including the ventromedial nucleus, suggesting that estrogen receptor-α may have a selective role in thalamocortical differentiation (Perez et al., 2003). A potential mechanism for the widespread effects of ATD on c-fos mRNA expression may be through the direction and amplification of information flow initiated by activation of somatosensory pathways (Li and
Sawchenko, 1998). If this were true, one might expect a selective influence of neonatal estrogen receptor-α antagonism to influence adult HPA axis activity under acute stress conditions.

5.3.4 Link to environment

Several processes influence the maturation and the response of the hypothalamic-pituitary-adrenal (HPA) axis. Manipulations that alter environmental variables, maternal-infant relationships, and sex steroid hormone levels during critical periods of development can permanently modify the HPA axis (Bale et al., 2010; Hellemans et al., 2010; Meaney et al., 1989; Meaney and Szyf, 2005a, b; Weinstock, 2005). Our findings suggest that any environmental perturbation that might impact the neonatal testosterone surge and/or brain aromatase may also cause changes in the network of brain regions involved in the endocrine, emotional and behavioral responses to stress. Evidence suggests that factors known to influence the development of the HPA axis, like prenatal stress and prenatal ethanol exposure, can also influence the onset and duration of the perinatal testosterone exposure (McGivern et al., 1993; Ward et al., 2003; Ward and Weisz, 1980). Future studies aimed at uncovering how changes in onset, duration and strength of the neonatal testosterone surge can impact on stress-neurocircuitry will lead to a better understanding of how interactions between environment and neonatal hormone milieu can lead to individual differences in stress responsivity.

Recently, much attention has been paid to the impact of endocrine-disrupting chemicals (EDCs) on biological processes in rodents, humans and other species (Diamanti-Kandarakis et al., 2009). EDCs are thought to influence the reproductive functions of humans and other animals, similar to the effects of steroid (or sex) hormones in both genders.
(Masuo and Ishido, 2011). Most of these so-called EDCs are derived from industrial products; however, several are also found in common household products, and many are detected in human umbilical cord and blood (Mori, 2001). Bisphenol A (BPA) is one of the most common EDCs, and its effects have been widely investigated (Willhite et al., 2008). Researchers have shown that BPA exposure can impact a variety of reproductive and nonreproductive behaviors such as rearing, anxiety, and learning and memory (Carr et al., 2003; Fujimoto et al., 2006; Kubo et al., 2003; Masuo and Ishido, 2011).

The role of sex hormones in sexual differentiation has long predicted that EDCs may impact brain development. Recently, Zsarnovszky et al. (2005) reported that a low dose of BPA affected the development of rat embryos, and that BPA acted as a highly potent pseudo-estrogen, disrupting the roles of estrogen in the development of the cerebellum. In addition to BPA, other endocrine disruptors have been shown to act as agonists or antagonists of androgens or estrogens, and/or components of steroidogenic enzyme pathways, including brain aromatase (Borgert et al. 2011). The work presented in this thesis here predicts that substances in the environment that change the hormonal milieu during development have the potential to impact HPA responses in adulthood. Studies examining the impact of these substances in the development of the HPA axis will contribute to our understanding of the possible health threats posed by increasing levels of pollutants in our environment.

Based on our findings, androgens and estrogens during development appear to play a critical role in the development of normal HPA function in adult male rats. They also appear to have the capacity to influence a vast and multimodal array of neural systems dedicated to regulating the HPA axis, including the limbic system. The potency by which testosterone acts throughout the central nervous system, clearly incorporates the gonadal system as compelling
link between abnormal HPA function and affective disease states. The potential link between testosterone and environment may provide an exciting avenue into furthering our understanding of the factors that lead to individual differences in stress responsiveness.
References


Dallman MF. Stress by any other name .....? Horm Behav, 2003; 43: 18-20; discussion 8-30.


Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM,


Drevets WC. Functional anatomical abnormalities in limbic and prefrontal cortical structures in major depression. Prog Brain Res, 2000; 126: 413-31.


Figueiredo HF, Dolgas CM, Herman JP. Stress activation of cortex and hippocampus is modulated by sex and stage of estrus. Endocrinology, 2002; 143: 2534-40.


Goldstein MZ. Depression and anxiety in older women. Prim Care, 2002; 29: 69-80, vi.


Hoffman GE, Lyo D. Anatomical markers of activity in neuroendocrine systems: are we all 'fos-ed out'? J Neuroendocrinol, 2002; 14: 259-68.

Houtsmuller EJ, Brand T, de Jonge FH, Joosten RN, van de Poll NE, Slob AK. SDN-POA
volume, sexual behavior, and partner preference of male rats affected by perinatal treatment with ATD. Physiol Behav, 1994; 56: 535-41.


Jaferi S, Bhatnagar S. Corticosterone can act at the posterior paraventricular thalamus to inhibit hypothalamic-pituitary-adrenal activity in animals that habituate to repeated stress. Endocrinology, 2006, 147: 4917-30


Kitay JI, Coyne MD, Swygert NH. Influence of gonadectomy and replacement with estradiol or testosterone on formation of 5 alpha-reduced metabolites of corticosterone by the adrenal gland of the rat. Endocrinology, 1970; 87: 1257-65.


Kovacs K, Kiss JZ, Makara GB. Glucocorticoid implants around the hypothalamic paraventricular nucleus prevent the increase of corticotropin-releasing factor and arginine vasopressin immunostaining induced by adrenalectomy. Neuroendocrinology, 1986; 44: 229-34.


Li HY, Sawchenko PE. Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels. J Comp Neurol, 1998; 393: 244-66.


Ma XM, Levy A, Lightman SL. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. Endocrinology, 1997; 138: 4351-7.


McGivern RF, Handa RJ, Rede E. Decreased postnatal testosterone surge in male rats exposed to ethanol during the last week of gestation. Alcohol Clin Exp Res, 1993; 17: 1215-22.


Polston EK, Gu G, Simerly RB. Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus. Neuroscience, 2004; 123: 793-803.


Schulz KM, Zehr JL, Salas-Ramirez KY, Sisk CL. Testosterone programs adult social behavior before and during, but not after, adolescence. Endocrinology, 2009b.

Seale JV, Wood SA, Atkinson HC, Bate E, Lightman SL, Ingram CD, Jessop DS, Harbuz MS. Gonadectomy reverses the sexually diergic patterns of circadian and stress-induced


Williamson M, Viau V. Androgen receptor expressing neurons that project to the paraventricular nucleus of the hypothalamus in the male rat. J Comp Neurol, 2007; 503: 717-40.


Xiao L, Jordan CL. Sex differences, laterality, and hormonal regulation of androgen receptor immunoreactivity in rat hippocampus. Horm Behav, 2002; 42: 327-36.


