Local glucocorticoid production by the mouse immune and nervous systems

by

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Abstract

Glucocorticoids are steroid hormones primarily produced by the adrenal glands and released into the bloodstream to coordinate animal development and a myriad of physiological processes. Adrenal glucocorticoid production greatly increases in response to stressors, except during a period in early development in altricial species, termed the stress hyporesponsive period. During the stress hyporesponsive period, blood glucocorticoid levels are very low and have a blunted response to some stressors. Glucocorticoids are also locally produced by organs such as the bone marrow, thymus, spleen, and brain. Within such organs, glucocorticoids can be synthesized from precursors or regenerated from the inactive metabolite; however, it is not known which route is more important. Local glucocorticoid production allows organs to independently regulate glucocorticoid levels based on demand and this may be of particular importance during the stress hyporesponsive period when blood glucocorticoid levels are low. In this dissertation, I present a series of studies examining production of glucocorticoids in lymphoid organs and brain across development and in response to an acute stressor. Briefly, I report that 1) glucocorticoids are locally elevated in lymphoid organs and specific brain regions in neonatal mice, but not adolescent or adult mice, 2) within the brain, glucocorticoid levels are more modular during early development and more coupled between regions during adulthood, 3) local glucocorticoid production increases greatly in response to a stressor during the stress hyporesponsive period, but less afterwards. Altogether, lymphoid organs and specific brain regions produce glucocorticoids. Local glucocorticoid production is of increased importance during the stress hyporesponsive period as it allows tissues to independently regulate local levels, provides benefits of high glucocorticoid levels where needed, and helps avoid the deleterious effects of glucocorticoids where they are not required.

Lay summary

Glucocorticoids (stress hormones) are steroid hormones that are powerful regulators of the immune and nervous systems. They are predominately produced by the adrenal glands, but also locally by tissues of the immune and nervous systems. Adrenal glucocorticoid production is well studied, but far less is known about local production. In this dissertation, I present a series of studies demonstrating that 1) glucocorticoid levels are higher in lymphoid organs than blood levels during early development, possibly due to local regeneration of a glucocorticoid metabolite, 2) discrete brain regions locally-produce glucocorticoids, particularly during neonatal development, and 3) local glucocorticoid production within immune tissues increases in response to stress during neonatal development. Together, these studies demonstrate that local glucocorticoid levels and production are dependent on tissue, age, and physiological context.

Preface

All of the work presented in this dissertation was conducted at the University of British Columbia, Point Grey campus. All projects and methods were approved by the University of British Columbia's Animal Care Committee (certificate numbers: A19-0059, A19-0060).

A version of Chapter 2 has been published [Hamden, J.E., Salehzadeh, M., Jalabert, C.J., O'Leary, T.P., Snyder, J.S., Gomez-Sanchez, C.E., Soma, K.K. 2019. Systemic and local levels of 11-dehydrocorticosterone in mice, rats, and birds: Effects of age, sex, and stress. General and Comparative Endocrinology. 281: 173-182.] I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as manuscript publication. Salehzadeh M. assisted in development for this study. Salehzadeh M. and Snyder J.S. assisted in the development the protocols. Salehzadeh M., Jalabert C.J., and O'Leary T.P. assisted in collection of tissue samples. Soma K.K was the supervisory author on this project and was involved throughout the project in concept formation and manuscript composition.

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List of abbreviations

11β-HSD1 – 11β-hydroxysteroid dehydrogenase type 1
11β-HSD2 – 11β-hydroxysteroid dehydrogenase type 2
µg – microgram
µl – microliter
µM – micromolar
ACTH – adrenocorticotropic hormone
ANOVA – analysis of variance
anti-DHC – anti-11-dehydrocorticosterone-3-carboxymethoxyoxime-thyroglobin
BCR – B cell receptor
BM – bone marrow
BSA – bovine serum albumin
ºC – degrees Celsius
CC – cerebral cortex
cm – centimeter
CI – confidence interval
CBG – corticosteroid binding globulin
cDNA – complementary DNA
CIHR – Canadian Institute of Health Research
Cq – qPCR quantification cycle
CYP11B1 – cytochrome P450 family 11, subfamily A, polypeptide 1
CV – coefficient of variation
DHC – 11-dehydrocorticosterone
DNA – deoxyribonucleic acid
DOC – 11-deoxycorticosterone
ELISA – enzyme-linked immunosorbent assay
g – gram
× g – earth's gravitational acceleration constant
GC – glucocorticoid
GR – glucocorticoid receptor
h, hr – hours
HPA – hypothalamic-pituitary-adrenal axis
HPC – hippocampus
HPH – high pH
HRP – Avidin - horseradish peroxidase
HYP – hypothalamus
IS – internal standard
LC – liquid chromatography
LC-MS/MS – liquid chromatograph tandem mass spectrometry
M – Molar
MeOH – methanol
min – minutes
mg – milligram
ml, mL – milliliter
MPA – mobile phase A
MPB – mobile phase B
MR – mineralocorticoid receptor
mRNA – messenger ribonucleic acid
m/s – meters per second
mV – millivolts

m/z – mass/charge ratio
N – normal
n – sample size
na – not applicable
NAD^+ – nicotinamide adenine dinucleotide
NADPH – nicotinamide adenine dinucleotide phosphate
nd – nondetectable
ng – nanograms
No – number
ns – nonsignificant
NSB – non-specific binding
NSERC – Natural Sciences and Engineering Research Council
OAZ1 – ornithine decarboxylase antizyme 1
PBS – phosphate buffered saline
pg – picograms
PND – postnatal day
qPCR – quantitative polymerase chain reaction
sec – seconds
SEM – standard error of the mean
SHRP – stress hyporesponsive period
sMRM – scheduled multiple reaction monitoring
SPE – solid phase extraction
SPL – spleen
TCR – T cell receptor
TH – thymus
TMB – tetramethylbenzidine liquid substrate solution
UBC – University of British Columbia
uHPLC – ultra high performance liquid chromatography
QC – quality control
WB – whole blood

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Chapter 1 Introduction

1.1 Overview

Hormones are signaling molecules produced by endocrine glands and secreted into the bloodstream to coordinate vertebrate physiology. Hormones are classified according to their chemical structure, mechanism of action, and/or source. Steroid hormones are a class of hormones secreted by the adrenals, testes, and ovaries. Once in the bloodstream, steroids act on target cells within tissues and exert pleiotropic effects depending on tissue identity and physiological context (Norris and Carr 2020). Based on this understanding of steroid production and action, the field of endocrinology has classically focused on systemic steroid levels and modulation of systemic steroid levels.

Over the last two decades, accumulating evidence indicates that in addition to their endocrine role, steroids can also act in a paracrine or autocrine fashion, acting on cells near the site of production or directly on the steroid-producing cells (Mittelstadt, et al. 2018; Schmidt, et al. 2008). These discoveries have shifted our fundamental perspective of studying steroid regulation from a systemic viewpoint to a local viewpoint. It is now accepted that many tissues that were exclusively viewed as passive recipients of steroids, have the ability to produce their own steroids to modulate local function (Ahmed, et al. 2019; Gathercole, et al. 2013; Taves, et al. 2011a). However, our understanding of tissue steroid production across development and in response to external stimuli is very limited.

Glucocorticoids have been a main focus when examining local steroid production due to their powerful and wide-ranging modulatory effects on various tissues (Sapolsky, et al. 2000). The main active glucocorticoid is cortisol in humans, and corticosterone in mice, rats, and birds. Glucocorticoids are predominantly produced by the adrenal cortex on a circadian cycle and increase in response to stressors. Glucocorticoids were named for their role in regulating energy expenditure, such as promoting gluconeogenesis in the liver (Exton 1979). However,

glucocorticoids exert effects on many other systems in the body as they act on virtually every cell type.

Glucocorticoids are particularly important in nervous and immune system development and function. Glucocorticoids have strong programming effects in neonates and modulate the immune system and behavior in adults, and adaptive outcomes are dependent on the level of steroid signaling (Sapolsky et al. 2000; Wada 2008). Blood glucocorticoid levels are high compared to other steroids in adulthood, but levels vary greatly across life. Blood glucocorticoid levels are very low in early life, and increase significantly in adulthood. As the field has traditionally focused on blood glucocorticoid levels, adrenal production and levels are well defined. In contrast, little is known about glucocorticoid levels within tissues or how tissues modulate local levels.

Due to limited knowledge of local glucocorticoid levels, it has been thought that tissue levels mirror blood levels, as free steroids are lipophilic and can diffuse into tissues. However, we now appreciate that there are more factors that modulate local steroid levels. Specifically, glucocorticoids can bind to corticosteroid binding globulin in the blood, which regulates glucocorticoid availability, and tissues possess the necessary machinery to produce steroids locally (Taves, et al. 2015; Viau, et al. 1996). My PhD work has investigated both systemic and local glucocorticoid production across life, organ systems, and physiological contexts. As blood glucocorticoid levels go from very low in early life to very high in adulthood, my studies focused on investigating whether tissue glucocorticoid levels mirrored this pattern, and whether the relationship between blood and local levels is static or plastic. Further, local glucocorticoid levels are higher than blood levels in early life within certain tissues, such as lymphoid organs and the brain (Taves et al. 2015). Early-life glucocorticoids have strong perinatal programming effects within these tissues, therefore my research focused particularly on local glucocorticoid production in early life. I hypothesized that, in early life when blood levels are low, tissues upregulate levels at baseline, and local production increases in response to stress. Further,

once blood levels increase after early life, tissues no longer upregulate levels as the blood provides sufficient levels. My first study established a novel method to measure 11-dehydrocorticosterone, the inactive metabolite of corticosterone, to determine if it is present endogenously in mice, rats, and songbirds, and whether levels are high enough to contribute to local corticosterone production, as previously suggested (Taves, et al. 2016a). My second study examined glucocorticoid production within glucocorticoid-sensitive brain regions across mouse development. My third study examined the effects of early life stress on blood and local glucocorticoid levels on mouse lymphoid organs.

1.2 Glucocorticoids

Glucocorticoids are a class of steroids produced by the adrenal glands. In mice, the adrenal glands are comprised of two distinct zones, the zona glomerulosa (mineralocorticoid production) and the zona fasciculata (glucocorticoid production). Within the zona fasciculata, glucocorticoids are synthesized from the common steroid precursor, cholesterol, and released into the blood to coordinate homeostasis and the stress response. Adrenal glucocorticoid production is under tight regulation by the hypothalamic-pituitary-adrenal axis. The hypothalamus produces corticotropin releasing hormone on a circadian cycle and increases production in response to a stressor. Then corticotropin releasing hormone travels via the hypophyseal portal system to the anterior pituitary and stimulates the production and release of adrenocorticotropin hormone into the bloodstream. Adrenocorticotropin hormone then binds to melanocortin receptor 2 in the zona fasciculata, to stimulate glucocorticoid production. Within the adrenal glands, glucocorticoids are synthesized de novo from cholesterol via a series of enzymatic reactions resulting in the release of the active glucocorticoid (Figure 1.1). Finally, glucocorticoids act on the glucocorticoid receptor found at both the hypothalamus and pituitary, to provide negative feedback on the system (Goncharova, et al. 2019).

In the blood, glucocorticoids are bound to corticosteroid binding globulin and unbound (free). In adult animals, corticosteroid binding globulin levels are high, 80-90% of glucocorticoids in the blood are bound, making the level of bioavailable glucocorticoids relatively low. Importantly, glucocorticoids bound to corticosteroid binding globulin can be cleaved by serine proteases at the site of tissues, making corticosteroid binding globulin an important regulator of tissue glucocorticoid levels. Unbound glucocorticoids can diffuse into target tissues and bind to the cytosolic glucocorticoid receptor or the higher affinity mineralocorticoid receptor. While the mineralocorticoid receptor has a relatively higher binding affinity to glucocorticoids than the glucocorticoid receptor, it is primarily bound by mineralocorticoids, such as aldosterone. Target tissues, particularly the liver, can metabolize the active glucocorticoid (corticosterone in mice) to the inactive 11-dehydrocoritocosterone via 11β-HSD2 activity or regenerate 11-dehydrocorticosterone into corticosterone via 11β-HSD1 activity to locally modulate receptor binding (Figure 1.1). Glucocorticoid receptor is a nuclear receptor that when bound to its ligand, acts as a transcription factor that regulates transcription of ~10-20% of the entire genome (Oakley and Cidlowski 2013). There is also evidence that glucocorticoids can exert non-genomic effects through binding to a membrane glucocorticoid receptor (Cottrell and Seckl 2009). The effects of glucocorticoids within target tissues are pleiotropic and change depending on tissue, age, and physiological context.

Glucocorticoids have profound impacts on primary lymphoid organs, such as bone marrow and thymus, and secondary lymphoid organs, such as spleen. Bone marrow is the site of B and T cell origin and of B cell development. T cells migrate from the bone marrow to the thymus to mature by undergoing positive and negative selection. During selection, T cells are subjected to apoptosis if their T cell receptor binds either too strongly or too weakly when presented with self-antigen (Klein, et al. 2014). T cell selection ensures that T cells react strongly enough to fight against pathogens, while preventing autoimmunity by binding strongly to self-antigens. B cells undergo a similar process in the bone marrow (Grimaldi, et al. 2005).

Glucocorticoids are highly immunosuppressive at high, prolonged levels, and induce lymphocyte apoptosis and prevent immune overshoot. When levels are moderate, glucocorticoids modulate T cell selection by promoting survival of T cells that would otherwise die during positive selection, and by promoting survival of more reactive T cells during negative selection (Taves and Ashwell 2020). Importantly, mineralocorticoid receptor expression is low in lymphoid organs, thus actions of glucocorticoids are primarily through glucocorticoid receptor signaling.

Glucocorticoids also have profound impacts on the nervous system. Glucocorticoid receptor and mineralocorticoid receptor are expressed ubiquitously throughout the brain across development (Madalena and Lerch 2017). As glucocorticoids have potent effects on gene transcription, high glucocorticoid levels may be harmful to the rapidly developing neonatal brain (Sapolsky and Meaney 1986; Sapolsky et al. 2000). Indeed, high glucocorticoid levels during early life associated with early life stress result in decreased body growth, decreased adult brain volume, altered hypothalamic-pituitary-adrenal axis responses to challenges, and memory and learning impairment in adulthood (Bilbo, et al. 2005; Bilbo, et al. 2008). In adult animals, glucocorticoids modulate other steroid levels by providing negative feedback on the hypothalamus and pituitary, and effect memory, decision making, cognition, and emotion (Bellavance and Rivest 2014; Franchimont 2004). Overall, the effects of glucocorticoids on the brain are region-specific and, similar to immune tissues, are age and context dependent.

1.3 Local glucocorticoid production

In addition to adrenal GC production, local glucocorticoid production has been demonstrated within numerous target tissues such as skin, lung, intestine, bone marrow, thymus, spleen, and brain (Taves et al. 2011a). Within tissues, glucocorticoids can be synthesized de novo from cholesterol, synthesized from other precursors such as 11-deoxycorticosterone, or regenerated from 11-dehydrocorticosterone via 11β-HSD1 activity. Importantly, it is unclear if synthesis from precursors, or regeneration from 11-

dehydrocorticosterone, is the greater contributor to local corticosterone levels. In lymphoid organs, the evidence for locally produced glucocorticoids includes expression of all necessary steroidogenic enzymes, in vivo corticosterone measurements, and studies of knockout models (Hamden, et al. 2019; Mittelstadt et al. 2018; Taves et al. 2016a; Taves, et al. 2016b; Taves et al. 2015). In the brain, the evidence for locally produced glucocorticoids includes expression of all necessary steroidogenic enzymes (Hamden, et al. 2021; Holmes and Seckl 2006; MacKenzie, et al. 2008; Mellon and Deschepper 1993; Moisan, et al. 1990; Taves et al. 2015), evidence that brain steroidogenic enzyme mRNA levels increase in response to adrenocorticotropic hormone administration (Ye, et al. 2008), brain corticosterone production in vitro (Higo, et al. 2011; MacKenzie, et al. 2000), in vivo corticosterone measurements within the brain after adrenalectomy that increase in response to stressors (Croft, et al. 2008; Higo et al. 2011), and studies of knockout models (Cobice, et al. 2013). Together, these experiments clearly demonstrate that lymphoid organs and brain can produce glucocorticoids. Local glucocorticoid production allows tissues to actively modulate local levels rather than being passive recipients of adrenally-produced glucocorticoids. Local production may be of increased importance during early development when adrenal glucocorticoid production is very low.

1.4 The stress hyporesponsive period

The stress hyporesponsive period is a time of greatly reduced adrenal glucocorticoid production. The stress hyporesponsive period occurs during early neonatal development of many altricial, but not precocial species (Taves et al. 2011a). In mice, the stress hyporesponsive period occurs from approximately postnatal days 2-12 (D'Amato, et al. 1992; Schmidt, et al. 2003). During the stress hyporesponsive period, total blood glucocorticoid levels are the lowest of any point in life and show greatly reduced adrenocortical response to stressors (McCormick, et al. 1998; Sapolsky and Meaney 1986; Stanton and Levine 1990; Wood and Walker 2015). The purpose of the stress hyporesponsive period is not well understood, but it may exist to

protect the developing brain and other developing tissues from deleterious effects of high glucocorticoid levels (Sapolsky and Meaney 1986), while allowing organs such as the thymus, where glucocorticoids are necessary for development, to increase levels via local production. Interestingly, during the stress hyporesponsive period, corticosterone levels are higher in bone marrow, thymus, spleen, and brain than blood levels at baseline (Hamden et al. 2019; Hamden et al. 2021; Taves et al. 2015). Locally increased corticosterone levels suggest an increased need for glucocorticoid receptor signaling and may provide insight into the purpose of the stress hyporesponsive period.

1.5 Early life stress and perinatal programming

During early neonatal development, stressors such as maternal separation and endotoxin exposure, produce small to moderate increases in circulating glucocorticoid levels during the stress hyporesponsive period, but have long-lasting programming effects on the immune system and brain. The effects of stress on early life programming are referred to as perinatal programming and specific effects are dependent on the type of stressor and age of the animal. The effects of neonatal stressors are typically seen following a second stressor in adulthood. The long-term effects of a neonatal stressor range from altered cytokine production in the periphery and brain, to altered hypothalamic-pituitary-adrenal axis activity, to deficits in learning and memory (Bilbo and Nelson 2003; Bilbo et al. 2005; Bilbo et al. 2008; Bowers, et al. 2008). It is unclear how stressors administered during the stress hyporesponsive period have such strong programming effects considering the greatly reduced adrenocortical response. As such, local glucocorticoid production may provide a potential mechanism for the programming effects neonatal stressors have on lymphoid organs and specific brain regions.

1.6 Objectives

In this dissertation, I present three studies examining tissue- and brain region-specific regulation of glucocorticoid levels at baseline and in response to a neonatal stressor.

In Chapter 2, I developed a novel enzyme-linked immunosorbent assay to measure 11-dehydrocorticosterone, because no commercially available method currently exists. I used my novel assay to measure 11-dehydrocorticosterone, alongside corticosterone, in the blood and lymphoid organs of mice at 3 ages, and in the serum/plasma of rats and songbirds after a stressor to establish if 11-dehydrocorticosterone is present at high enough levels in vivo to contribute to local corticosterone production. The novel 11-dehydrocorticosterone assay performed well, demonstrating excellent sensitivity, accuracy, precision, and specificity in all tissue types. 11-Dehydrocorticosterone was measurable in all samples. Specifically, 11-dehydrocorticosterone was ~10 fold lower than corticosterone in blood and lymphoid tissues of mice at all ages and was the lowest during the stress hyporesponsive period. In the serum/plasma of both rats and songbirds, 11-dehydrocorticosterone was lower than corticosterone and increased in response to stress. These data demonstrate that 11-dehydrocorticosterone is present in vivo in mice, rats, and songbirds and might be released from the adrenal glands along with corticosterone. Further, 11-dehydrocorticosterone is present in the lymphoid organs of mice and was highest in the bone marrow, as was corticosterone, suggesting that it may contribute to the local production of corticosterone.

In Chapter 3, I developed a novel liquid chromatography tandem mass spectrometry method to measure a panel of 6 glucocorticoids and the glucocorticoid precursor progesterone in the blood and discrete brain regions of mice at 3 ages. I also measured mRNA levels of steroidogenic enzymes to provide in vivo evidence for local corticosterone production in the mouse brain. Transcripts of steroidogenic enzymes were present in all brain regions at all ages, and local glucocorticoid levels were both brain region- and age- specific. In neonatal animals (during the stress hyporesponsive period), glucocorticoids were higher than blood in 2 out of 3 brain regions, suggesting that the brain can upregulate glucocorticoids with fine spatial resolution, where they may be beneficial for development. Further, glucocorticoids within brain regions were highly correlated but they were not correlated between brain regions or with

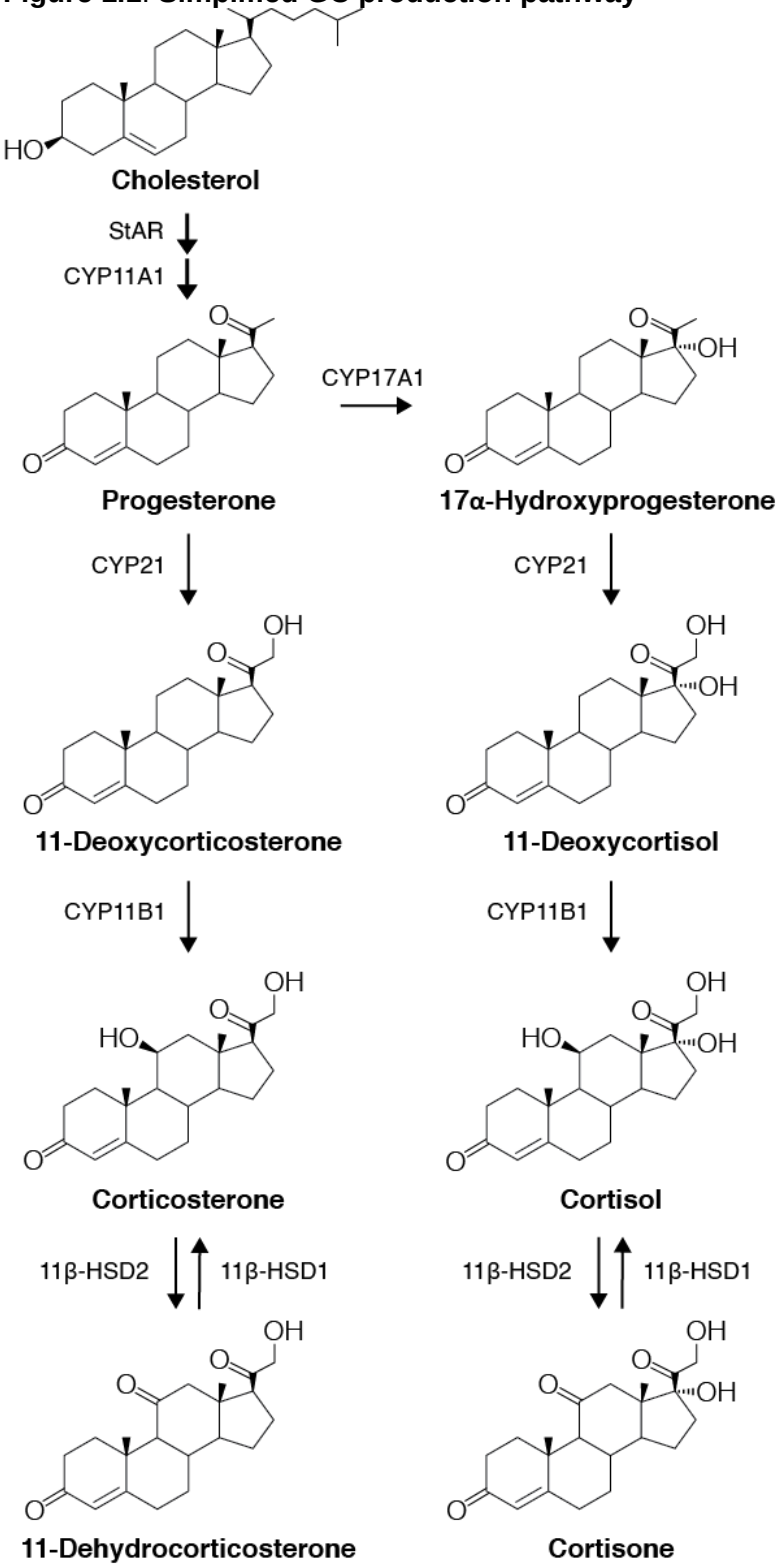
glucocorticoids in the blood. After the stress hyporesponsive period, glucocorticoid levels were lower in all brain regions than in the blood and glucocorticoids were tightly correlated between regions and with the blood. Together, these data suggest that the neonatal brain produces glucocorticoids in a region-dependent fashion and that brain glucocorticoids become more tightly coupled with age.

In Chapter 4, I investigated the effects of a stressor on local glucocorticoid levels in blood and lymphoid organs of neonatal mice at 4 ages to determine if glucocorticoid production changes over the course of the stress hyporesponsive period. The relationship between blood and lymphoid glucocorticoid levels was age specific. Overall, levels were highest before the stress hyporesponsive period, lowest at the start and increased thereafter. During the stress hyporesponsive period, blood glucocorticoid levels increased slightly in response to stress, while lymphoid glucocorticoid increased greatly and surpassed blood increases. These data demonstrate that lymphoid organs can greatly increase glucocorticoid levels even when adrenal production is minimal, and provide a mechanism by which neonatal stressors can modulate lymphocyte development.

Together these studies contribute to a growing body of evidence that lymphoid organs and individual brain regions are not passive recipients of circulating glucocorticoids, but rather modulate local levels based on specific physiological needs. Further, tissue glucocorticoid levels do not reflect blood glucocorticoid levels and can be either higher, similar, or lower, depending on tissue, age, and physiological context. These findings of tissue- and region-specific glucocorticoid regulation demonstrate that the effects of a stressor are not equal on all tissues and that blood glucocorticoid measurements are not reflective of local glucocorticoid signaling.

1.7 Tables and figures

Figure 1.1: **Simplified GC production pathway**



Steroid names (bold) and steroidogenic enzymes involved in glucocorticoid regulation

Chapter 2 Systemic and local levels of 11-dehydrocorticosterone in mice, rats, and birds: Effects of age, sex, and stress

2.1 Introduction

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal glands to coordinate a myriad of physiological processes (Landys, et al. 2006; Munck, et al. 1984; Sapolsky et al. 2000; Toufexis, et al. 2014; Wada 2008). Corticosterone is the predominant circulating GC in many species, including mice, rats, and songbirds (Gong, et al. 2015; Landys et al. 2006; Taves, et al. 2017; Taves et al. 2016b; Taves et al. 2015; van Weerden, et al. 1992). Corticosterone can be converted to the inactive 11-dehydrocorticosterone (DHC) within tissues by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Cottrell and Seckl 2009). Conversely, corticosterone can be regenerated from DHC by 11β-HSD1 (Chapman, et al. 2013) (Figure 2.1). During development, 11β-HSD1 and 2 show marked changes in expression in tissues, including lymphoid organs (Taves et al. 2016a). Furthermore, during neonatal development, rodents display the stress hyporesponsive period (SHRP), in which they have very low levels of circulating GCs and minimal effects of stressors on circulating GCs (Gunnar and Donzella 2002; Sapolsky and Meaney 1986; Stanton and Levine 1990; Wood and Walker 2015). In mice, the SHRP begins at postnatal day (PND) 2 and ends at PND12 (D'Amato et al. 1992; Schmidt et al. 2003).

GCs are powerful regulators of immunity, and bone marrow, thymus, and spleen are particularly sensitive to the effects of GCs. For example, GCs modulate thymocyte selection *via* glucocorticoid receptor binding (Iwata, et al. 1991; Vacchio, et al. 1999). In addition, short-term increases in systemic GCs enhance immunity by promoting proliferation and trafficking of immune cells in the early stages of an immune response (Bowers et al. 2008; Dhabhar, et al. 1996; Wiegers, et al. 1994). In contrast, long-term elevation of GCs suppresses the immune system, as demonstrated by involution of the thymus and spleen (Selye 1936). GCs promote

thymic involution, in part, by increasing apoptosis of developing and mature T cells (Ashwell, et al. 2000).

In mice, lymphoid organs such as bone marrow, thymus, and spleen locally produce GCs, allowing for tissue-specific regulation of GC levels (Schmidt and Soma 2008; Schmidt et al. 2008; Taves et al. 2011a). Evidence for the local production of corticosterone in mice includes expression of all GC-synthetic and -regenerative enzymes, *in vitro* production of corticosterone by thymic epithelial cells, and local elevation of corticosterone during the SHRP (Mittelstadt et al. 2018; Pazirandeh, et al. 1999; Qiao, et al. 2009; Taves et al. 2016a; Taves et al. 2015; Vacchio, et al. 1994). Interestingly, when either the corticosterone precursor, 11-deoxycorticosterone, or the corticosterone metabolite, DHC, are provided *in vitro* at a high concentration (1µM) to bone marrow, thymus, or spleen, all three tissues produce more corticosterone *via* regeneration from DHC than *via* synthesis from 11-deoxycorticosterone (Taves et al. 2016a). However, little is known about endogenous DHC levels in mice during early development, although a few studies have examined adult mice (Alberts, et al. 2005; Cobice et al. 2013; Harris, et al. 2001; Hundertmark, et al. 2002a; Tagawa, et al. 2007; Verma, et al. 2018; Yau, et al. 2001). Thus, it remains unclear to what extent corticosterone is locally regenerated from DHC *in vivo*, especially during the SHRP. Importantly, very little is known about endogenous DHC levels because no immunoassays are currently available to measure DHC.

In addition to mice, rats and songbirds are important animal models for the study of stress physiology. Numerous studies in rats and birds have demonstrated that circulating corticosterone levels rise in response to acute stressors (Romero 2002; Sapolsky et al. 2000), but few studies have measured DHC in rats (Hay and Mormède 1997; Hundertmark, et al. 2002b; Obut, et al. 2004; Tagawa et al. 2007), and no study has measured DHC in birds.

Here, we developed and validated a sensitive and specific enzyme-linked immunosorbent assay (ELISA) to measure DHC. After assay validation, we measured DHC in

circulation and in tissues of mice, rats, and songbirds. In Study 1, corticosterone and DHC were measured in the blood and lymphoid organs of mice at three ages across development. In Studies 2 and 3, circulating corticosterone and DHC were measured before and after restraint stress in adult rats and songbirds, respectively, to determine whether an acute stressor increases DHC levels.

2.2 Materials and methods

2.2.1 DHC assay materials

Sheep anti-11-dehydrocorticosterone-3-carboxymethoxyoxime-thyroglobin (anti-DHC) and biotin-labeled DHC were produced by Dr. Celso Gomez-Sanchez. DHC (Q3690-000) was purchased from Steraloids Inc. (Newport, USA) and dissolved in 100% ethanol (and stored at -80 C). High-binding microtiter plates (82050-720) were purchased from VWR (Edmonton, Canada). Boric acid (B6768), bovine serum albumin (BSA) (A1933), tetramethylbenzidine liquid substrate solution (TMB) (T4444), sulfuric acid (28105), Tween 20 (P1379), ProClin 200 (48171-U), and sodium chloride (S5886) were purchased from Sigma-Aldrich (Oakville, Canada). Tris (161-0716) was purchased from Bio-Rad Laboratories (Mississauga, Canada). Avidin horseradish peroxidase (HRP-Avidin) (A-2014) was purchased from Vector Labs (Burlingame, USA). Sheep IgG (7409005) and donkey anti-sheep IgG (7453800) were purchased from Lampire Biological Laboratories (Pipersville, USA).

2.2.2 DHC assay development and validation

We validated the DHC immunoassay for sensitivity, accuracy, precision, and specificity using multiple approaches.

2.2.2.1 Standard curve development

To develop a standard curve with excellent sensitivity, various concentrations of DHC, primary and secondary antibodies, and biotin-labeled DHC were tested. The standards in the standard curve were 0, 1, 2, 4, 8, 16, 32, and 64 pg/well.

2.2.2.2 Accuracy and intra- and inter-assay variation

To assess accuracy and precision, we extracted (using solid phase extraction (SPE), see Section 2.9 below) and measured two quality controls (QCs) (2pg and 16pg, n=10 each) across multiple assays (n=10).

2.2.2.3 Antibody cross-reactivity

Cross-reactivity of the anti-DHC antibody was tested for closely-related steroids: progesterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol, and cortisone (Table 2.1). Steroids were dissolved in ethanol, diluted to 5% ethanol using ELISA buffer, and then extracted and resuspended as described in Section 2.9.

2.2.2.4 Recovery

Matrix effects were assessed by creating blood, serum, plasma, and tissue pools and then comparing unspiked and spiked samples from the same pool (Table 2.2). Blood, serum, plasma and tissue pools were divided into 2 groups, unspiked and spiked. Exogenous DHC and corticosterone were added to spiked pools; vehicle was added to unspiked pools. Pools were then extracted and resuspended as described in Section 2.9.

2.2.2.5 Serum serial dilution

Matrix effects were also assessed by serial diluting one pool of mouse serum and examining parallelism with the standard curve (n=2/dilution). Serially diluted serum was extracted via SPE and resuspended as described in Section 2.9.

2.2.2.6 Comparison with liquid chromatography tandem mass spectrometry (LC-MS/MS)

We compared the immunoassay with a LC-MS/MS assay. We serially diluted one pool of mouse serum, and serum was extracted via SPE, as described in Section 2.9. DHC was measured by both ELISA and LC-MS/MS (n=2/method/dilution). For LC-MS/MS analysis, dried SPE eluates were resuspended in 50µl 50% MeOH and injected into a Nexera X2 UHPLC system, as previously described (Tobiansky et al., 2018). Steroids were measured by scheduled multiple reaction monitoring, and data were acquired on a Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (Tobiansky, et al. 2018). Picograms of DHC measured by both ELISA and LC-MS/MS were compared. The DHC concentration at each serum dilution was also calculated (n=8/method).

2.2.3 DHC assay procedure

High-binding 96-well microtiter plates were coated with 100µl/well sheep IgG (5µg/µl) in coating buffer (deionized H_2O, 0.1M borate, pH 9.6). Plates were placed on an orbital shaker for 1h at room temperature, and then left overnight at 4°C. Plates were washed three times using a plate washer with 200µl/well washing buffer (phosphate buffered saline (PBS), 0.05% Tween 20). Plates were then blocked with 200µl/well blocking buffer (PBS, 3% BSA) and incubated for 1h at room temperature. Blocking buffer was removed, and 100µl/well donkey anti-sheep IgG in ELISA buffer (deionized H_2O, 1.2% Tris, 0.05% Tween 20, 0.05% ProClin 200, 0.29% NaCl, 0.25% BSA, pH 7.4) was added and plates were incubated for 1h at room temperature. Plates were washed three times with washing buffer. Then 50µl/well of anti-DHC diluted in ELISA buffer was added to all wells, except for the non-specific binding (NSB) wells, in which 50µl/well of ELISA buffer was added. Afterwards, 50µl/well of standards, controls, and samples were added to appropriate wells. Blank and NSB wells received 50µl/well of ELISA buffer. Next 50µl/well of biotin-labeled DHC in ELISA buffer was added to all wells. Plates were shaken for 1h at room temperature. Plates were washed three times with washing buffer. Then, 100µl/well

(HRP-Avidin) in ELISA buffer was added to all wells and plates were shaken for 30min at room temperature. Plates went through a final wash as above. 100µl/well TMB solution was added to all wells and plates were incubated in the dark for 30min at room temperature. The reaction was halted by 100µl/well 1N sulfuric acid. Absorbance at 450nm was read using a plate reader (Victor^3 multilabel reader, Perkin Elmer) within 10min of stopping the reaction.

2.2.4 Corticosterone assay

Corticosterone was quantified using a radioimmunoassay (07120103) from MP Biomedicals (Santa Ana, USA). The lowest standard was further diluted to increase assay sensitivity to 1.56pg/tube (Schmidt and Soma 2008). Corticosterone recovery was estimated by creating blood, serum, plasma, and tissue pools and then comparing unspiked and spiked samples from the same pool (Table 2.2). Due to the low concentration of corticosterone in PND5 mice, recovery was estimated separately because a greater tissue amount was used than for PND23 and PND90 mice (Table 2.2).

2.2.5 Subjects

Study 1. Subjects were male and female C57BL/6J mice at PND5, PND23, and PND90 (PND86-90) (n=10 for PND5 and PND23, n=9 for PND90), with PND0 defined as the first day pups were present in the cage. PND5 was selected because it is within the SHRP, and at PND5, corticosterone levels are higher in lymphoid organs than in whole blood (hereafter "blood") (Taves et al. 2015). Sex of PND5 mice was determined *via* genotyping at the University of British Columbia Genotyping Facility. After weaning at PND19, mice were housed with 2-5 same-sex mice per cage. Mice were housed in a specific pathogen-free colony in the Centre for Disease Modeling at the University of British Columbia. Colony rooms were maintained between 20-22ºC with 40-70% relative humidity. Mice were housed in filter top cages, with beta-chip bedding, under a 14:10 light:dark cycle (lights on 0600-2000h), with free access to water (purified by reverse osmosis and sterilized by chlorination) and food (Teklad Diet 2919 for

breeders and Teklad Diet 2918 after weaning at PND19). A red translucent hut and nestlet were placed in each cage for enrichment.

Study 2. Subjects were adult (9-weeks-old) male and female Long-Evans rats purchased from Charles River (n=9 and 10, respectively). Rats were housed in a conventional animal facility at the University of British Columbia. Rats were housed in same-sex pairs in polyurethane cages, with aspen-chip bedding, under a 12:12 light:dark cycle (lights on 0700-1900h), with free access to tap water and food (Rat Diet 4012, Land O'Lakes, Inc). A small polyvinyl chloride tube was placed in each cage for enrichment.

Study 3. Subjects were free-living adult male song sparrows, *Melospiza melodia* (n=7). They were captured using conspecific song playback and mist nets in October to November (nonbreeding season) in Vancouver, BC (49° 12N, 123° 01W).

All procedures were in compliance with the Canadian Council on Animal Care and protocols were approved by the University of British Columbia Animal Care Committee.

2.2.6 Study 1. Mouse tissue collection

Mouse tissues were collected between 0900-1100h to reduce diurnal variation in steroids. Mice were rapidly and deeply anesthetized with 5% isoflurane (2L/min) and euthanized by rapid decapitation (<3 min from touching the cage). Blood, femurs, tibia, thymus, and spleen were collected in microcentrifuge tubes and immediately frozen on dry ice. Tissues were stored at −80°C until steroids were extracted. Due to the low concentrations of DHC, separate age-matched cohorts of mice were used to quantify corticosterone and DHC. Steroids were extracted from 0.42-25mg or µl of tissue or blood, depending on the age of the subject and the steroid being measured. Bone marrow was extracted from femurs prior to steroid extraction.

2.2.7 Study 2. Rat restraint stress and serum collection

Animals were handled 5min/day for 5 days before serum collection. Prior to restraint, rats were moved to a separate holding room and allowed to habituate for 2h. Individual cages

were then transported to an adjacent room, where rats were placed into acrylic restraint tubes (PLAS Labs, Lansing, MI). Restraint tubes for males (6.35cm inner diameter, 21.6cm length) were larger than restraint tubes for females (5.1cm inner diameter, 20.3cm length). Blood samples were obtained by placing the rat's tail in warm water, making a small incision on a visible tail vein using a scalpel (No. 11), and collecting blood in microcentrifuge tubes. Baseline blood was collected after rats were put into restraint tubes, but within 3min from approaching the cage. Rats remained in restraint rubes, were placed back into their home cages and moved to another room for the duration of the restraint period. An additional blood sample was obtained for all animals after 60min of restraint. Serum was isolated by centrifuging blood at 10,000g for 1min and frozen within 30min of collection, then stored at −80°C until steroids were extracted. Steroids were extracted from 0.1-3µl of serum, depending on the sex of the subject and steroid being measured.

2.2.8 Study 3. Song sparrow restraint stress and plasma collection

Blood samples were obtained from the brachial vein by venipuncture using a sterile needle into heparinized capillary tubes as previously described (Newman, et al. 2008). Initial blood samples were collected within 3min from the time of capture. Birds were then restrained in an opaque cloth bag for 30min and a second blood sample was collected. Capillary tubes were kept cool until centrifugation (within 5h). After centrifugation, plasma was stored at −80°C until steroids were extracted. Steroids were extracted from 2-4µl of plasma, depending on the steroid being measured.

2.2.9 Steroid extraction

Steroids were extracted from all samples by solid-phase extraction (SPE) using C_{18} columns as previously described in detail (Tobiansky et al. 2018). Briefly, tissue samples were weighed and then homogenized in acetonitrile with 0.01% formic acid using a bead mill homogenizer at 4m/s for 30sec. After centrifugation of homogenates, supernatants were

transferred to 7mL glass scintillation vials, dried at 40°C in a vacuum centrifuge, and resuspended in 500µL HPLC-grade methanol. SPE columns were primed with 3mL HPLC-grade hexane, 3mL HPLC-grade acetone, and then 3mL HPLC-grade methanol prior to sample loading. Samples (500µL) were loaded onto columns, and the eluates were collected into glass vials. Then 2mL HPLC-grade methanol was added to the columns, and the eluates were collected into the same vials. The total eluate (2.5mL) was dried at 40°C in a vacuum centrifuge. Samples were resuspended by adding ethanol directly to the dried residues, vortexed briefly, and diluted with buffer to 5% ethanol. Resuspended samples were stored at −20ºC overnight. Corticosterone was quantified using a radioimmunoassay (07120103) from MP Biomedicals (Santa Ana, USA), and DHC was quantified using an enzyme-linked immunosorbent assay (ELISA) as described above.

2.2.10 Statistical analysis

Non-detectable samples (samples below the lowest standard on the standard curve) were set to the average of the water blanks + 2 standard deviations (Taves et al. 2015). To make comparisons between tissues, 1mL of blood was considered to weigh 1g (Schmidt and Soma 2008; Taves, et al. 2011b). The correlation between ELISA and LC-MS/MS values of DHC was determined by Pearson correlation. When necessary, data were log transformed prior to analysis. In Study 1, male and female mice showed similar steroid levels, so sexes were pooled and subjects were analyzed by one-way analysis of variance separately at each age, followed by Dunnett's multiple comparison test. In Study 2, data were analyzed by two-way analysis of variance, and in Study 3, data were analyzed by paired t-tests. All data were analyzed using GraphPad Prism 6 and R version 3.3.3 (Another Canoe). All graphs are presented using the non-transformed data.

2.3 Results

2.3.1 DHC ELISA development and validations

To develop the DHC ELISA, we established a standard curve, and then validated our assay by examining intra- and inter-assay variation, cross-reactivity, recovery, serum serial dilution, and comparison with LC-MS/MS.

2.3.1.1 Standard curve development

The range of the standard curve was optimized to be 1 to 64 pg/well (Figure 2.2), which demonstrates excellent sensitivity. The extracted water blanks across all assays (n=10) were consistently below the lowest standard (1 pg/well).

2.3.1.2 Intra- and inter- assay variation

We used two QCs (2pg and 16pg) and observed average values of 2.08pg and 15.59pg, demonstrating excellent accuracy even for the low QC. The intra-assay variation for the two QCs was 5% and 9%, respectively (n=6). To assess inter-assay variation, we used the same two QCs and measured them across multiple assays (n=8). Inter-assay variation for the two QCs was 15% and 10%, respectively.

2.3.1.3 Antibody cross-reactivity

Cross-reactivity was tested for six closely-related steroids: progesterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol, and cortisone, at 100pg and 1000pg. These steroids were all non-detectable at 100pg. At 1000pg, we detected slight cross-reactivity for 11-deoxycorticosterone (0.51%), corticosterone (0.36%), and cortisone (0.49%) (Table 2.1).

2.3.1.4 Recovery

Recovery was calculated by subtracting unspiked tissue values from spiked tissue values and dividing by the amount of exogenous steroid added. Corticosterone and DHC recovery was species- and tissue-specific (Table 2.2), as seen previously (Taves et al., 2015).

2.3.1.5 Serum serial dilution

Serial diluted serum was plotted as percent bound and was parallel to the standard curve, demonstrating a lack of matrix effects (Figure 2.2).

2.3.1.6 Comparison with liquid chromatography tandem mass spectrometry (LC-MS/MS)

Analysis of DHC in mouse serum by ELISA and LC-MS/MS showed a significant positive linear correlation between the methods (r^2=0.9928; p=0.0036; Figure 2.3A). The serum DHC concentration as measured by ELISA (0.65 ± 0.04 ng/mL) was slightly higher than the serum DHC concentration as measured by LC-MS/MS (0.39 ±0.01 ng/mL) (Figure 2.3B). This is consistent with previous studies that compare immunoassays and LC-MS/MS for other steroids (Fanelli, et al. 2011; Moal, et al. 2007). The primary antibody used in the ELISA shows a slight cross-reactivity with 11-deoxycorticosterone (0.51%), corticosterone (0.36%), and cortisone (0.49%). Only corticosterone (76.4 ± 1.5 ng/mL) and 11-deoxycorticosterone (4.5 ± 0.03 ng/mL) levels were high enough in this pool of serum, as measured by LC-MS/MS, to significantly affect DHC ELISA values. Cortisone was non-detectable. This explains the slight difference in DHC values between LC-MS/MS and ELISA.

2.3.2 Study 1: Systemic and local levels of corticosterone and DHC in mice

In PND5 mice, as expected, corticosterone levels were higher in bone marrow, thymus and spleen than in blood ($F_{3,27}$=90.53; p<0.0001; Figure 2.4A). Also at PND5, DHC levels were higher in bone marrow than in blood, and DHC levels were lower in thymus than in blood ($F_{3,27}$=79.70; p<0.0001; Figure 2.4B).

In PND23 mice, corticosterone levels were higher in blood than in bone marrow, thymus, and spleen ($F_{3,27}$=92.41; p<0.0001; Figure 2.4C). Also at PND23, DHC levels were higher in bone marrow and lower in spleen than in blood ($F_{3,27}$=18.29; p<0.0001; Figure 2.4D).

In PND90 mice, corticosterone levels were higher in blood than in bone marrow ($F_{3,24}$=44.41; p<0.0001; Figure 2.4E). Also at PND90, DHC levels were higher in bone marrow and thymus than in blood ($F_{3,24}$=10.87; p<0.0001; Figure 2.4F).

Circulating corticosterone levels were lowest at PND5, which is consistent with PND5 being in the SHRP (Figure 2.4A, C, E). Unlike corticosterone, DHC levels in blood and tissues generally decreased with age (Figure 2.4B, D, F).

Corticosterone concentrations were generally higher than DHC concentrations. At PND5, corticosterone was approximately 1.5-fold higher than DHC in blood and 2- to 5-fold higher than DHC in lymphoid organs. At PND23, corticosterone was approximately 85-fold higher than DHC in blood and 18- to 53-fold higher than DHC in lymphoid organs. At PND90, corticosterone was approximately 110-fold higher than DHC in blood and 12- to 37-fold higher than DHC in lymphoid organs. Thus, the relationship between corticosterone and DHC is tissue- and age-specific.

2.3.3 Study 2: Systemic levels of corticosterone and DHC in rats

For serum corticosterone levels in rats, there were main effects of Sex ($F_{1,16}$=30.85; P<0.0001) and Restraint ($F_{1,16}$=119.8; p<0.0001) and there was a Sex × Restraint interaction ($F_{1,16}$=6.403; p=0.0223; Figure 2.5A). Corticosterone levels were higher in females than in males and increased more in females than in males after restraint (Figure 2.5A).

For serum DHC levels in rats, there were main effects of Sex ($F_{1,16}$=42.04; p<0.0001) and Restraint ($F_{1,16}$=148.4; p<0.0001) and there was a Sex × Restraint interaction ($F_{1,16}$=14.61; p=0.0015; Figure 2.5B). DHC levels were higher in females than in males and increased more in females than in males after restraint (Figure 2.5B).

At baseline, corticosterone levels were approximately 7-fold higher than DHC levels in male rats and 13-fold higher than DHC levels in female rats. After restraint, corticosterone levels were approximately 12-fold higher than DHC levels in male rats and 20-fold higher than DHC levels in female rats.

2.3.4 Study 3: Corticosterone and DHC in song sparrows

Plasma corticosterone levels in male song sparrows increased in response to restraint stress (t_6=11.08; p<0.0001; Figure 2.6A). Plasma DHC levels also increased in response to restraint stress (t_6=11.07; p<0.0001; Figure 2.6B). Overall, corticosterone levels were approximately 5-fold higher than DHC levels at baseline and approximately 9-fold higher than DHC levels after restraint stress.

2.4 Discussion

Despite the importance of DHC for local regeneration of corticosterone and thus for stress physiology, DHC levels in circulation have rarely been reported in rodents and have never been reported in birds. No immunoassays are currently available to measure DHC. Here, we developed and validated a sensitive and specific ELISA to measure DHC. In Study 1, we quantified corticosterone and DHC in mouse blood, bone marrow, thymus, and spleen across 3 ages. Interestingly, each tissue showed a specific pattern of local steroid regulation. In Study 2, we measured circulating corticosterone and DHC in rats at baseline and after restraint stress. Corticosterone levels were higher than DHC levels in both males and females, and both corticosterone and DHC levels increased more in females than in males after restraint. In Study 3, we measured circulating corticosterone and DHC in male songbirds at baseline and after restraint stress. Both corticosterone and DHC levels increased after restraint. To our knowledge, this is the first study to report 1) DHC levels in neonatal mice, 2) DHC levels in lymphoid organs, 3) local regulation of DHC levels, 4) a sex-specific increase in DHC levels in response to an acute stressor in rats, and 5) DHC levels in any bird.

2.4.1 DHC ELISA development and validations

We validated our DHC ELISA on four key elements: accuracy, precision, sensitivity, and specificity. To assess accuracy, we measured two standards (2pg and 16pg) and observed values within 4% of expected values. To assess precision, we measured the same two standards repeatedly. The intra-assay variation was 5% and 9% for the 2pg and 16pg standards, respectively, and the inter-assay variation was 15% and 10%, respectively. The sensitivity of our ELISA is indicated by the low detection limit of 1pg/well (Figure 2.2). To assess specificity, we used six closely-related steroids. We detected low cross-reactivity to 11-deoxycorticosterone, corticosterone, and cortisone at 0.51%, 0.36%, and 0.49%, respectively. Progesterone, 11-deoxycortisol, and cortisol were non-detectable, even at the 1000pg level (Table 2.2.1). Moreover, a serial dilution of serum showed parallelism with the standard curve, demonstrating that the extraction protocol effectively removed possible matrix effects.

To further assess the accuracy, precision, and specificity of our ELISA, we directly compared it against a LC-MS/MS assay. One pool of mouse serum was serial diluted and measured by both LC-MS/MS and ELISA. The amount of DHC in serum samples as measured by both methods showed a significant positive linear correlation (r^2=0.9928; p=0.0036). As expected, the DHC concentration in mouse serum was slightly higher when measured by ELISA, relative to LC-MS/MS, as seen in comparisons of other steroid assays (Fanelli et al. 2011; Moal et al. 2007). Higher DHC concentrations obtained by ELISA than LC-MS/MS may be explained by the slight cross-reactivity with corticosterone (0.36%), which is present at high levels, and with 11-deoxycorticosterone (0.51%), which is present at low levels. Nonetheless, all of these validations taken together indicate that the DHC ELISA is accurate, precise, sensitive, and specific, and it is a useful tool for measuring DHC levels. This immunoassay is especially useful because there are currently no other DHC immunoassays available.

2.4.2 Study 1: Local regulation of corticosterone and DHC in mouse immune tissues

Local production of GCs may be of particular importance during the SHRP, when circulating GCs are low and tissues are rapidly developing. A growing body of evidence suggests many tissues locally regulate GCs (Taves et al. 2017). GCs play an important role in lymphocyte development, most commonly studied in T-cells. Studies over the last three decades have shown that GCs modulate T-cell affinity for antigens, responsiveness, and proliferation (Mittelstadt, et al. 2012; Pazirandeh et al. 1999; Vacchio and Ashwell 1997; Vacchio et al. 1999). Furthermore, recent studies with mice lacking CYP11B1 in either thymocytes or thymic epithelial cells reveal that thymic epithelial cells, rather than thymocytes, are responsible for most of the locally-produced, bioactive corticosterone. However, this study examined only synthesis of corticosterone from 11-deoxycorticosterone, not regeneration of corticosterone from DHC (Mittelstadt et al. 2018). GCs are less studied in B-cell development and function, but GCs modulate B-cell development and cause apoptosis of B-cells at high concentrations (Cortez, et al. 1996; Garvy, et al. 1993; Gruver-Yates, et al. 2014; Trottier, et al. 2008a). Finally, *in vitro* experiments administering a high concentration (1μM) of either 11-deoxycortiocsterone or DHC suggest that corticosterone is preferentially produced from DHC rather than 11-deoxycorticosterone (Taves et al. 2016a), but DHC levels in blood and lymphoid tissues were previously unknown.

The current data demonstrate that DHC is present in the blood and lymphoid tissues of mice. In bone marrow, both corticosterone and DHC were locally elevated at PND5, but only DHC was locally elevated at PND23 and neither were locally elevated at PND90. Corticosterone levels were lower in bone marrow than blood at PND23 and PND90. In thymus, corticosterone was locally elevated only at PND5 and was lower than in blood at PND23 and PND90. In contrast, DHC levels were lower in thymus than in blood at PND5. DHC was also elevated in thymus at PND90. In spleen, corticosterone was locally elevated at only PND5 and lower than

blood at all other ages. DHC was not locally elevated in spleen at any age and was lower in spleen than blood at PND90 (Figure 2.4). The local elevation of corticosterone in all lymphoid organs at PND5 is consistent with previous findings (Taves et al. 2015). Additionally, our DHC levels at PND90 are consistent with recently reported DHC levels in circulation of adult mice measured by LC-MS/MS and our own LC-MS/MS data (Verma et al. 2018).

The local elevation of DHC in bone marrow and the local reduction of DHC in thymus at PND5 could be explained in three different ways. 1) Tissues might increase local DHC levels by sequestering DHC from blood, in order to be locally regenerated to corticosterone. In this scenario, tissues create a "reservoir" of DHC that can be locally regenerated to corticosterone *via* 11β-HSD1. However, there are no known mechanisms to sequester DHC. 2) Local DHC levels might be a result of local corticosterone synthesis and subsequent metabolism to DHC *via* 11β-HSD2. In this scenario, high local levels of DHC simply reflect high local inactivation of corticosterone. 3) Local DHC levels might be a result of both uptake of DHC from blood and local corticosterone synthesis with subsequent metabolism to DHC *via* 11β-HSD2. This scenario allows for the greatest control over local corticosterone levels by providing a mechanism for both local synthesis and regeneration of corticosterone.

In vitro experiments suggest that tissues increasingly regenerate corticosterone from DHC with age (Taves et al. 2016a), but until now, DHC levels across age in mice were unknown. As expected, baseline corticosterone levels in the blood were very low at PND5 and increased approximately 75-fold at PND23. Surprisingly, DHC levels did not increase in the blood or in tissues after PND5 and were lowest in blood at PND90. These data indicate that DHC is present in circulation and tissues, but might not be at high enough levels outside of the SHRP to substantially contribute to local corticosterone production. It is possible that tissues rely more heavily on regeneration from DHC to contribute to local elevation of corticosterone during the SHRP and more on synthesis afterwards. These data are also in agreement with the idea that the SHRP reduces the harmful effects of circulating GCs on developing tissues, and

thus allows for tissue-specific upregulation of corticosterone based on need (Sapolsky and Meaney 1986; Taves et al. 2015).

Corticosterone locally synthesized from 11-deoxycorticosterone by thymic epithelial cells modulates T-cell development (Mittelstadt et al. 2018), but no study has examined whether corticosterone locally regenerated from DHC is also biologically active. Studies using genetic models, such as 11β-HSD1$^{-/-}$ mice, will be helpful in determining if intracellular regeneration is important for lymphocyte selection (Cobice et al. 2013).

2.4.3 Study 2: Corticosterone and DHC levels after restraint stress in rats

Despite the widespread use of rats in studies of stress physiology, few studies have measured DHC in rats and only one has measured DHC after a stressor (Hundertmark et al. 2002b; Obut et al. 2004; Tagawa et al. 2007). Here, we present data on circulating corticosterone and DHC in rats at baseline and after 60min of restraint stress (Figure 2.5). Our data demonstrate that both corticosterone and DHC levels increase in response to restraint in a sex-specific fashion. Similar to corticosterone, DHC increased in response to stress and increased to a greater extent in females than in males. Additionally, our reported DHC levels at baseline are in line with previously reported values in rats of the same age (Tagawa et al. 2007).

These data indicate that DHC increases in serum in response to restraint stress. Systemic DHC levels may increase in response to stress for two reasons: 1) increased conversion of corticosterone to the inactive DHC, thus protecting tissues from harmful effects of corticosterone or 2) provide a reservoir for specific tissues to intracellularly regenerate DHC to corticosterone. Evidence for the latter includes high levels of 11β-HSD1 in the rat brain (Diaz, et al. 1998; Holmes and Seckl 2006; Wyrwoll, et al. 2011) and prolonged stress-induced increases in blood corticosterone in 11β-HSD1-deficient mice (Harris et al. 2001). Together, the data suggest that 11β-HSD1 plays a pivotal role in HPA axis regulation by utilizing systemic DHC to

intracellularly regenerate corticosterone, to provide increased negative feedback on the HPA axis and prevent a heightened and prolonged stress response.

2.4.4 Study 3: Corticosterone and DHC production after restraint stress in song sparrows

Song sparrows show increases in corticosterone levels in response to restraint stress (Newman and Soma 2009; Newman et al. 2008). While the corticosterone response to restraint is well characterized in song sparrows and many other birds, DHC has yet to be measured in any bird species. Here, we present data on both corticosterone and DHC in male song sparrows at baseline and 30min after restraint. Corticosterone levels were higher than DHC levels at baseline and restraint, and both corticosterone and DHC increased in response to restraint stress (Figure 2.6).

Little is known about 11β-HSD1 in song sparrow tissues, but a recent study in zebra finches was unable to detect 11β-HSD1 mRNA in adult brain, while mRNAs for 11β-HSD2, mineralocorticoid receptor, and glucocorticoid receptors were all detected in brain regions (Rensel, et al. 2018). Another study in birds found no evidence of local GC production in the brain of European starlings but did report evidence of local GC production in lymphoid organs of zebra finches (Schmidt, et al. 2009). These data may indicate that inactivation of corticosterone to DHC by 11β-HSD2, to keep local corticosterone levels low, may be important in the brain of birds, but not in immune tissues. Additional studies in birds of DHC across season, age, and species will be useful.

2.4.5 Possible implications for human health

Cortisol, and its metabolite cortisone, are the predominant circulating GCs in humans (Levine, et al. 2007). A wide array of human tissues express 11β-HSD1—including intestine, adipose tissue, and brain—and intracellular regeneration of cortisol from cortisone via 11β-HSD1 might be important in type 2 diabetes mellitus and neurodegenerative diseases (Rask, et

al. 2001; Walker 2007). Interestingly, corticosterone and DHC are also present at low levels in human blood, and human neuronal cells can convert DHC to corticosterone *in vitro* (Branchaud, et al. 1969; Morris and Williams 1953; Rajan, et al. 1996; Weichselbaum and Margraf 1960). These data raise the possibility that DHC and corticosterone are relevant for human health.

2.5 Conclusions

We developed and validated a sensitive and specific ELISA for measurement of DHC and compared our immunoassay with a LC-MS/MS assay. After validation, we used this DHC immunoassay to measure circulating and local DHC levels in mice and circulating DHC levels in rats and song sparrows. In mice, DHC levels in the blood and lymphoid organs vary by age and tissue. For example, at PND5, DHC was locally elevated in bone marrow, relative to the blood. DHC levels decreased with age, in contrast to corticosterone. These data suggest that age-related changes in DHC levels impact local regeneration of corticosterone from DHC in lymphoid organs. In rats and song sparrows, circulating DHC and corticosterone levels increase in response to restraint stress. This novel assay and these fundamental data on DHC levels provide a basis for future studies of DHC, an important but relatively neglected component of GC physiology.

2.6 Tables and figures

Table 2.1: Cross-reactivities of the anti-11-dehydrocorticosterone (DHC) antibody.

	Progesterone	11-Deoxycorticosterone	11-Deoxycortisol	Corticosterone	Cortisol	Cortisone
100pg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1000pg	n.d.	5.10	n.d.	3.56	n.d.	4.89
Cross-reactivity	< 0.1%	0.51%	< 0.1%	0.36%	< 0.1%	0.49%

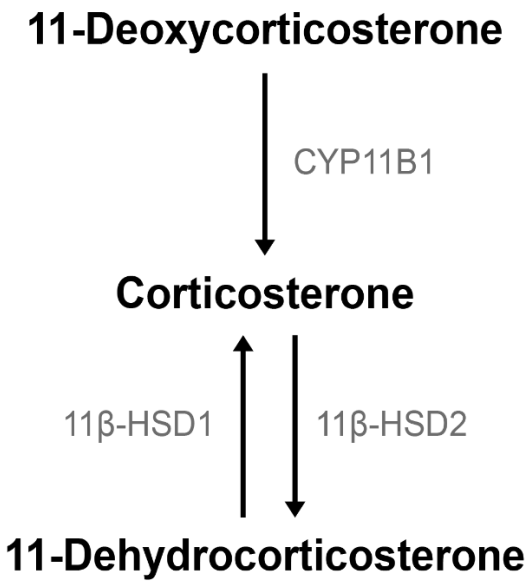
Data represent the mean (n=5) relative to DHC binding (100%), n.d. = not detectable.

Table 2.2: Steroid recovery from different sample types.

	Whole Blood	Serum/ Plasma	Bone Marrow	Thymus	Spleen
Mouse PND5 Corticosterone	129%	n.a.	64%	103%	94%
Mouse PND23 and PND90 Corticosterone	81%	n.a.	81%	76%	90%
Rat Corticosterone	n.a.	50%	n.a.	n.a.	n.a.
Song sparrow Corticosterone	n.a.	63%	n.a.	n.a.	n.a.
Mouse DHC	94%	79%	124%	106%	97%
Rat DHC	n.a.	49%	n.a.	n.a.	n.a.
Song sparrow DHC	n.a.	47%	n.a.	n.a.	n.a.

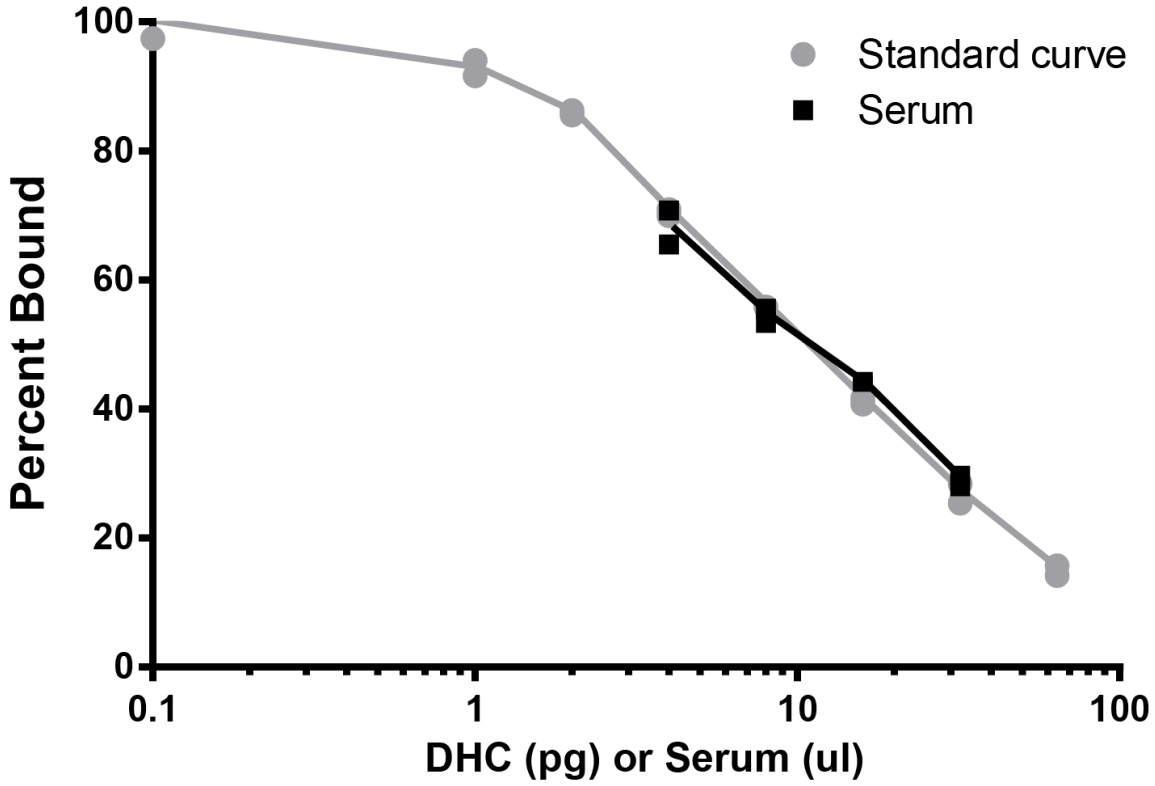
Data represent the mean steroid recovery (n=3). PND = postnatal day; DHC = 11-dehydrocorticosterone; n.a. = not applicable. For mouse DHC, values represent recovery for all ages.

Figure 2.1: **Simplified GC production pathway**



Steroid names in black and steroidogenic enzyme names in grey italics.

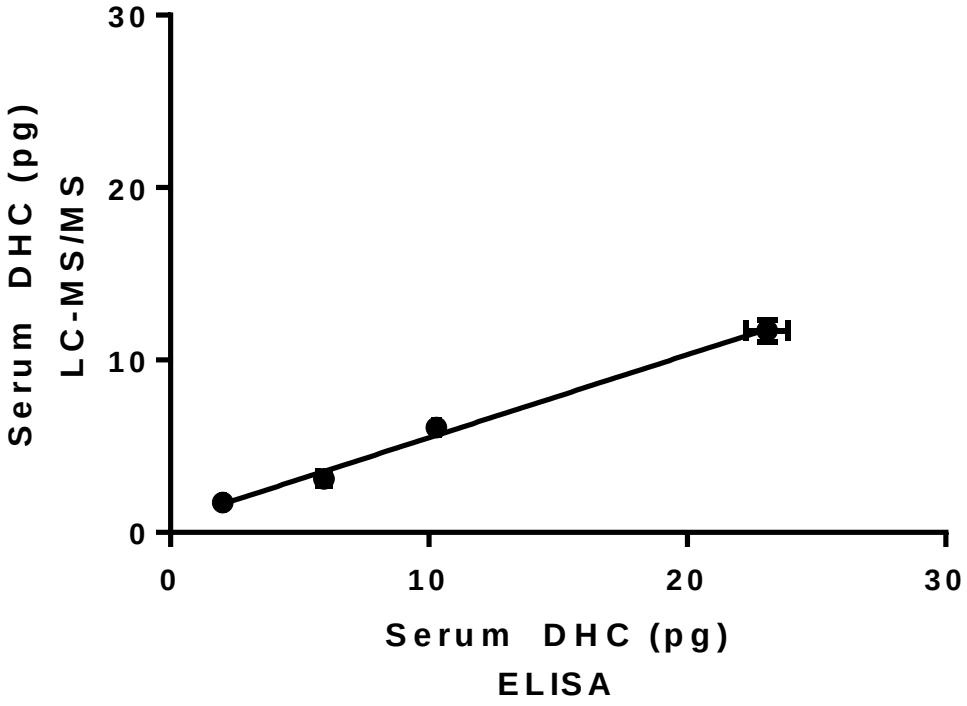
Figure 2.2: Representative 11-dehydrocorticosterone (DHC) standard curve and serum serial dilution.



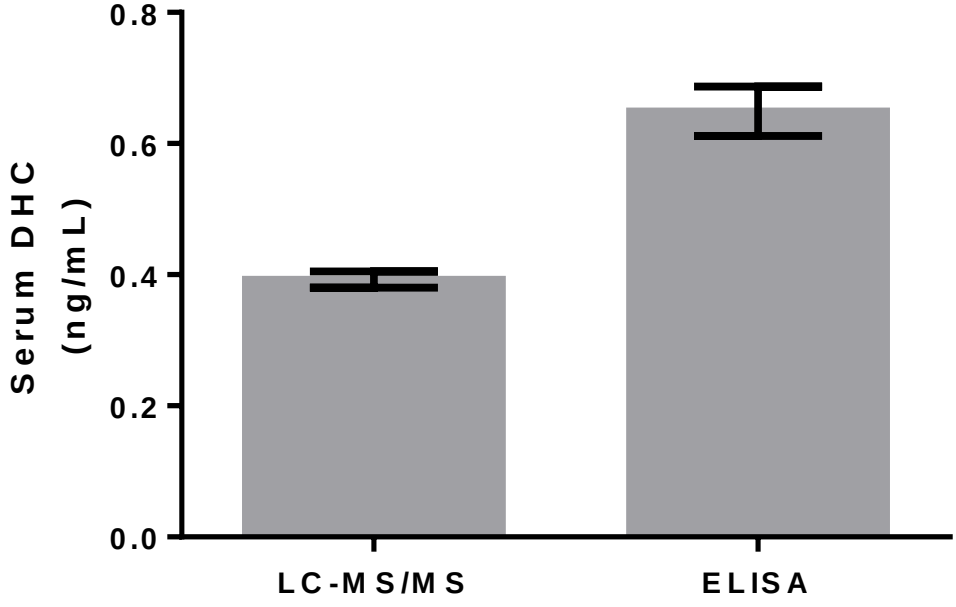
Curves were generated by plotting the percent of biotin-labeled DHC bound versus the concentration of DHC/well on a logarithmic scale. Standard curves and serum were run in duplicate and mean percent bound was determined for each concentration. The lowest standard was 1pg/well, demonstrating excellent assay sensitivity. Serial diluted serum was parallel to the DHC standard curve, indicating a lack of matrix effects. n=2/dilution for both the standard curve and serum serial dilution.

Figure 2.3: Picograms of 11-dehydrocorticosterone (DHC) in serial diluted mouse serum

A

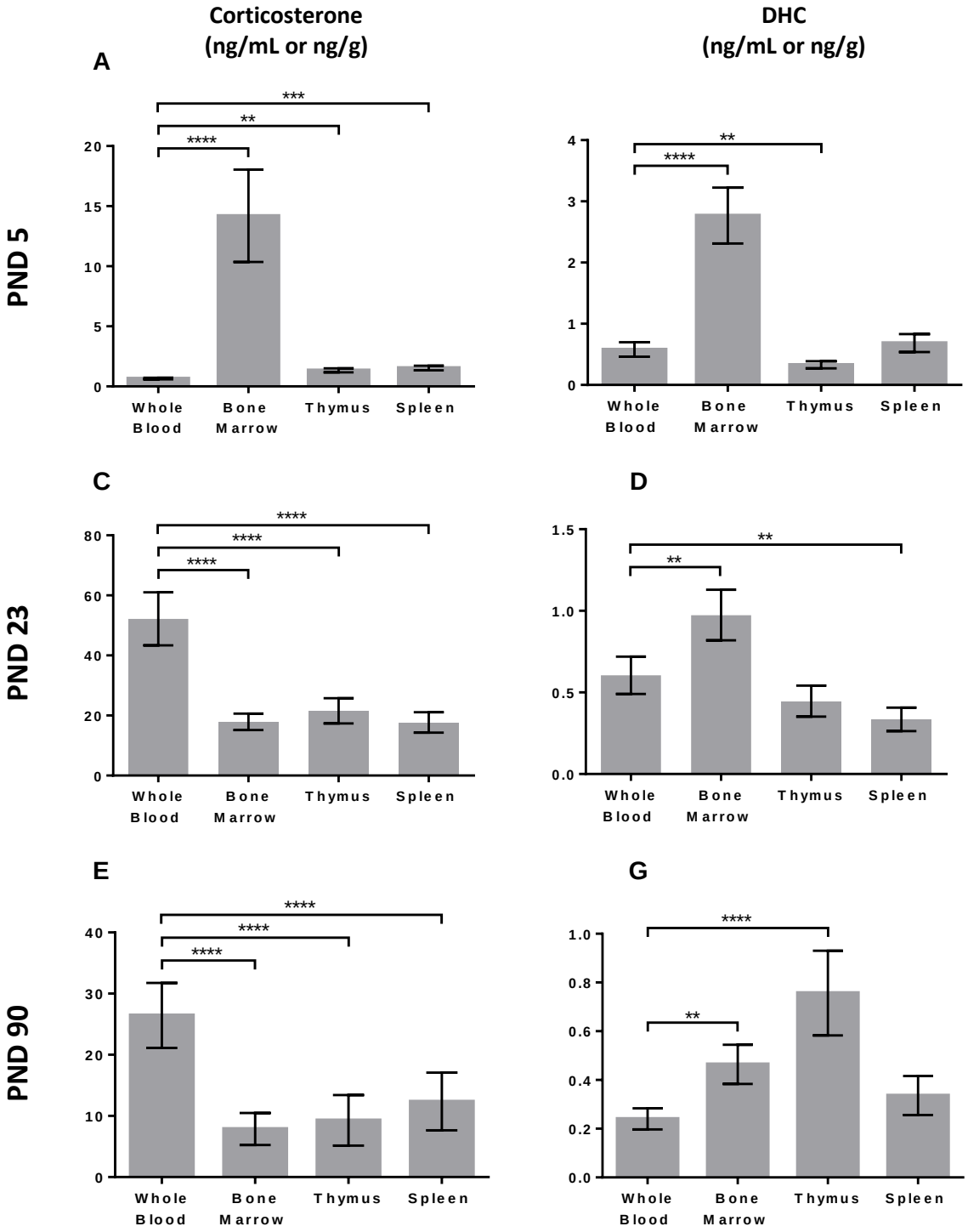


B



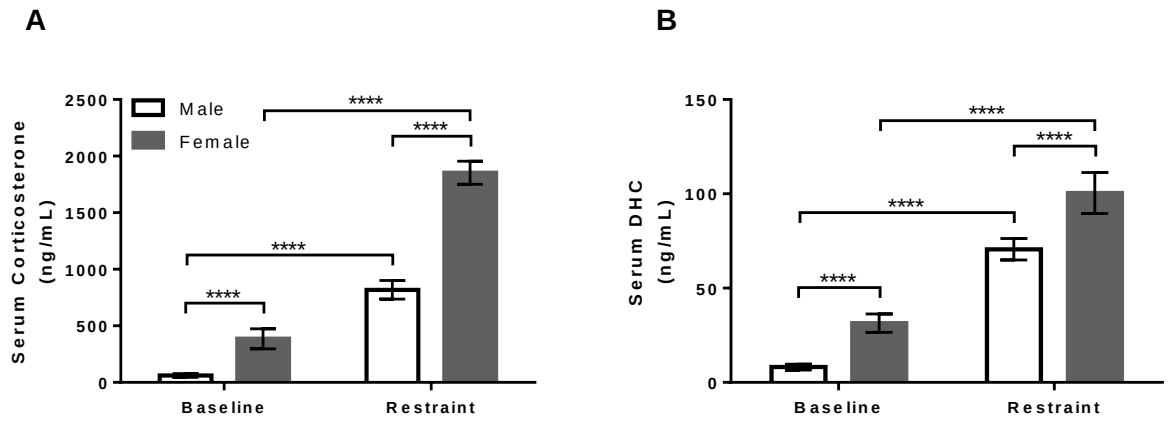
LC-MS/MS and ELISA (n=2/method/dilution) (A) and concentraton of DHC in the same mouse serum measured by LC-MS/MS and ELISA (n=8/method) (B). DHC concentration for each method was determeined by first calculating the average conentration of each volume of serum volume and then calculating the average of all serum DHC concentrations. Data are expressed as mean ± SEM.

Figure 2.4: Concentrations of corticosterone or 11-dehydrocorticosterone in whole blood, bone marrow, thymus, and spleen of mice.



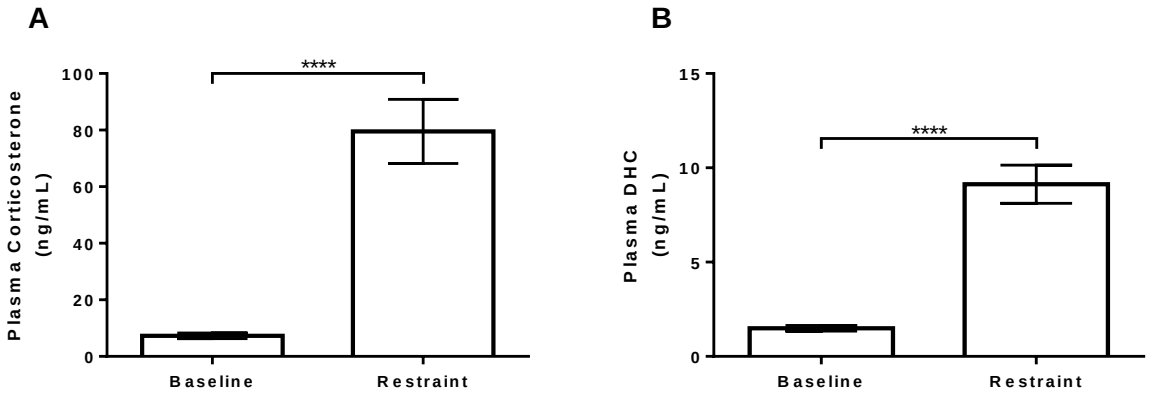
Concentrations of corticosterone (A, C, E) or 11-dehydrocorticosterone (DHC) (B, D, G) in whole blood, bone marrow, thymus, and spleen of mice at PND5 (A and B), PND23 (C and D), and PND90 (E and G). Data are shown as mean ± SEM and tissue levels different from blood levels are indicated as follows * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001. n=10 for both steroids and all tissues at PND5 and PND23, n=9 for both steroids and all tissues at PND90.

Figure 2.5: The effects of sex and restraint for 60min on concentrations of corticosterone or 11-dehydrocorticosterone in serum of adult rats.



The effects of sex and restraint for 60min on concentrations of corticosterone (A) or 11-dehydrocorticosterone (DHC) (B) in serum of adult rats. Data are shown as mean ± SEM. **** P≤0.0001. n=9 for males and females for baseline and restraint.

Figure 2.6: The effect of restraint for 30min on concentrations of corticosterone or 11-dehydrocorticosterone in plasma of free-living non-breeding male song sparrows.



The effect of restraint for 30min on concentrations of corticosterone (A) or 11-dehydrocorticosterone (DHC) (B) in plasma of free-living non-breeding male song sparrows. Data are shown as mean ± SEM. **** P≤0.0001. n=7 for both baseline and restraint.

Chapter 3 Steroid profiling of glucocorticoids in microdissected mouse brain across development

3.1 Introduction

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal glands into circulation to coordinate a myriad of physiological processes (Jacobson and Sapolsky 1991; Munck et al. 1984; Sapolsky et al. 2000; Wada 2008). In mice, corticosterone is the predominant GC secreted into the blood after activation of the hypothalamic-pituitary-adrenal (HPA) axis (Taves et al. 2017; Taves et al. 2016a; Taves et al. 2015). Within target cells, corticosterone binds to intracellular glucocorticoid and mineralocorticoid receptors to modulate transcription of numerous genes (10-20% of the genome) (Oakley and Cidlowski 2013). In addition, corticosterone binds to membrane-associated receptors to exert non-genomic effects (Oakley and Cidlowski 2013). Within tissues, corticosterone can be locally converted to the inactive metabolite 11-dehydrocorticosterone (DHC), by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Cottrell and Seckl 2009), or corticosterone can be locally regenerated from DHC by the enzyme 11β-HSD1 (Figure 1) (Chapman et al. 2013).

In addition, corticosterone can be locally synthesized within numerous tissues from cholesterol or precursors such as 11-deoxycorticosterone (DOC) *via* 11β-hydroxylase (CYP11B1) activity (Davies and MacKenzie 2003; Hamden et al. 2019; Schmidt and Soma 2008; Schmidt et al. 2008; Taves et al. 2011a; Taves et al. 2015; Tsutsui, et al. 1999). Local GC production allows for tissue-specific regulation of GC levels in an array of tissues such as skin, lung, intestine, and thymus (Hostettler, et al. 2012; Kostadinova, et al. 2014; Nikolakis and Zouboulis 2014; Skobowiat, et al. 2011; Talabér, et al. 2013).

There is also evidence for local GC production in the brain. Specific brain regions, particularly the hippocampus (HPC), express all of the necessary GC-synthetic and -regenerative enzymes in rodents and humans (Holmes and Seckl 2006; MacKenzie et al. 2008; Mellon and Deschepper 1993; Moisan et al. 1990; Taves et al. 2015). In adult rats, ACTH

administration increases *Cyp11b1* mRNA in the hypothalamus (HYP) and cerebral cortex (CC) (Ye et al. 2008). *In vitro* experiments have demonstrated production of corticosterone from progesterone by brain tissue (Higo et al. 2011; MacKenzie et al. 2000). Furthermore, corticosterone is still detectable within the brain after adrenalectomy and increases in response to various stressors, suggesting local production of corticosterone (Croft et al. 2008; Higo et al. 2011; Hojo, et al. 2011). Finally, 11β-HSD1-deficient mice have lower levels of corticosterone, and higher levels of DHC in the HPC and CC, relative to wild-type mice (Cobice et al. 2013). These experiments strongly suggest that discrete brain regions locally produce GCs but focused on adult animals.

Local GC production could be of particular importance during neonatal development, as altricial rodents undergo a period of greatly reduced adrenal GC production termed the stress hyporesponsive period (SHRP). The SHRP is characterized by very low blood GC levels, with little to no increase in response to stressors (Gunnar and Donzella 2002; Sapolsky and Meaney 1986; Stanton and Levine 1990; Wood and Walker 2015). In mice, the SHRP begins at postnatal day (PND) 2 and ends approximately at PND12 (D'Amato et al. 1992; Schmidt et al. 2003). The purpose of the SHRP is poorly understood, but it might reduce harmful effects of GCs on body growth and the developing brain (Sapolsky and Meaney 1986). Interestingly, during the SHRP, GC levels are higher in some organs (e.g. thymus) than in blood (Hamden et al. 2019; Taves et al. 2017; Taves et al. 2015). These data raise the exciting possibility that the SHRP does not exist solely to protect organs like the brain from harmful effects of GCs, but to allow for tissue-specific regulation of GCs during development.

Here, in two studies with separate groups of mice, we assessed 1) systemic and local levels of progesterone, corticosterone, its precursor DOC, and its metabolite DHC, as well as cortisol, its precursor 11-deoxycortisol, and its metabolite cortisone and 2) mRNA levels of the steroidogenic enzymes CYP11B1, 11β-HSD1, and 11β-HSD2. We focused on the HPC, CC, and HYP at three key ages in mouse development, PND5 (within the SHRP), PND21 (post-

weaning), and PND90 (adult). Importantly, all three regions express high levels of both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (De Kloet, et al. 1998; McEwen 2012). Specifically, the HPC was selected because of its critical roles in memory and learning (Abrari, et al. 2009; Barbazanges, et al. 1996; Jacobson and Sapolsky 1991); the CC was selected of its high density of GR and relative immaturity at birth (Lee, et al. 2007); and the HYP was selected because of its importance in the HPA axis. We combined the use of the Palkovits punch technique with state-of-the-art liquid chromatography tandem mass spectrometry (LC-MS/MS) and qPCR, in order to measure steroids and steroidogenic enzyme mRNA in discrete brain regions at three major stages of mouse development.

3.2 Materials and methods

3.2.1 Subjects

Two cohorts of female and male (sexes were pooled, see “Data Analysis” below for details) C57BL/6J mice were used in this study — one cohort for steroids (n=10/age) and one cohort for mRNA (n=7/age). Mice were collected at PND5, PND21 and PND90, with PND0 defined as the first day pups were present in the cage. Mice were weaned on PND19. PND5 (neonate within the SHRP), PND21 (post-weaning), and PND90 (adult) were selected because they represent critical points in development. Additionally, previous studies have shown that systemic GC levels and local GC levels within GC-synthetic tissues, such as the thymus, change across these ages (Hamden et al. 2019; Taves et al. 2015). Sex of PND5 mice was determined *via* genotyping at the University of British Columbia Genotyping Facility.

Mice were housed in a specific pathogen-free colony in the Centre for Disease Modeling at the University of British Columbia. Colony rooms were maintained between 20-22°C with 40-70% relative humidity. Mice were housed in ventilated Ehret polysulfone Type IIL filter top cages, with beta-chip bedding, under a 14:10 light:dark cycle (lights on 0600-2000 h), with free access to water (purified by reverse osmosis and sterilized by chlorination) and food (Teklad

Diet 2919 for breeders and Teklad Diet 2918 after weaning). A red translucent hut and nestlet were placed in each cage for enrichment. All procedures complied with the Canadian Council on Animal Care and protocols were approved by the University of British Columbia Animal Care Committee.

3.2.2 Tissue collection

Tissues were collected between 0900-1100 h to reduce possible diurnal variation in steroids (Taves et al. 2015). Mice were rapidly and deeply anesthetized with 5% isoflurane (2 L/min) and euthanized by rapid decapitation (<3 min from disturbing the cage). Trunk whole blood (hereafter “blood”) was collected in a microcentrifuge tube, the brain was rapidly extracted from the skull, and tissues were immediately frozen on crushed dry ice and stored at −80°C. Brains were sectioned into 300µm coronal sections using a MicroHM525 cryostatic microtome (Thermo Fisher Scientific) at −14°C and mounted on Fisherbrand Superfrost™ Plus microscope slides (12-550-17) (Taves et al., 2011; Tobiansky et al., 2018). Using major neuroanatomical landmarks, tissue was microdissected samples from the HPC, CC, and HYP using the Palkovits punch technique with a 1.0 mm inner diameter biopsy punch tool (0.245 mg tissue/punch) (Fisher Scientific, 12-460-402) (Palkovits, 1973). HPC and HYP punches were taken throughout each region. CC punches were taken directly above HPC punches and contain tissue from the retrosplenial area, primary and secondary motor areas, and primary somatosensory cortex (Figure 2). Brain tissue was collected bilaterally. Due to the small brain size at PND5, microdissected samples are enriched with HPC, CC, or HYP, but might contain tissue from adjacent regions. Sections were stained with cresyl violet to verify correct punch placement. No samples were excluded due to incorrect punch placement. Blood (5 µl) and microdissected brain punches (1.715 - 4.165 mg) were placed in 2 mL polypropylene bead ruptor tubes (Sarstedt, 72.694.007) with five ceramic zirconium oxide beads (1.4 mm diameter) (Fisher Scientific, 15340159) for mechanical tissue homogenization with a bead homogenizer

(see below) and stored at −80°C until steroid or mRNA extraction (Supplemental Tables 1 and 2 for data on tissue mass).

3.2.3 Steroid extraction

To track recovery for each sample, 50 µL of deuterated internal standards (progesterone-d_9, corticosterone-d_8, and cortisol-d_4, C/D/N Isotopes Inc., Pointe-Claire, Canada) in 50:50 HPLC-grade methanol:MilliQ water were added to the samples. Progesterone-d_9 was used to track recovery for progesterone; corticosterone-d_8 was used to track recovery for 11-deoxycortisol, DOC, corticosterone, and DHC; cortisol-d_4 was used to track recovery for cortisol and cortisone. Proteins were precipitated and total steroids (bound and unbound) were extracted by adding 1 mL HPLC-grade acetonitrile and homogenizing tissues using a bead mill homogenizer at 4 m/s for 30 sec (Omni International Inc., Kennesaw, GA). Samples were centrifuged for 5 min (16,100 × g), and 1 mL of the supernatant was transferred to a pre-cleaned borosilicate glass culture tube (washed twice with 1 mL HPLC-grade MeOH). Then 500 µL HPLC-grade hexane was added to each culture tube, vortexed, and centrifuged for 2 min (3,200 × g). The hexane was then removed and discarded. Samples were dried in a vacuum centrifuge (60°C for 45 min; ThermoElectron SPD111V). Dried residues were resuspended in 55 µL of 25% HPLC-grade methanol:MilliQ water, vortexed, and centrifuged for 1 min (3,200 × g). The supernatant was then transferred to a 0.6 mL polypropylene microcentrifuge tube and centrifuged for 2 min (16,100 × g). 50 µL of the supernatant was transferred to a glass LC vial insert and stored overnight at −20°C until steroid analysis.

Samples were processed along with blanks and calibration curves. Calibration curves for progesterone, DOC, corticosterone, 11-deoxycortisol, cortisol, and cortisone were made from certified reference standards (Cerilliant Co., Round Rock, TX), and the calibration curve for DHC was made by dissolving crystalline DHC (Steraloids, Q3690-000) in HPLC-grade methanol. Curves were prepared in 50:50 HPLC-grade methanol:MilliQ water.

3.2.4 Steroid analysis by LC-MS/MS

Samples were loaded into an autoinjector at 15°C, and 45 µL of each resuspended sample was injected into a Nexera X2 UHPLC system (Shimadzu Corp., Japan), passed through an in-line filter and a Poroshell 120 HPH C_{18} guard column (2.1 mm), and then separated on a Poroshell 120 HPH C_{18} column (2.1 × 50 mm; 2.7 µm; at 40°C) using 0.1 mM ammonium fluoride in MilliQ water as mobile phase A and HPLC-grade methanol as mobile phase B (MPB). The flow rate was 0.4 mL/min. During loading, MPB was at 10% for 0.5 min, and then the gradient profile began at 42% MPB for 3.5 min before being ramped to 60% MPB until 9.4 min. From 9.4 to 9.5 min the gradient was 60-70% MPB, then it was ramped to 98% MPB until 11.9 min, and finally a column wash at 98% MPB until 13.4 min. The MPB was then returned to starting conditions for 1 min. Total run time was 14.9 min. The autoinjector needle was rinsed externally before and after each sample injection with 100% isopropanol.
Steroids were detected with scheduled multiple reaction monitoring with two mass transitions for progesterone, DOC, corticosterone, DHC, 11-deoxycortisol, cortisol, and cortisone and one mass transition for each internal standard. In some cases, at low concentrations, additional transitions were used to confirm the identity of the steroid. Steroid concentrations were acquired on an AB Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (AB Sciex LLC, MA) in positive electrospray ionization mode for all steroids.

To examine matrix effects, linearity, parallelism, recovery, accuracy, and precision of the LC-MS/MS method, we used several validations. To demonstrate the lack of matrix effects, we created pools of blood and brain tissue, which were serial diluted and compared to the calibration curves to assess parallelism of slopes (Table 3.1). To assess recovery, tissue pools were split into two groups (n=5/group), unspiked and spiked with known amount of steroid. Unspiked tissue pool values were subtracted from spiked tissue pool values and divided by the amount of steroid added. Accuracy and precision were assessed by measuring 2 pg and 200 pg quality controls in triplicate in each assay.

3.2.5 Reverse-transcription quantitative polymerase chain reaction

Total RNA was extracted from microdissected brain tissue (and water blanks) using 1 mL TRIzol (Life Technologies Corporation, Carlsbad, CA). RNA was extracted from samples according to manufacturer's specifications with the additions of 1) 50 µl vacuum grease to the TRIzol to create clear separation between the TRIzol and chloroform and 2) 2 µl glycogen to the isopropanol RNA precipitation step to maximize yield (Tobiansky et al., 2018). RNA quantity and quality were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was reverse transcribed from 200 to 500 ng of total RNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 436814).

Relative expression levels of the genes of interest were assessed using 5'-nuclease probe-based qPCR assays (Integrated DNA Technologies, Inc.) (Taves, Plumb, et al., 2016). The qPCR was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444964) and run at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C (15 seconds) to 60°C on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA) with ROX as a reference dye. All samples were run in duplicate. A reference gene (*Oaz1*) was measured in each sample (de Jonge et al., 2007; Taves, Plumb, et al., 2016). All samples from animals of a given age were run on a single 96-well plate, along with inter-assay controls to allow for comparisons across plates. Relative expression of the genes of interest were calculated and graphed relative to the expression levels of *Cyp11b1* in the HYP for each age separately. Amplification efficiencies of all primers were excellent (90.0% to 91.4%). All technical replicates were quantifiable and CV between duplicates was <10%. Negative controls (water blanks, no template, no reverse transcriptase) all confirmed the specificity of the assays.

3.2.6 Data analysis

The linearity of LC-MS/MS calibration curves was analyzed by Pearson correlation.

For steroid analyses, a value was considered non-detectable if neither transition was present. For groups with less than 40% non-detectable measurements, data were imputed (Tobiansky, et al. 2020; Wei, et al. 2018). Data were imputed for each age and steroid independently. At least 60% of measurements for progesterone, DOC, corticosterone, and DHC were above the lower limit of detection in all groups. To make comparisons between blood and tissues, 1 mL of blood was considered to weigh 1 g (Hamden et al. 2019; Schmidt and Soma 2008; Taves et al. 2015). The relationships between tissue and circulating steroid levels were similar in females and males, and data from both sexes were pooled for further analysis, as before (Taves et al. 2015). The data for each sex are presented separately in Supplemental Tables 3, 4, and 5.

We analyzed our steroid data in complementary ways. First, permutational analysis of variance revealed that steroid levels differ by Age and Steroid, but there was no Age × Steroid interaction, so further analysis focused on steroids at each age separately. Second, at each age, data were analyzed for an effect of Tissue Type by one-way analysis of variance, followed by Dunnett's multiple comparison test (comparisons between blood and each region only). Third, at each age, correlations between steroid levels were examined using Spearman's rho correlations with Benjamini-Hochberg correction for multiple comparisons (Lattin, et al. 2015). Fourth, at each age, multiple linear regression was used to determine factors that predict local corticosterone concentrations within each brain region. Progesterone was not correlated with DOC, corticosterone, or DHC, and thus was removed from correlational and multiple linear regression analyses. Data were converted to nmol/L prior to correlational and multiple linear regression analyses. To ensure homogeneity of variance, data were log-transformed prior to analysis when necessary. All graphs are presented using non-transformed data.

For gene expression data, the mean of the reference gene duplicates for each sample were used to calculate the ΔCq. Statistics were run using the ΔΔCq, whereas the graphs are presented as relative expression levels (relative to the *Cyp11b1* in the HYP of each age) using

the $2^{-\Delta\Delta Cq}$ transformation to aid in visual interpretation (Schmittgen & Livak, 2008). Data were analyzed by two-way analysis of variance followed by Tukey multiple comparisons test. All data were analyzed using GraphPad Prism 8 and R versions 3.5.3 (Great Truth) and 3.6.2 (Dark and Stormy Night).

3.3 Results

3.3.1 LC-MS/MS assay development and validation

The use of two mass transitions and a unique retention time for each analyte provided excellent specificity (Figure 3, Supplemental Table 6). The calibration curve range for each steroid was optimized to be 0.2 to 1000pg per tube and showed excellent linearity for progesterone (r^2=0.9996, p<0.0001), DOC (r^2=0.991, p<0.0001), corticosterone (r^2=0.9998, p<0.0001), DHC (r^2=0.9998, p<0.0001), 11-deoxycortisol (r^2=0.9995, p<0.0001), cortisol (r^2=0.9995, p<0.0001), and cortisone (r^2=0.9966, p<0.0001) (Figure 3.4A and B). To assess matrix effects, tissue pools were created for blood and brain separately, and pools were serial diluted. The slopes for serial-diluted tissue pools and calibration curves were similar for all steroids (Table 3.1). Recovery was assessed by subtracting unspiked tissue pools from spiked tissue pools and dividing by the amount of steroid added (n=5/tissue) (Table 3.1). Recovery was within 17% of the expected value for all steroids in both blood and brain tissue. The assay demonstrated high accuracy and precision, with all measurements within 16% of the expected value (Table 3.1) and an intra-assay CV less than 7% and inter-assay CV less than 5%. Water blanks produced no signal for any steroid in any assay.

3.3.2 Steroid levels

11-Deoxycortisol, cortisol, and cortisone were non-detectable in all blood and brain samples at the three ages. In contrast, progesterone, DOC, corticosterone, and DHC were detectable in nearly all samples (Supplemental Table 7).

Using permutational analysis of variance, steroid levels varied by Age ($F_{1,112}$=127.513; p<0.0001) and Steroid ($F_{3,112}$=30.463; p<0.0001), but there was no Age × Steroid interaction ($F_{3,112}$=0.474; p=0.85). As expected, progesterone levels were highest at PND90, and DOC, corticosterone, and DHC levels were lowest at PND5 (Supplemental Figure 1). Also as expected, DOC and DHC levels were lower than corticosterone levels at all ages. Based on this initial analysis, subsequent analyses focused on the effects of Tissue Type within each age for each steroid.

At PND5, there was no significant difference in progesterone levels between the blood and brain regions ($F_{3,27}$=0.856; p=0.48; Figure 3.5A). Similarly, there was no significant difference in DOC levels between the blood and brain regions ($F_{3,27}$=1.977; p=0.14; Figure 3.5B). For corticosterone levels, there was a significant effect of Tissue Type ($F_{3,27}$=7.153; p=0.001; Figure 3.5C). Relative to corticosterone levels in the blood, levels were higher in the HPC (p=0.0004) and HYP (p=0.007), but not the CC (p=0.07). For DHC, there was also a significant effect of Tissue Type ($F_{3,27}$=5.363; p=0.005; Figure 3.5D). Relative to DHC levels in the blood, levels were lower in the HPC (p=0.04) and HYP (p=0.003), but not the CC (p=0.64).

At PND21, for progesterone, there was a significant effect of Tissue Type ($F_{3,27}$=59.74; p<0.0001; Figure 3.6A). Relative to progesterone levels in the blood, levels were lower in the HPC (p=0.0003), CC (p<0.0001), and HYP (p<0.0001). For DOC, there was a significant effect of Tissue Type ($F_{3,27}$=4.547; p=0.01; Figure 3.6B). Relative to DOC levels in the blood, levels were lower in the CC (p=0.007) and HYP (p=0.01), but not the HPC (p=0.22). For corticosterone, there was a significant effect of Tissue Type ($F_{3,27}$=49.720; p<0.0001; Figure 3.6C). Relative to corticosterone levels in the blood, levels were lower in all brain regions (p<0.0001 in all 3 cases). For DHC, there was a significant effect of Tissue Type ($F_{3,27}$=29.96; p<0.0001; Figure 3.6D). Relative to DHC levels in the blood, levels were lower in the HYP (p<0.0001), but not the HPC (p=0.06) or the CC (p=0.08).

At PND90, there was no significant difference in progesterone levels between the blood and brain regions ($F_{3,27}$=1.438; p=0.25; Figure 3.7A). Similarly, there was no significant difference in DOC levels between the blood and brain regions ($F_{3,27}$=0.100; p=0.96; Figure 3.7B). For corticosterone, there was a significant effect of Tissue Type ($F_{3,27}$=11.970; p<0.0001; Figure 3.7C). Relative to corticosterone levels in the blood, levels were lower in the HPC (p=0.0007), CC (p<0.0001) and HYP (p<0.0001). There was no significant difference in DHC levels between the blood and brain regions ($F_{3,27}$=2.821; p=0.06; Figure 3.7D).

Pairwise correlations (Spearman's rho with Benjamini-Hochberg correction) between DOC, corticosterone, and DHC levels in blood, HPC, CC, HYP within each age are presented as correlation matrices (Figure 3.8). Two main patterns emerged. First, DOC, corticosterone, and DHC levels were more correlated within and between brain regions than between brain regions and blood. Second, correlations within and between brain regions became stronger with age. Progesterone was not correlated with DOC, corticosterone, or DHC in the blood or brain regions; thus, it was excluded from correlational analysis and multiple linear regression (below).

Using multiple linear regression, we were able to significantly predict local corticosterone levels in the HPC, CC, and HYP at each age. The model included corticosterone levels in the blood as well as DOC and DHC levels in the blood and brain regions. The significant predictors of local corticosterone levels are presented in Table 3.2. In all regions and for all ages, local corticosterone levels were predicted by the full model that included, blood DOC, blood corticosterone, blood DHC, brain DOC, and brain DHC levels. At PND5, local DOC levels were the best predictor of local corticosterone levels in the HPC, CC, and HYP. Outside of the SHRP, local DHC levels were the best predictors of local corticosterone levels in the HPC and CC at PND21 and CC at PND90.

3.3.3 Steroidogenic enzyme mRNA levels

A second cohort of animals was used to measure the expression of *Cyp11b1, Hsd11b1,* and *Hsd11b2* in the HPC, CC, and HYP at PND5, PND21, and PND90. We were able to detect these 3 transcripts in all samples. Overall, *Hsd11b1* mRNA levels were higher than *Cyp11b1* and *Hsd11b2* mRNA levels. The mRNA levels varied across age for each transcript separately (Supplemental Figure 2). Interestingly, *Hsd11b1* mRNA levels increased by PND21 in the HPC and CC, but not in the HYP. *Cyp11b1* mRNA levels increased with age in only the HYP, and *Hsd11b2* mRNA levels decreased with age in only the HPC.

At PND5, there were main effects of Region ($F_{2,54}$=8.172; p=0.0008) and Gene ($F_{2,54}$=368.6; p<0.0001) as well as a Region × Gene interaction ($F_{4,54}$=15.17; p<0.0001; Figure 3.9A). *Hsd11b1* mRNA levels were higher than *Hsd11b2* and *Cyp11b1* mRNA levels in the HPC, CC, and HYP (p<0.0001, in all cases). *Hsd11b2* mRNA levels were higher than *Cyp11b1* mRNA levels in the HPC (p<0.0001) and CC (p=0.006), but not in the HYP (p=0.98) (Figure 3.9A).

At PND21, there were main effects of Region ($F_{2,54}$=3.194; p=0.05) and Gene ($F_{2,54}$=684.8; p<0.0001) as well as a Region × Gene interaction ($F_{4,54}$=27.20; p<0.0001; Figure 3.9B). *Hsd11b1* mRNA levels were higher than *Hsd11b2* and *Cyp11b1* mRNA levels in the HPC, CC, and HYP (p<0.0001, in all cases). *Hsd11b2* mRNA levels were higher than *Cyp11b1* mRNA levels in the HYP (p=0.002), but not in the HPC (p=0.89) or CC (p=0.13) (Figure 3.9B).

At PND90, there was no significant main effect of Region ($F_{2,54}$=1.820; p=0.17), but there was an effect of Gene ($F_{2,54}$=519.4; p<0.0001) and a Region × Gene interaction ($F_{4,54}$=20.180; p<0.0001; Figure 3.9C). *Hsd11b1* mRNA levels were higher than *Hsd11b2* and *Cyp11b1* mRNA levels in the HPC, CC, and HYP (p<0.0001, in all cases). *Hsd11b2* mRNA levels were lower than *Cyp11b1* mRNA levels in the CC (p<0.0001), but not in the HPC (p=0.61) or HYP (p=0.82) (Figure 3.9C).

3.4 Discussion

Here, we developed an ultrasensitive and specific LC-MS/MS method to quantify 7 steroids in a single small sample. We then used this LC-MS/MS method to measure steroids in the blood and 3 microdissected brain regions of mice at 3 major stages in development. Corticosterone levels are higher in the HPC and HYP than in the blood at PND5, but higher in the blood than in brain at PND21 and PND90. At each age, local corticosterone levels are more closely correlated with local DOC, corticosterone, and DHC levels within and between brain regions than with systemic DOC, corticosterone, and DHC levels in the blood. Correlations within and between brain regions increase with age. We were able to predict local corticosterone levels from local DOC and DHC levels, but not from blood DOC, corticosterone, or DHC levels at any age. Finally, transcripts for the steroidogenic enzymes, *Cyp11b1*, *Hsd11b1,* and *Hsd11b2* are present in all brain regions and at all ages, but *Hsd11b1* mRNA levels are the highest. Together, these data support the hypothesis that corticosterone is locally produced in the mouse brain, particularly during early development.

3.4.1 LC-MS/MS development and validations

Our ultra-sensitive LC-MS/MS method combined with use of the Palkovits punch allows for simultaneous measurement of a panel of 7 steroids in a single sample with as little as 1.7 mg tissue. By microdissecting brain tissue, we are able to measure steroids in discrete brain regions even when the brain is very small, such as mice at PND5. To our knowledge, this is the first study to measure steroids in microdissected mouse brain, during early development or adulthood. Microdissection allows for greatly increased spatial specificity and reproducibility, but yields small amounts of tissue per sample and requires a very sensitive technique for steroid measurement, such as LC-MS/MS (Jalabert, et al.).

We validated our LC-MS/MS method, including assessments of sensitivity, matrix effects, recovery, specificity, accuracy, and precision. The calibration curves span a wide range

(0.2 to 1000 pg per sample) and are linear even at very low steroid concentrations (Figure 3.4). The lower limit of quantification is 0.2 pg per sample, which is 10 times more sensitive than most immunoassays (Newman and Soma 2009; Schmidt et al. 2009; Taves et al. 2015) (Figure 3.4). The lack of matrix effects is demonstrated by the parallelism of serially diluted tissue to the calibration curves. Moreover, recovery from blood and brain tissue was excellent for all steroids (Table 3.1). Specificity is ensured by using two mass transitions and a unique retention time for each steroid. Lack of specificity is a common problem with immunoassays, especially at low analyte levels (Hamden et al. 2019; Jalabert et al. ; Moal et al. 2007). Accuracy and precision are very good for all steroids.

LC-MS/MS allows for the simultaneous measurement of multiple analytes in a single sample, potentially generating large data sets. This rapid liquid-liquid extraction protocol, combined with the short run time and lack of need for analyte derivatization, generates data on numerous steroids in a short time. The measurement of active GCs as well as their precursors and metabolites provides greater insight into the systemic and local steroid environments, and the large data set permits additional statistical approaches as described below (Lattin et al. 2015; Storbeck, et al. 2019). In the future, we will include additional steroids such as aldosterone and allopregnanolone, as they are also neurosteroids (Brunton, et al. 2012; Brunton, et al. 2009; Varga, et al. 2013).

3.4.2 Steroids and steroidogenic enzymes at PND5

Consistent with previous reports, corticosterone levels at PND5 are very low (D'Amato et al. 1992; Schmidt et al. 2003). Here, corticosterone is elevated specifically in the HPC and HYP, but not in the CC, revealing the spatial heterogeneity of brain GCs at PND5. A previous study also observed local elevation of corticosterone in the brain at PND5, but this study used the whole brain rather than microdissected regions (Taves et al. 2015). Progesterone levels are low and show no significant difference between the blood and any brain region. Interestingly, DHC

is locally reduced in the HPC and HYP, but not in the CC. Together, these data suggest that corticosterone is locally elevated in the HPC and HYP by 1) lower metabolism by 11β-HSD2, and/or 2) higher regeneration by 11β-HSD1.

To clarify the mechanisms underlying the local elevation of corticosterone in the HPC and HYP, we performed correlational and multiple linear regression analyses. At PND5, DOC, corticosterone, and DHC levels are more highly correlated within each brain region than with each steroid's level in the blood. Within each brain region, corticosterone is positively correlated with both DOC and DHC. If elevated corticosterone levels within the HPC and HYP were solely due to regeneration from DHC, we might expect a negative correlation between local corticosterone and local DHC. Additionally, the regression analysis revealed that local DOC levels are a significant predictor of local corticosterone levels in the HPC, CC, and HYP. These data suggest local production of corticosterone from DOC and reduced metabolism to DHC.

As there was no elevation of corticosterone levels in the CC, we hypothesized that CC corticosterone is primarily derived from the blood and, thus, would be strongly and positively correlated with blood corticosterone. However, CC corticosterone levels are more closely correlated with CC DOC and CC DHC levels than with blood DOC, blood corticosterone, and blood DHC levels. The regression model could only predict CC corticosterone levels when including systemic DOC, corticosterone, and DHC as well as local DOC and DHC levels. Taken together, the correlational and regression analyses suggest that CC corticosterone levels are influenced by both systemic steroid levels and local corticosterone production and inactivation by steroidogenic enzymes.

For local production and inactivation of GCs, each brain region must contain *Cyp11b1*, *Hsd11b1*, and/or *Hsd11b2*. All 3 transcripts were present in all 3 regions. *Hsd11b1* mRNA levels are much higher than *Cyp11b1* and *Hsd11b2* mRNA levels in all 3 regions. Given the region-specific elevation of corticosterone, we expected regional differences in transcript levels (e.g. lower levels of *Hsd11b1* or higher levels of *Hsd11b2* in the CC). These regional differences

were not observed. However, mRNA levels may not correlate with protein levels (Anderson and Seilhamer 1997). Moreover, steroidogenic enzyme activity is modulated by a variety of factors, including post-translational modifications (e.g. phosphorylation) and cofactor availability (Arakane, et al. 1997; Stewart and Krozowski 1999). Nonetheless, these gene expression data complement the steroid data and further support the hypothesis that these regions produce corticosterone at PND5.

The SHRP is traditionally thought to protect the brain from the deleterious effects of high GC levels; however, GCs are crucial for brain development (Matthews 2001; Sapolsky and Meaney 1986). The present data raise the hypothesis that the SHRP may not exist to protect the entire brain from GCs during development, but rather to allow for region-specific corticosterone levels. These results may help to explain the region-specific effects of neonatal stress on brain development. Numerous studies have demonstrated the adverse effects of early-life stress, such as dysregulation of the HPC and HYP, resulting in impaired memory and HPA axis activity (Bath, et al. 2016; Bilbo et al. 2005; Naninck, et al. 2015). Selective GC production in specific regions may allow corticosterone to exert greater effects where it is needed, particularly during stress, while protecting other regions such the CC. Future studies will examine other regions (e.g. amygdala and prefrontal cortex) and the effects of an acute stressor on local corticosterone production.

3.4.3 Steroids and steroidogenic enzymes at PND21 and PND90

Outside of the SHRP, PND21 and PND90 subjects have high systemic corticosterone levels, and thus local corticosterone synthesis might be less important. Accordingly, corticosterone levels are lower in the brain than in the blood at PND21 and PND90, with similar levels across regions. These data are in agreement with a previous report, which measured corticosterone in the whole brains of adult mice and rats (Butte, et al. 1972). However, another study reported no difference between blood and brain corticosterone levels in PND15 and

PND60 mice (Taves et al., 2015). Progesterone levels are lower in the brain than in the blood at PND21 but not at PND90. DOC and DHC levels are low, compared to the high corticosterone levels at both ages. Brain DOC and DHC levels show small differences relative to blood levels at PND21, but not at PND90. Together, these data raise the hypothesis that brain corticosterone at PND21 and PND90 is primarily derived from circulating steroids in the blood (but see below). Importantly, we measured total (bound and unbound) corticosterone levels in this study. Circulating corticosteroid binding globulin (CBG) levels are very low at PND5 and higher in adulthood (Viau et al. 1996). Due to higher CBG levels at PND21 and PND90, the levels of free corticosterone in the blood may be lower than the levels of corticosterone in the brain.

If brain corticosterone is primarily derived from blood corticosterone at PND21 and PND90, then there would be strong correlations between blood and brain corticosterone in all regions. However, correlations were stronger between brain corticosterone, brain DOC, and brain DHC than between brain corticosterone and blood DOC, blood corticosterone, and blood DHC in all three regions. Correlations between DOC, corticosterone, and DHC within a brain region suggest local production of corticosterone. Furthermore, only local DHC is a significant individual predictor of local corticosterone in the HPC at PND21 and in the CC at PND21 and PND90. These results suggest that brain corticosterone levels at PND21 and PND90 are a result, in part, of local production rather than simply passive receipt of corticosterone from the circulation.

At both PND21 and PND90, all 3 regions express all 3 GC-modulating enzymes, suggesting the ability for local corticosterone production and inactivation, similar to previous reports in adult rats (Sakai, et al. 1992; Ye et al. 2008). *Hsd11b1* levels are the highest in all 3 regions and similar between PND21 and PND90, consistent with previous studies. The high expression of *Hsd11b1* is somewhat surprising given the low DHC levels in both the blood and brain. Rather, we expected to see high expression of *Hsd11b2* to metabolize corticosterone in the brain (Robson, et al. 1998). *Cyp11b1* and *Hsd11b2* transcript levels are generally similar,

with some minor region- and age- specific differences as previously reported (Taves et al. 2015). As mentioned previously, enzyme transcript levels might differ from protein levels, and steroidogenic enzymes are subject to post-translational modifications that rapidly affect enzyme activity (Pandey and Miller 2005). In addition, activities of steroidogenic enzymes (e.g. 11β-HSD1 and 11β-HSD2) are dependent on cofactors such as $NADP^+$ and NADPH (Monder and White 1993). Finally, 11β-HSD1 and 11β-HSD2 also regulate levels of androgens and estrogens (Barnard, et al. 2020), which may explain why transcripts of these enzymes did not correspond with GC levels in this study. Future studies should investigate the activities of steroidogenic enzymes in these brain regions.

There are at least 4 major mechanisms by which brain regions can achieve a particular level of corticosterone. First, free corticosterone can passively diffuse into the brain from the blood. Second, brain regions can locally synthesize corticosterone from cholesterol. Third, brain regions can locally metabolize corticosterone to DHC or regenerate corticosterone from local DHC. Fourth, systemic DOC and DHC can enter the brain and be locally converted to corticosterone. At PND21 and PND90, our data suggest that a combination these 4 mechanisms is most likely, with regeneration from local DHC being most important. Additionally, studies in *Hsd11b1*-deficient mice have demonstrated lower corticosterone levels and higher DHC levels in the brain compared to wildtype mice (Cobice et al. 2013). As corticosterone levels are higher in the blood than in any brain region at both PND21 and PND90, local production from either precursors or metabolites would seem to be unnecessary. Nevertheless, these data suggest local production of corticosterone occurs even in adult animals, perhaps allowing for rapid modulation of corticosterone levels within specific brain regions. Studies of adrenalectomized animals might elucidate the roles of systemic DOC, corticosterone, and DHC in the local production of corticosterone within the brain. If brain regions locally produce corticosterone *de novo* from cholesterol, then adrenalectomized animals should have detectable corticosterone within the brain, as has been shown in the HPC of adult rats (Higo et al. 2011).

However, if corticosterone is not locally produced *de novo* from cholesterol, then corticosterone should not be detectable in the brain of adrenalectomized animals. Adrenalectomy in neonatal mice is very difficult due to their small size, but one study has reported successful adrenalectomy in mice at PND3 (Ogasawara, et al. 1983). Adrenalectomy has been commonly performed in neonatal rats (Inomata and Nakamura 1989; Walker, et al. 1990) and in adult mice and rats.

Consistent with previous reports, systemic and local corticosterone levels increase dramatically after the SHRP, while progesterone, DOC, and DHC levels increase slightly (Hamden et al. 2019; Taves et al. 2015). A comparison of the correlational matrices across ages shows that correlations between brain DOC, brain corticosterone, and brain DHC *within* a region become more frequent and stronger with age. This suggests that local corticosterone production is still important in adulthood when circulating corticosterone levels are high. Further, correlations of brain DOC, brain corticosterone, and brain DHC *between* brain regions also become more frequent and stronger. These data suggest that the immature PND5 brain displays greater regional heterogeneity, or modularity, with respect to GC signaling, while the mature PND90 brain displays greater regional connectivity, or coupling. Finally, multiple linear regression analyses show that local DOC and local DHC are significant predictors of local corticosterone levels at PND5, while local DHC levels only are significant predictors at PND21 and PND90. Previous studies of corticosterone production in adult mouse immune tissues also suggest that DHC is more important than DOC in local corticosterone production (Taves et al. 2016a). The increases in *Hsd11b1* mRNA levels with age are also consistent with the importance of DHC. Similarly, using the whole mouse brain, a previous study reported higher *mRNA* levels for *Hsd11b1* than *Cyp11b1* or *Hsd11b2* at adulthood, and an increase in *Hsd11b1 mRNA* levels outside of the SHRP (Taves et al., 2015). Changes in *mRNA* levels have also been shown for other steroidogenic enzymes in the rat during development (Kohchi, et al. 1998; Taves et al. 2015). In conjunction with the data presented here, these studies suggest local

corticosterone production in the rodent brain varies across regions and throughout development.

3.5 Conclusions

LC-MS/MS is currently the “gold standard” in steroid analysis, providing the greatest sensitivity and specificity as well as enabling the simultaneous measurement of multiple steroids in a single small sample. Our LC-MS/MS method combined with the Palkovits punch technique for mouse brain allows for the first targeted analysis of GCs, GC precursors, and GC metabolites during key points in development. The collection of numerous data points from a single subject is amenable to novel methods of data analysis (Lattin et al. 2015). Taken together, these data clearly demonstrate that corticosterone levels within the brain are not a simple reflection of corticosterone levels in the blood. Moreover, the relationships between systemic and local DOC, corticosterone, and DHC differ across development, with greater spatial heterogeneity of brain corticosterone levels in early development and greater coupling in adulthood.

3.6 Tables and figures

Table 3.1: LC-MS/MS assay accuracy, recovery, and lack of matrix effects

		Neat	Blood (1-10µl)	Brain (1-5mg)
Progesterone	**Slope** (%Δ)	**0.074** (0.00)	**0.072** (-2.83)	**0.071** (-4.15)
	%Recovery (%CV)	**112.27** (2.83)	**104.88** (2.95)	**101.47** (5.91)
11-Deoxycorticosterone	**Slope** (%Δ)	**0.354** (0.00)	**0.353** (-0.11)	**0.306** (-13.62)
	%Recovery (%CV)	**116.40** (5.70)	**112.13** (2.34)	**93.75** (7.04)
Corticosterone	**Slope** (%Δ)	**0.135** (0.00)	**0.137** (1.11)	**0.140** (3.34)
	%Recovery (%CV)	**111.30** (2.37)	**101.79** (1.39)	**97.01** (3.28)
11-Dehydrocorticosterone	**Slope** (%Δ)	**0.101** (0.00)	**0.118** (16.21)	**0.098** (-3.33)
	%Recovery (%CV)	**94.85** (3.45)	**96.68** (2.20)	**107.06** (2.09)

Note: To calculate steroid recovery in each matrix, unspiked (n=5) homogenate steroid values were subtracted from spiked (n=5) homogenate values.

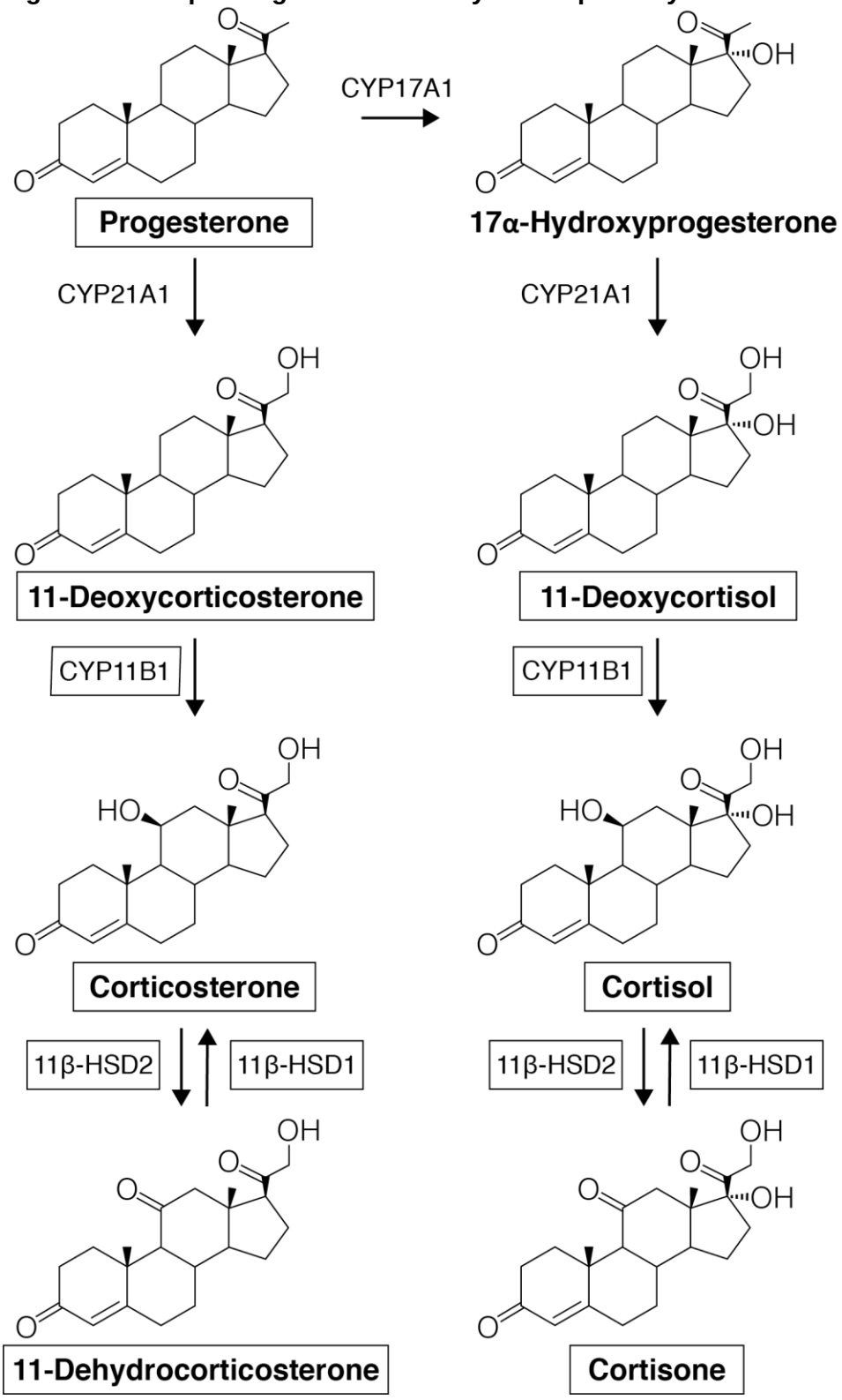
Table 3.2: Multiple linear regression analysis examining the factors that predict local corticosterone levels

Age	Region	r^2	$F_{(5,4)}$	p	Predictor	$t_{(4)}$	p
PND5	HPC	0.94	27.95	0.003	HPC DOC	8.33	0.001
					HPC DHC	4.17	0.01
	CC	0.88	14.05	0.01	CC DOC	4.03	0.02
	HYP	0.90	16.45	0.009	HYP DOC	2.87	0.05
PND21	HPC	0.94	28.33	0.003	HPC DHC	4.46	0.01
	CC	0.91	18.51	0.007	CC DHC	4.53	0.01
	HYP	0.87	12.96	0.01	n.s.	n.s.	n.s.
PND90	HPC	0.85	11.31	0.02	n.s.	n.s.	n.s.
	CC	0.98	102.54	<0.001	CC DHC	3.30	0.03
	HYP	0.92	22.59	0.005	n.s.	n.s.	n.s.

Note: Systemic levels of DOC, corticosterone, and DHC were not significant predictors in any case.

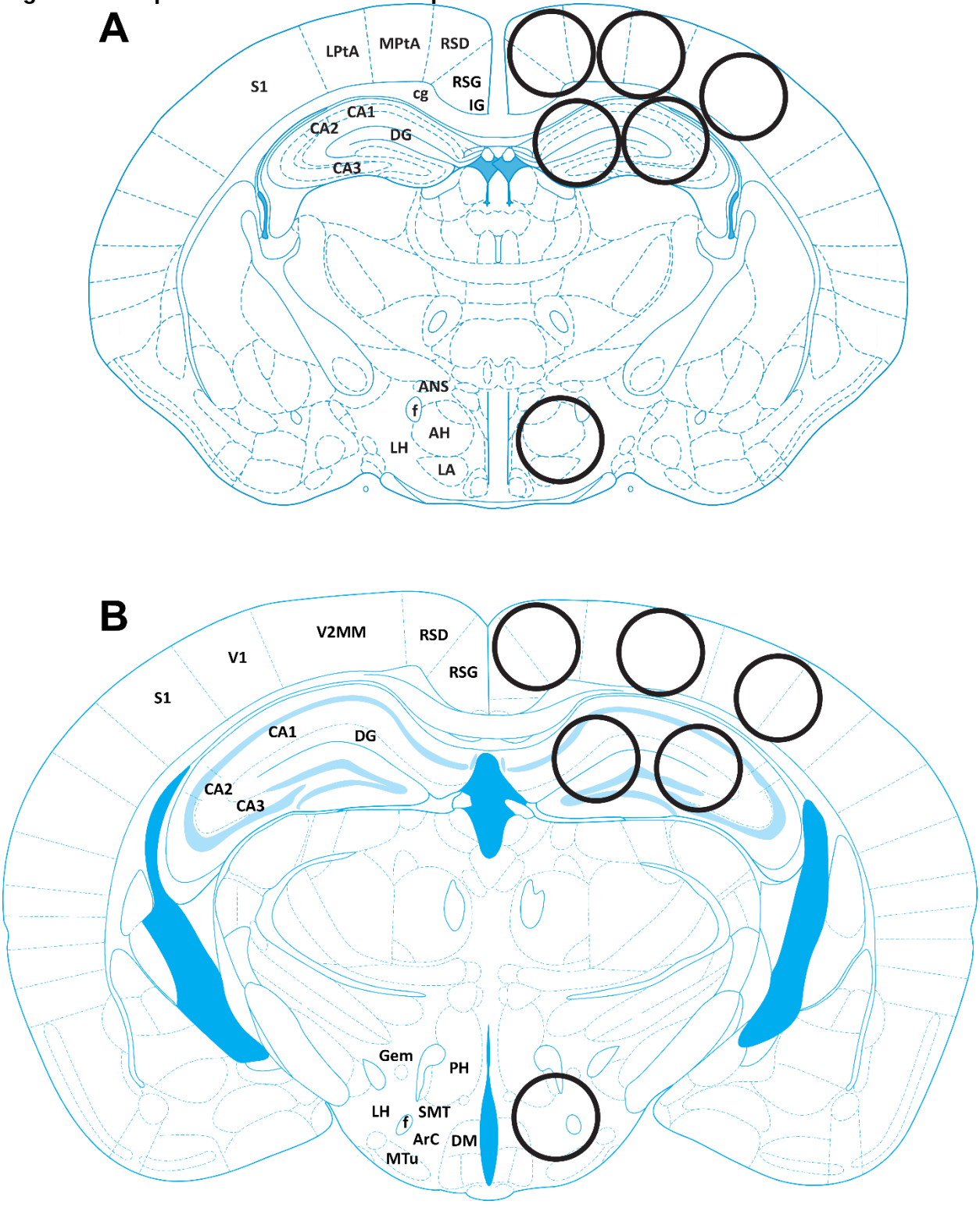
Abbreviations: PND, post-natal day; HPC, hippocampus; CC, cerebral cortex; HYP, hypothalamus. n.s. = no significant predictor

Figure 3.1: Simplified glucocorticoid synthetic pathway.



Boxes indicate steroids and steroidogenic enzymes measured in this study.

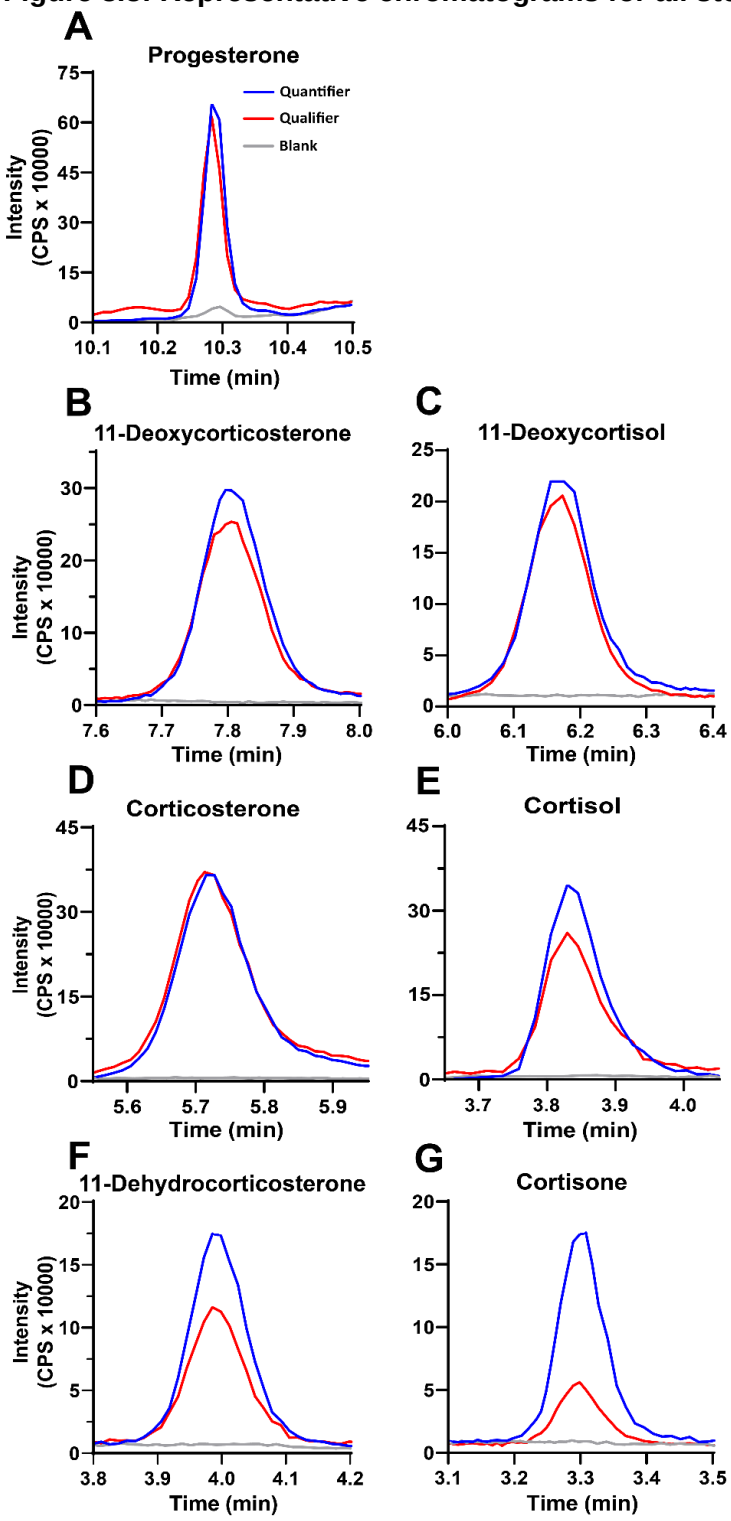
Figure 3.2: Representative locations of punches for tissue microdissection.



(A) PND5 and (B) PND21 and PND90. Each circle represents one punch from each brain region. For simplicity of illustrating punch locations, the circles are shown approximately to scale. The PND5 brain diagram was adapted from (Paxinos, et al. 2007) and the PND21/90 brain diagram was adapted from (Franklin and Paxinos 2007).

Abbreviations: (A) **AH**, Anterior hippocampus; **ANS**, Accessory neurosecretory nuclei **ArC**, Arcuate hypothalamic nucleus; **CA1**, Field CA1 area of the hippocampus; **CA2**, Field CA2 of the hippocampus; **CA3**, Field CA3 of the hippocampus; **cg**, cingulum; **DG**, Dentate gyrus; **DM**, dorsomedial hypothalamic nucleus; **f**, fornix; **IG**, indusium griseum; **LH**, lateral hypothalamic area; **LPtA**, lateral parietal association cortex; **MPtA**, medial parietal association cortex; **MTu**, medial tuberal nucleus; **ns**, nigrostriatal tract; **PH**, posterior hypothalamic nucleus; **RSD**, Retrosplenial dysgranular area; **RSG**, Retrosplenial granular cortex; **S1**, somatosensory cortex; **SMT**, submammillothalamic nucleus; **V1**, Primary visual cortex; **V2MM**, Secondary visual cortex-mediolateral area

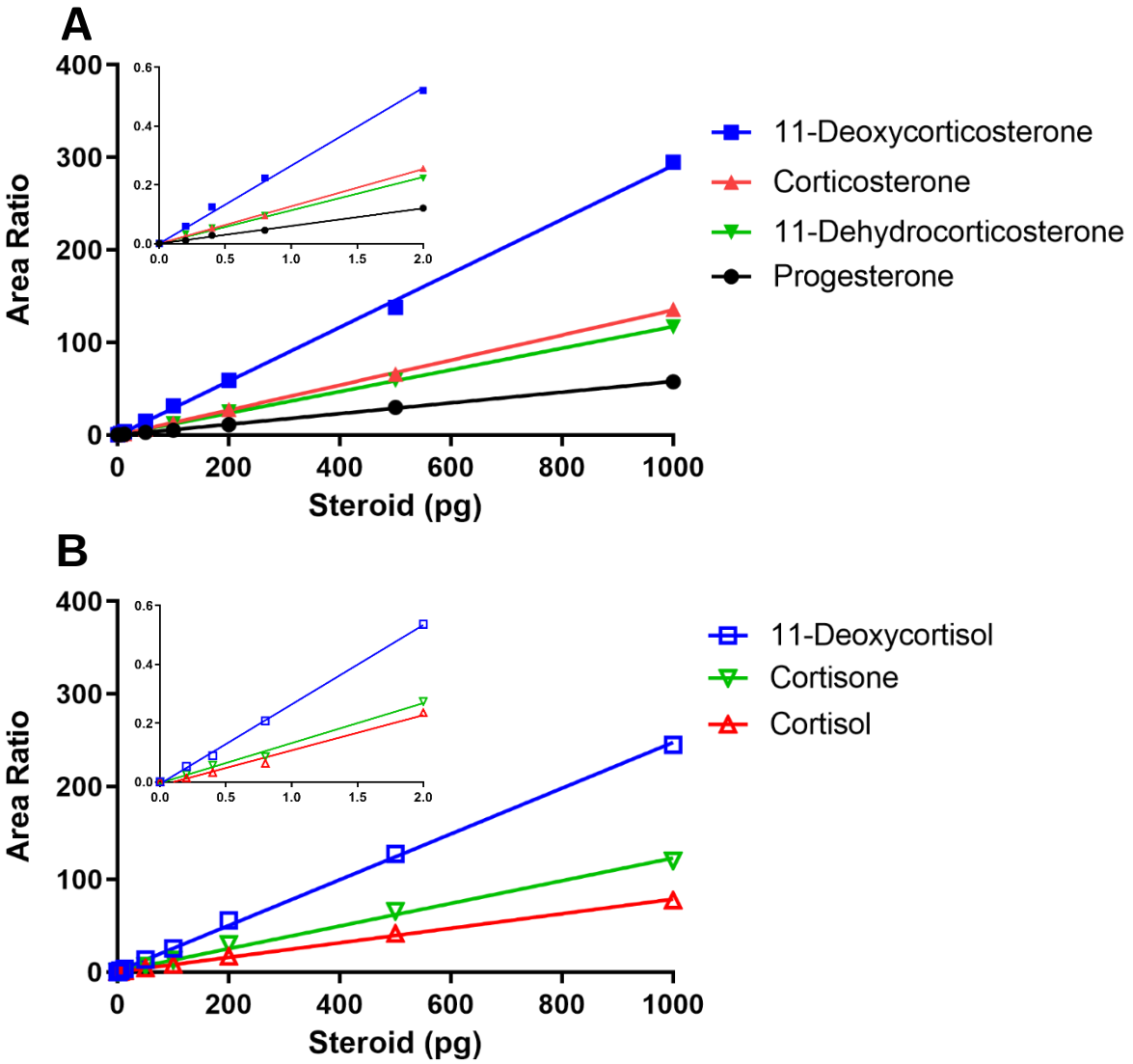
Figure 3.3: Representative chromatograms for all steroids measured by LC-MS/MS.



Quantifier transition (blue) and qualifier transition (red) are shown over the water blank (grey).

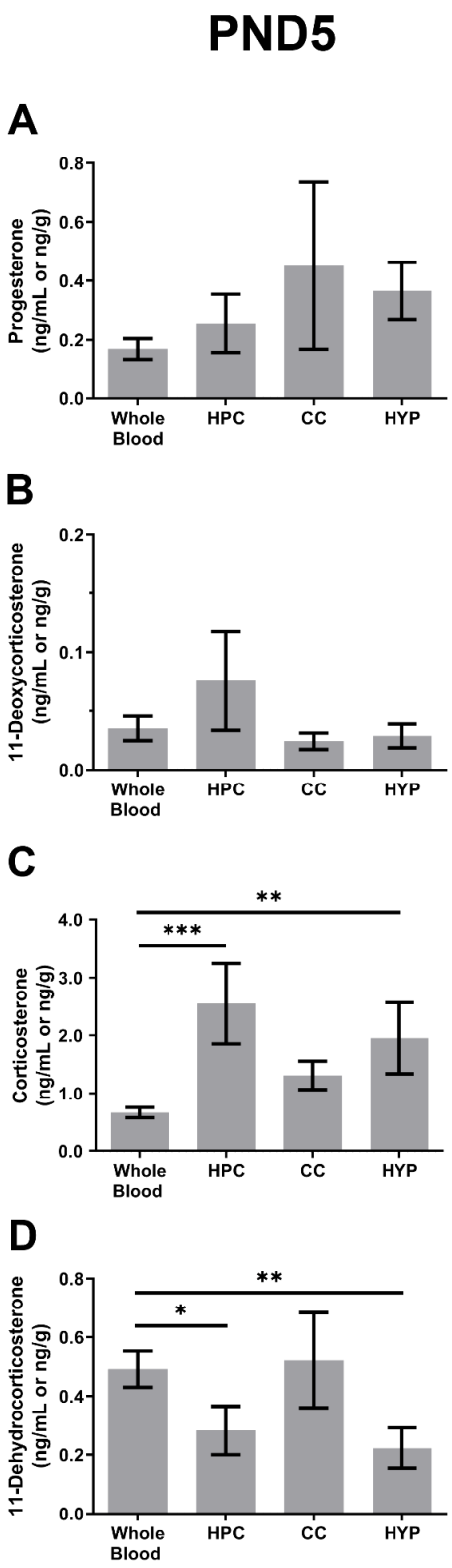
Standards shown for corticosterone and cortisol are 5pg, all others are 2pg.

Figure 3.4: Calibration curves for detection of low levels of steroids by LC-MS/MS.



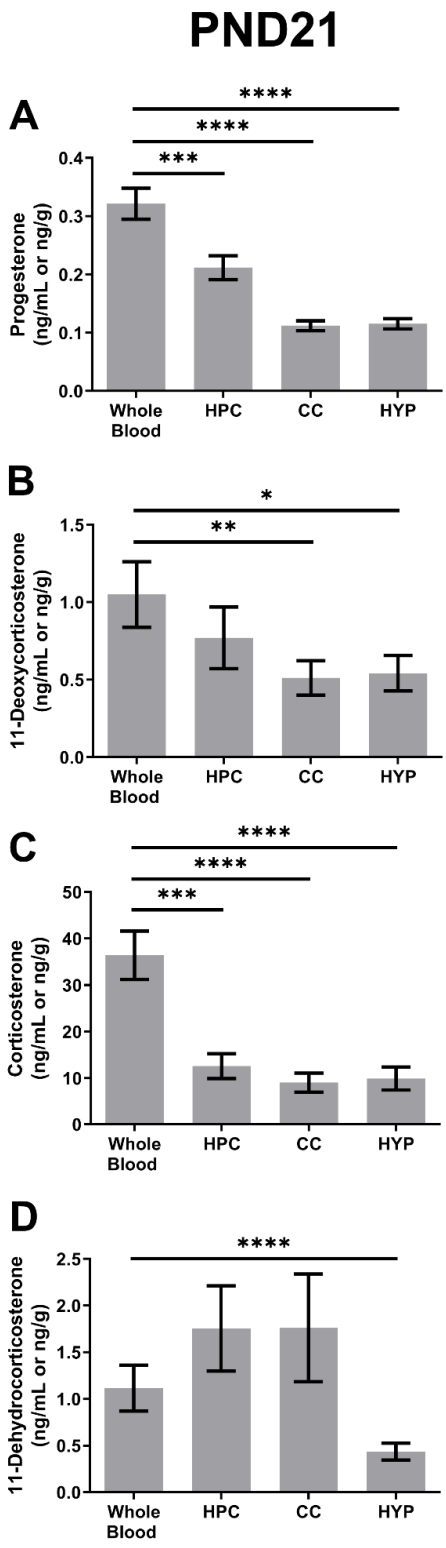
(A) Steroids detectable in samples and (B) steroids non-detectable in samples. Area ratio is calculated by dividing the peak area of the analyte by the peak area of the corresponding deuterated internal standard in the same sample. Calibration curve range was 0.2 to 1000 pg for all steroids. Insets display the lowest standards on the curve.

Figure 3.5: Steroid concentrations in mouse whole blood and microdissected brain at PND5.



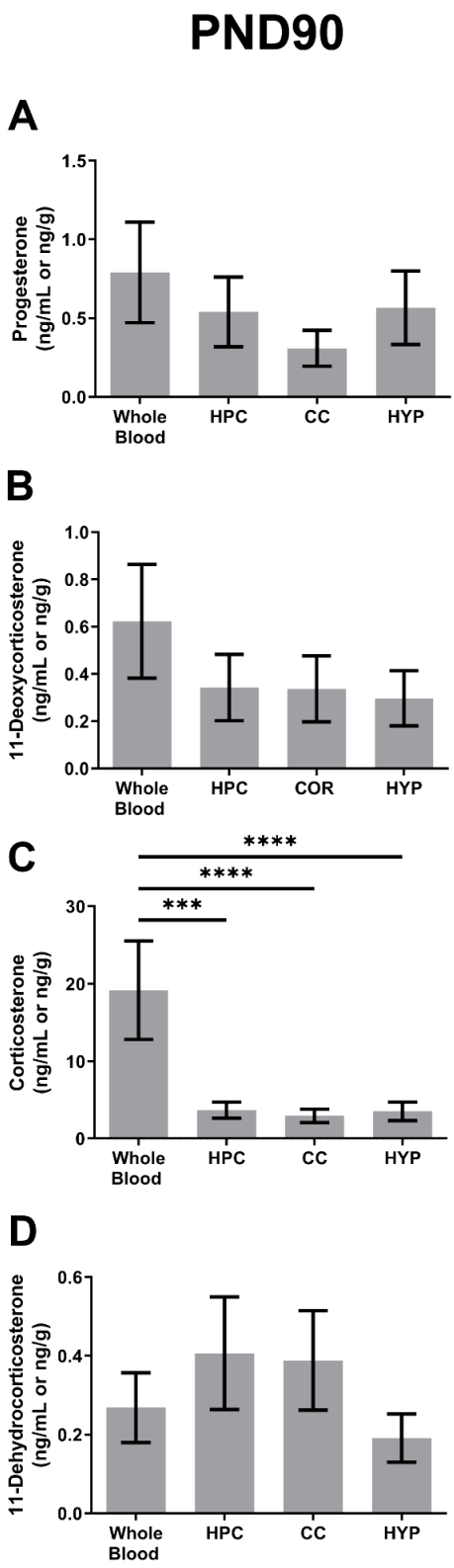
In post-natal day 5 (PND5) mice, concentrations of (A) progesterone, (B) 11-deoxycorticosterone, (C) corticosterone, (D) 11-dehydrocorticosterone in whole blood, hippocampus (HPC), cerebral cortex (CC), and hypothalamus (HYP). Data are shown as mean ± SEM. Tissue levels different from blood levels are indicated as follows: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. n=10 for all steroids and tissues.

Figure 3.6: Steroid concentrations in mouse whole blood and microdissected brain at PND21.



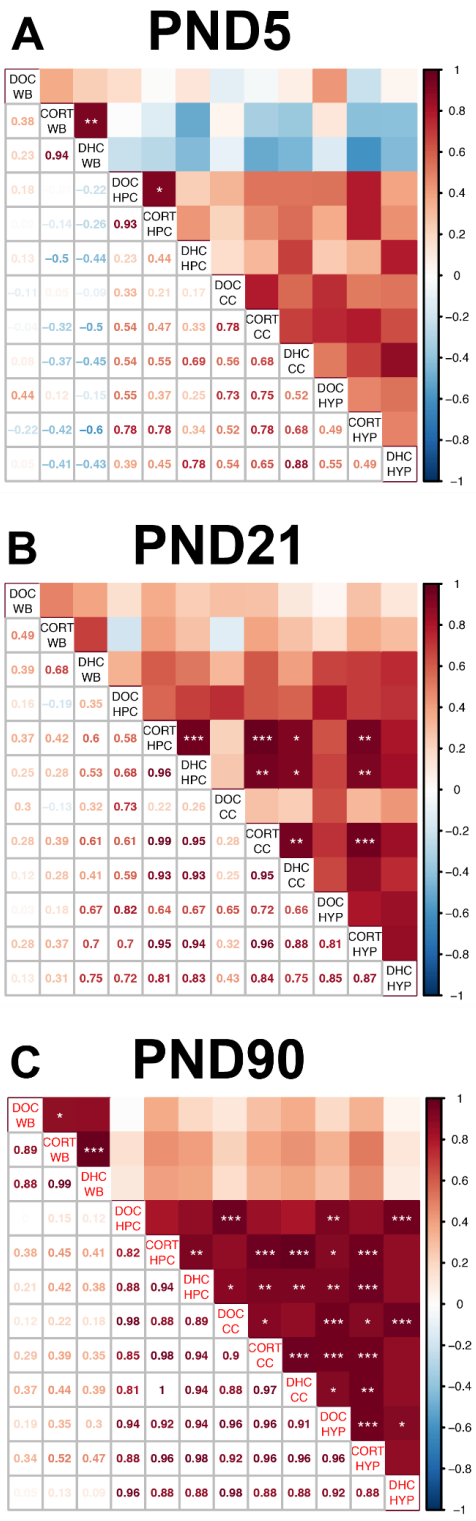
In post-natal day 21 (PND21) mice, concentrations of (A) progesterone, (B) 11-deoxycorticosterone, (C) corticosterone, (D) 11-dehydrocorticosterone in whole blood, hippocampus (HPC), cerebral cortex (CC), and hypothalamus (HYP). Data are shown as mean ± SEM. Tissue levels different from blood levels are indicated as follows: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. n=10 for all steroids and tissues.

Figure 3.7: Steroid concentrations in mouse whole blood and microdissected brain at PND90.



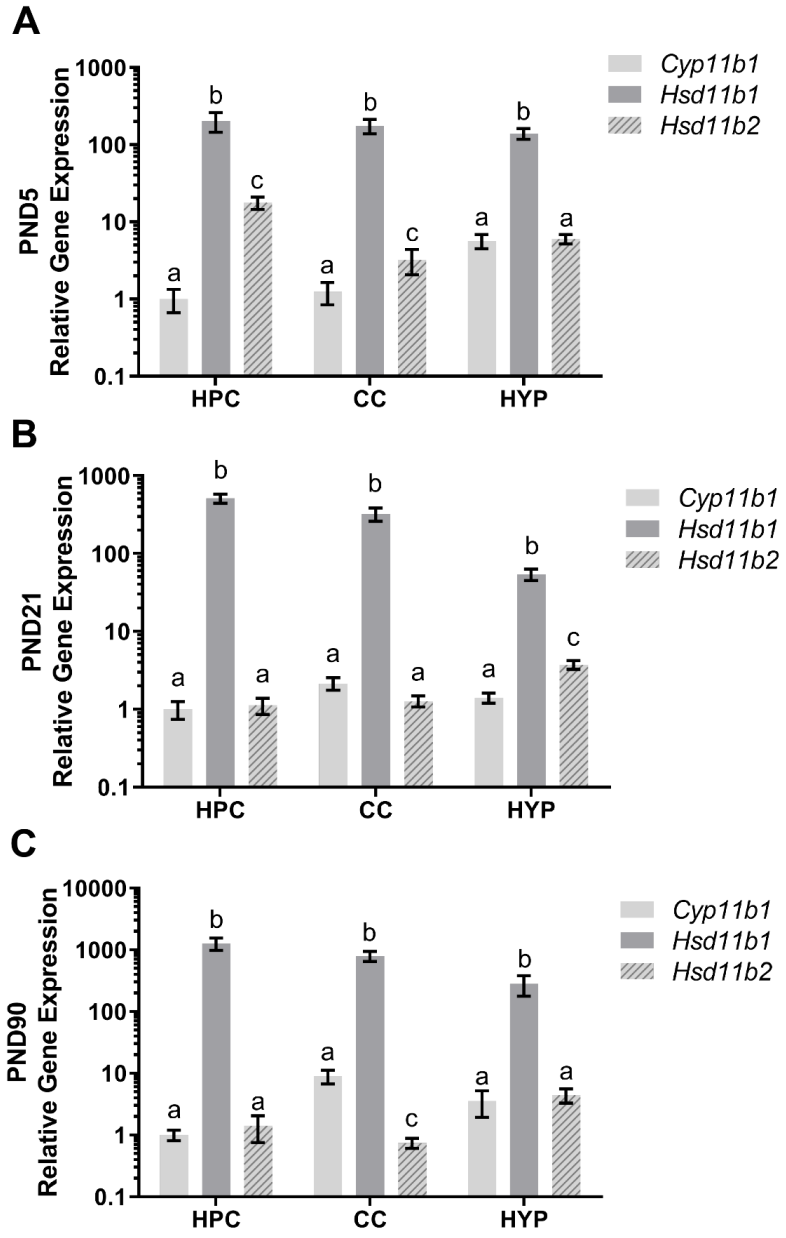
In post-natal day 90 (PND90) mice, concentrations of (A) progesterone, (B) 11-deoxycorticosterone, (C) corticosterone, (D) 11-dehydrocorticosterone in whole blood, hippocampus (HPC), cerebral cortex (CC), and hypothalamus (HYP). Data are shown as mean ± SEM. Tissue levels different from blood levels are indicated as follows: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. n=10 for all steroids and tissues.

Figure 8: Matrices of all pairwise Spearman Rho correlations between steroid levels in whole blood and each brain region.



(A) post-natal day 5 (PND5), (B) PND21, and (C) PND90. All values are expressed in Spearman's rho and are corrected for multiple comparisons using the Benjamini-Hochberg method. WB = whole blood, HPC = hippocampus, CC = cerebral cortex. HYP = hypothalamus, DOC = 11-deoxycorticosterone, CORT = corticosterone, DHC = 11-dehydrocorticosterone. *p≤0.05, **p≤0.01, ***p≤0.001.

Figure 9: Relative expression of steroidogenic enzyme mRNA in microdissected mouse brain.



hippocampus (HPC), cerebral cortex (CC), and hippocampus (HYP) of mice at (A) postnatal day 5 (PND5), (B) PND21, and (C) PND90. Data are shown as mean ± SEM relative to *Cyp11b1* in the HPC at each age. Differences within each brain region are denoted by letters. n=7 for all enzymes and regions

Chapter 4 Isoflurane stress induces glucocorticoid production in mouse lymphoid organs

4.1 Introduction

Glucocorticoids (GCs) are steroids produced by the adrenal glands and within lymphoid organs such as the bone marrow, thymus and spleen (Taves et al. 2017). In mice, corticosterone is the primary active GC. Evidence for lymphoid production of GCs includes expression of steroidogenic enzymes, in vitro production of corticosterone, in vivo corticosterone measurement, and studies of knockout mouse models (Hamden et al. 2019; Mittelstadt et al. 2018; Taves et al. 2016a; Taves et al. 2015). Within lymphoid tissues, corticosterone can be synthesized from cholesterol or other precursors, or regenerated from the inactive metabolite 11-dehydrocorticosterone (DHC) (Taves et al. 2016a) (Figure 4.4.1). Importantly, the environmental factors that drive local corticosterone production are unknown, but might be of greater importance during early life, when adrenal GC production is low in altricial species, termed the stress hyporesponsive period (SHRP).

The SHRP is characterized by very low circulating corticosterone levels at baseline and relatively small increases in circulating corticosterone levels in response to stressors (D'Amato et al. 1992; Henning 1978; Meaney, et al. 1985; Schmidt, et al. 2002; Schmidt et al. 2003). The SHRP in mice ranges from approximately postnatal day 2 (PND2) to PND12 (with PND0 defined as the day of birth) (Schmidt et al. 2003). There is a similar developmental period in humans, from ~4 months to ~5 years (Gunnar and Donzella 2002). Studies of the SHRP in mice and rats demonstrate that some stressors such as ether, shock, cold, and novelty produce little to no increase in circulating corticosterone levels, while other stressors such as endotoxin and maternal separation (≥ 4 hours) produce slightly larger increases in circulating cortisterone levels (Butte, et al. 1973; Cirulli, et al. 1997; Cirulli, et al. 1994; Cote and Yasumura 1975; D'Amato et al. 1992; del Rey, et al. 1996; Gray 1971; Haltmeyer, et al. 1966; Schmidt et al. 2002; Schmidt et al. 2003; Spinedi, et al. 1997; Tang and Phillips 1977; Walker, et al. 1991;

Walker et al. 1990). The purpose of the SHRP is not well understood, but may be protect rapidly developing tissues from deleterious effects of high GC levels (Sapolsky and Meaney 1986). Interestingly, during the SHRP, GC levels are higher in lymphoid organs than in blood, suggesting local GC production may facilitate lymphocyte development (Hamden et al. 2019; Taves et al. 2015).

During chronic stress, high levels of corticosterone are prolonged and highly immunosuppressive, but corticosterone is also a modulator of lymphocyte development when levels are moderate or low. Corticosterone is necessary for a competent T cell repertoire and modulates T cell development by altering T cell selection (Lu, et al. 2000; Mittelstadt et al. 2012; Mittelstadt et al. 2018; Taves and Ashwell 2020). Increased GC signaling leads to the positive selection of more strongly reactive T cell antigen receptors (Iwata et al. 1991; Taves, et al. 2019). Further, increased GC signaling results in more T cells surviving negative selection (Vacchio and Ashwell 1997; Vacchio et al. 1994; Vacchio et al. 1999). Together these data clearly demonstrate that increased corticosterone signaling modulates thymocyte development by shifting the window for positive selection in favor of more reactive T cells and allowing more cells to survive negative selection (Van Laethem, et al. 2001). Less is known about the role of corticosterone in B cell development. Developing B cells express GR and undergo GC-induced apoptosis (Garvy et al. 1993; Trottier, et al. 2008b). Chronic elevation of GCs reduces all B cell subset populations in the bone marrow (Gruver-Yates et al. 2014; Taves et al. 2016a). GCs also inhibit B cell receptor (BCR) signaling in leukemia cells in vitro, suggesting a role for GCs in BCR function similar to their role in TCR selection (Cain, et al. 2020; Cortez et al. 1996).

Endotoxin exposure and maternal separation during the SHRP have long-lasting programming effects on the immune system, referred to as perinatal programming . These stressors produce widespread changes in the immune system and HPA axis, usually seen after a second “hit” in adulthood, deemed the “two-hit model” (Avitsur and Sheridan 2009; Bilbo et al. 2005; Hodgson, et al. 2001; Shanks, et al. 2000; Walker, et al. 2010). The effects of other

stressors such as ether, shock, cold, and novelty on perinatal programming are less studied. This is likely due to the lack of increases in systemic corticosterone levels associated with these stressors and thus, a seemingly limited ability to impact development. However, local production of corticosterone within lymphoid tissues may allow tissues to increase local levels in response to stressors and permit seemingly mild stressors to have strong perinatal programming effects.

To determine if a stressor administered before, during, or after the SHRP induces local corticosterone production in lymphoid organs, we treated mice at 4 ages with isoflurane (stressor), vehicle (oxygen; vehicle control), or neither (baseline). Isoflurane is an inhalable anesthetic and a halogenated ether, similar to anesthetics previously used for SHRP studies (Butte et al. 1972; Gray 1971; Tang and Phillips 1977). We measured a panel of 7 steroids in the blood, bone marrow, thymus, and spleen by liquid chromatography tandem mass spectrometry (LC-MS/MS). By collecting data on corticosterone, its precursors, and metabolites in the blood and lymphoid organs we can better understand the contributions of adrenally produced and locally produced GCs to local GC levels before and after stress.

4.2 Materials and methods

4.2.1 Subjects

C57BL/6J breeding mouse pairs (n=12) were housed in a specific pathogen-free colony in the Centre for Disease Modeling at the University of British Columbia. Mouse pups were housed undisturbed in their home cage with both parents until treatment. Cages were checked for new litters twice a day, and litter sizes ranged from 5-11 animals. Colony rooms were maintained between 20-22°C with 40-70% relative humidity. Mice were housed in ventilated Ehret polysulfone Type IIL filter top cages, with beta-chip bedding, under a 14:10 light:dark cycle (lights on 0600-2000 h), with free access to water (purified by reverse osmosis and sterilized by chlorination) and food (Teklad Diet 2919). A red translucent hut and nestlet were placed in each cage for enrichment. All procedures complied with the Canadian Council on

Animal Care and protocols were approved by the University of British Columbia Animal Care Committee.

4.2.2 Isoflurane treatment and tissue collection

On the first day pups were present in the cage (PND0), pups were randomly assigned to a treatment (Baseline, Oxygen, or Isoflurane) and experimental age (PND1, 5, 9, or 13) (n=10 pups/treatment/age). The Oxygen group was included as a vehicle control. Each treatment group at a particular age was comprised of 2-3 pups from the same litter and incorporated at least 4 litters from different breeding pairs. PND1 (pre-SHRP), PND5 (early-SHRP), PND9 (late-SHRP), and PND13 (post-SHRP) were selected to provide ages before, during, and after the SHRP in mice (Schmidt et al. 2003). Both systemic and local GC levels have been shown to change dramatically across this early developmental period (Hamden et al. 2019; Schmidt et al. 2003; Taves et al. 2015). All experiments were conducted between 0900-1100 h to reduce diurnal variation in steroid levels.

On the day of treatment, animals assigned to the Baseline group were euthanized by rapid decapitation, without the use of anesthetic, less than 1 min after initial disturbance of the cage. Animals assigned to the Oxygen or Isoflurane treatments were placed in an induction chamber with a small amount of nesting material from their home cage and a heating pad under the chamber. For the Oxygen treatment, oxygen was turned on at 2 L/min prior to placing animals in the induction chamber. Animals were left in the chamber for 30 min and euthanized by rapid decapitation without the use of isoflurane. For the Isoflurane treatment, oxygen was turned on at 2 L/min with 5% isoflurane prior to placing animals in the induction chamber. After 3 min of exposure, isoflurane was turned off and animals were left in the chamber with oxygen for 27 min (total time 30 min) and euthanized by rapid decapitation without the use of further anesthetic. The 3 min isoflurane exposure is similar to previous studies using ether (Butte et al. 1972; Gray 1971; Tang and Phillips 1977) and 30 min is a common endpoint in neonatal stress

studies and when circulating corticosterone levels peak (D'Amato et al. 1992; Gray 1971; Schmidt et al. 2002; Schmidt et al. 2003). Whole blood (hereafter “blood”), femurs, thymus, and spleen were collected and immediately frozen on dry ice and stored at −70ºC until steroid extraction. Sex of mice was determined by the University of British Columbia Genotyping Facility using PCR of tail clips.

4.2.3 Steroid analysis by LC-MS/MS

Steroids were extracted from the blood, femur bone marrow, thymus, and spleen via liquid-liquid extraction and analyzed by LC-MS/MS as before (Hamden et al. 2021) with slight modification. The limit of detection for progesterone, 11-deoxycorticosterone (DOC), corticosterone, DHC, 11-deoxycortisol, cortisol, and cortisone was improved 4-fold, from 0.2 pg to 0.05 pg. Standard curves, controls, blanks (solvent with deuterated internal standard), and double blanks (solvent only) were extracted and analyzed alongside all samples. Pooled mouse serum was used as an inter-assay control (n=3/assay) to allow for comparison across all assays used in this study. A steroid was considered non-detectable if the quantifier and qualifier transitions were not present.

4.2.4 Statistical analysis

For groups with less than 40% non-detectable measurements, the data for non-detectable samples were imputed (Tobiansky et al. 2020; Wei et al. 2018). Data were imputed for each age, treatment, and steroid independently. At least 60% of measurements for progesterone, DOC, corticosterone, and DHC were detectable in all groups. To make comparisons between blood and tissues, 1 mL of blood was considered to weigh 1 g (Hamden et al. 2019; Schmidt and Soma 2008; Taves et al. 2011b; Taves et al. 2015). There was no effect of sex in baseline or stressed steroid levels and no interaction between sex and any other factor ($p \leq 0.83$, in all cases) in these pre-pubertal animals, and thus data from both sexes were

pooled for further analysis, as before (Hamden et al. 2021; McCormick et al. 1998; Spinedi et al. 1997; Taves et al. 2015).

Data were analyzed in three complementary ways. First, data were analyzed for an effect of Treatment and Tissue for each steroid separately at each age by mixed-effects model (α=0.05 for all statistical tests). When there was a significant main effect of Treatment or a Tissue × Treatment interaction, post hoc analyses were performed by Tukey's multiple comparison test to determine differences between treatment groups within each tissue. Second, to compare the difference between blood and lymphoid organ steroid levels across treatment and age, blood steroid values were subtracted from tissue steroid values. These difference scores were analyzed for an effect of Treatment and Age by two-way analysis of variance followed by Tukey's multiple comparison test. Third, multiple linear regression was used to elucidate which systemic and local steroids were most important in contributing to local corticosterone levels at each age and treatment. To ensure homogeneity of variance, data were log transformed prior to analysis when necessary. For multiple linear regression, steroid levels were converted to molarity to allow comparisons across steroids. All graphs are presented using non-transformed data as mean ± standard error of the mean. All data were analyzed using GraphPad Prism 9 (version 9.0.0) and R (version 4.0.2 "Taking Off Again").

4.3 Results

4.3.1 Steroid levels

Progesterone, DOC, corticosterone, and DHC were detectable in 100%, 91%, 100%, and 95% of samples, respectively. In contrast, 11-deoxycortisol, cortisol, and cortisone were non-detectable in all samples.

4.3.1.1 PND1

For progesterone, DOC, corticosterone, and DHC, there were main effects of Tissue ($p \leq 0.02$ in all cases), but no main effects of Treatment or Tissue × Treatment interactions (Figure 4.4.2). Steroid levels were higher in bone marrow, thymus, and spleen than in blood.

4.3.1.2 PND5

For progesterone, DOC, corticosterone and DHC, there were main effects of Tissue ($p \leq 0.0001$ in all cases), main effects of Treatment ($p < 0.01$ in all cases), and Tissue × Treatment interactions ($p < 0.0001$ in all cases) (Figure 4.4.3). In all groups, progesterone levels were higher in all tissues than in blood. For DOC, there was a significant increase in all tissues after isoflurane compared to baseline. For corticosterone and DHC, there was a significant increase in all tissues after oxygen and isoflurane compared to baseline. DOC, corticosterone, and DHC levels increased more in bone marrow, thymus and spleen than in blood (Figure 4.4.3; Table 4.1).

4.3.1.3 PND9

For progesterone and DHC, there were main effects of Tissue ($p < 0.0001$ in both cases), main effects of Treatment ($p \leq 0.01$ in both cases), and Tissue × Treatment interactions ($p < 0.0001$ in both cases). For DOC and corticosterone, there were main effects of Tissue ($p < 0.0001$ in both cases) and main effects of Treatment ($p < 0.0001$ in both cases), but no Tissue × Treatment interactions (Figure 4.4). For progesterone, levels increased after isoflurane compared to baseline in blood, thymus and spleen, but not in bone marrow. For DOC, corticosterone, and DHC, there were significant increases in steroid levels after isoflurane compared to baseline and oxygen, and the increases were similar across all tissues (Figure 4.4; Table 4.1).

4.3.1.4 PND13

For progesterone, DOC, and DHC, there were main effects of Tissue ($p \leq 0.04$ in all cases), main effects of Treatment ($p < 0.0001$ in all cases), but no Tissue × Treatment interactions. For corticosterone, there was a main effect of Tissue ($p < 0.0001$), a main effect of Treatment ($p < 0.0001$), and a Tissue × Treatment interaction ($p = 0.04$) (Figure 4.5). There were significant increases in progesterone, DOC, and corticosterone levels after isoflurane compared to baseline and oxygen, but no difference between oxygen and isoflurane for corticosterone levels in any tissue (Figure 4.5; Table 4.1).

4.3.2 Difference in blood and lymphoid tissue corticosterone levels

To determine if the relationship between blood and tissue steroid levels at baseline and after stress changes across age, we subtracted blood steroid levels from tissue steroid levels. For these difference scores, there was a main effect of Treatment for bone marrow only ($p = 0.04$), a main effect of Age in all tissues ($p < 0.0001$ in all cases), and a Treatment × Age interaction in all tissues ($p < 0.0001$ in all cases) (Figure 4.6; Table 4.2). There were no significant differences across ages in the baseline group for any tissue. Additionally, there were no significant differences across ages in the oxygen group for bone marrow and spleen; PND5 and PND9 were different from PND13 for thymus. In the isoflurane group, PND5, PND9, and PND13 were all different from each other for all tissues. At PND5, difference scores were positive, and 95% confidence intervals of the mean (CIs) did not cross zero (Table 4.3), indicating that tissue levels were greater than blood levels. At PND9, tissue levels were similar to blood levels, and 95% CIs cross zero, indicating that tissue levels are no different than blood levels (Table 4.3). At PND13, difference scores were negative, and 95% CIs do not cross zero, indicating that tissue levels were lower than blood levels (Table 4.3). Overall, the relationship between blood and tissue steroid levels is complex and varies with age and stressor exposure.

4.3.3 Predictors of local corticosterone levels

Using multiple linear regression, we were able to predict local corticosterone levels within lymphoid organs at each age. For each lymphoid organ, the best model included corticosterone levels in the blood as well as DOC and DHC levels in the blood and each lymphoid organ, respectively, but not progesterone levels in the blood or lymphoid organs. Results of the regression analysis and significant predictors are presented in Table 4.4. Briefly, at PND1, we were able to predict local corticosterone levels in all tissues for all treatments (except baseline bone marrow). At PND5, we were able to predict local corticosterone levels in all tissues at baseline and in the spleen after oxygen. At PND9, we were able to predict local corticosterone levels in the thymus and spleen at baseline, and in all tissues after oxygen. At PND13, we were able to predict corticosterone levels in all tissues at baseline, in the thymus and spleen after oxygen, and in the spleen after isoflurane. Overall, our model was effective at predicting local corticosterone levels at baseline and after oxygen but less effective at predicting local levels after isoflurane.

4.4 Discussion

The SHRP has long been thought to protect developing tissues from the deleterious effects of high GC levels. Emerging evidence on the local production of GCs during the SHRP, may refine this idea by allowing tissues that require GCs for development to locally increase levels. These data provide further evidence to support this hypothesis by demonstrating that an acute stressor (isoflurane anesthesia) during neonatal development causes small, age-dependent increases in systemic corticosterone levels, but can cause large increases in local levels within the SHRP. At PND1, mice are non-responsive to isoflurane stress and adrenal GC production increases with age thereafter. Lymphoid GC levels were greater than blood GC levels at PND1 but did not increase with stress. Baseline levels were similar to blood levels at PND5, similar to blood at PND9, lower than blood at PND13, and increased with stress at all

three ages. Counter to our results on blood GC levels, local levels peaked at PND5 and were similar at PND9 and PND13. Together, these data suggest that lymphoid organs increase local production of GCs during the SHRP in response to stress and that local production may be particularly important at PND5. Local GC production at PND5 has important implications for rodent models of early-life stress, as stressors are commonly administered at this age.

4.4.1 Systemic glucocorticoid levels

The SHRP has been characterized by ultra-low baseline corticosterone concentrations in the blood and relatively small increases in response to a variety of stressors (Cirulli et al. 1997; Cirulli et al. 1994; D'Amato et al. 1992; Schmidt et al. 2002; Schmidt et al. 2003; Spinedi et al. 1997). Here we report very high corticosterone levels at PND1, as seen before in C57Bl/6J mice (Taves et al. 2015), but not in CD1 mice that have very low PND1 corticosterone levels (Schmidt et al. 2002). This might reflect a strain difference. Importantly, PND0 is defined as the day of birth in all three studies. Consistent with this study, neither strain showed an increase in corticosterone levels in response to stress (Schmidt et al. 2002). At PND1, the large variability in blood corticosterone levels might be explained by varying times after birth (Boksa 1997). At PND5, blood corticosterone levels were very low at baseline and showed a very small increase in response to isoflurane stress (<2 ng/mL increase). At PND9, blood corticosterone levels were still very low at baseline, but had a greater increase in response to stress (~10 ng/ml) than on PND5. These data at PND5 and 9 are comparable to previous studies using ether, a similar inhalant anesthetic (Gray 1971; Schoenfeld, et al. 1980; Tang and Phillips 1977). At PND13, blood corticosterone levels were higher at baseline (although lower than adult levels) (Hamden et al. 2019) and levels increased greatly after stress, consistent with the end of the SHRP (Schmidt et al. 2003). These data suggest that rather than developing in two distinct phases, the HPA axis may develop gradually, with full maturation occurring after PND13.

To our knowledge, these are the first data from animals in the SHRP on blood progesterone, DOC, and DHC levels after a stressor. Baseline blood progesterone levels were highest at PND1 and similar across the other ages, as previously reported (Taves et al. 2015). Progesterone levels responded as did corticosterone levels, with greater increases in response to stress in older animals. As expected, DOC and DHC levels followed the same developmental pattern as corticosterone levels. The highest levels were observed at PND1, the lowest levels at PND5, and levels increased similarly to corticosterone in response to stress. Baseline DOC and DHC levels at PND5 are in agreement with previous reports (Hamden et al. 2019; Hamden et al. 2021; Taves et al. 2015). Together, these data show that circulating progesterone, DOC, corticosterone and DHC levels follow a similar developmental pattern and that blood steroid levels increase minimally in response to stress during the SHRP. Thus, during the SHRP, progesterone, DOC, and corticosterone are all likely secreted from the adrenal.

Sex differences in blood GC levels are well known in adult rodents, with females having higher baseline levels and greater increases in response to stressors. In contrast, we found no sex differences in circulating GC levels at baseline or after stress in neonatal mice, as in previous work (Hamden et al. 2019; Hamden et al. 2021; McCormick et al. 1998; Spinedi et al. 1997; Taves et al. 2015). Three studies also reported no effects of sex in older, but still pre-pubescent, mice (Hamden et al. 2019; Hamden et al. 2021; Taves et al. 2015). These data suggest that sex differences in GC levels may be driven by an increase in gonadal sex steroids at puberty.

4.4.2 Local glucocorticoid levels

Similar to blood steroid levels, local steroid levels increased in response to isoflurane stress after PND1. However, after stress, lymphoid steroid levels were much higher than blood steroid levels at PND5, similar to blood steroid levels at PND9, and lower than blood steroid levels at PND13.

These data provide further evidence of local production of GCs within lymphoid organs, particularly at PND5. Previous studies demonstrated that neonatal lymphoid organs possess all the necessary synthetic and regenerative enzymes to produce corticosterone, produce corticosterone *in vitro* from precursors and DHC, and have locally elevated corticosterone levels at baseline *in vivo* (Mittelstadt et al. 2018; Taves et al. 2015; Vacchio et al. 1994). Here, we demonstrate that an acute stressor dramatically increases GC levels within lymphoid organs at PND5, despite a small increase in blood GC levels. Given the wealth of evidence that lymphoid organs produce GCs, these data suggest that isoflurane stress rapidly increases local corticosterone production in lymphoid organs.

It is unclear what factor(s) stimulates local GC production, but adrenocorticotropin hormone (ACTH) is a likely candidate. The SHRP is caused by a combination of high negative feedback, low circulating ACTH levels, and low adrenal responsiveness to ACTH (Schmidt 2019). Interestingly, lymphocytes in the bone marrow, thymus, and spleen express the ACTH receptor (MC2R), suggesting they respond to circulating ACTH (Clarke and Bost 1989; Johnson, et al. 2001). These data raise the possibility that, during the neonatal period, circulating ACTH mediates the effects of isoflurane stress on lymphoid GC production. Such a mechanism would increase spatial specificity of GC signaling and prevent deleterious effects of high systemic GC levels on somatic growth and brain development during this critical period (Nishi, et al. 2014) .

Alternatively, locally elevated corticosterone levels after stress could be due to sequestration rather than local production, but this is not likely. If corticosterone were not locally produced, we would expect that local GC levels would closely reflect blood GC levels, unless corticosterone is sequestered by binding to the glucocorticoid receptor (GR) higher affinity mineralocorticoid receptor (MR), or corticosteroid binding globulin within tissues. However, glucocorticoid binding of MR in lymphoid tissues is very low (Miller, et al. 1990). Further, DOC and DHC are also higher within lymphoid organs than in blood, and DOC and DHC have lower

binding affinity than corticosterone to GR and MR. Overall, the most likely explanation of locally increased DOC, corticosterone, and DHC is local production of GCs.

Contrary to previous reports (Hamden et al. 2019; Taves et al. 2015), we do not report local corticosterone elevation in the lymphoid organs at baseline compared to blood at PND5. Here, baseline animals were euthanized without anesthesia, and in previous studies, animals were briefly (<3 min) anesthetized with isoflurane before euthanasia (Hamden et al. 2019; Taves et al. 2015). It is well accepted that systemic GC levels in the blood require at least 3 min from the onset of a stressor to show significant increases above baseline levels (Romero and Reed 2005b), but systemic ACTH levels increase more quickly. If ACTH stimulates local GC production, then it is possible that local GC levels rise before 3 min. This is an important methodological consideration for future studies of local GC levels.

The transition to similar stress-induced blood and local steroid levels at PND9, and to blood levels exceeding local levels at PND13, is consistent with increased responsiveness of the adrenals. As the adrenals increase in responsiveness, tissues have less need to locally produce corticosterone, as it can be more easily derived from the blood. Further, the greatest increases in lymphoid GC levels were observed at PND5, whereas greater increases in blood GC levels were observed at PND9 and PND13. Together these data indicate that lymphoid production of corticosterone is greatest when adrenal production of corticosterone is lowest.

Multiple regression analysis using blood DOC, corticosterone, DHC and local DOC and DHC as predictors of local corticosterone levels demonstrates that the factors predicting local corticosterone levels change with age and stress. The strong ability of the model to predict local corticosterone levels at PND1 regardless of treatment, combined with the similar steroid levels across treatments, suggests that the same factors modulate both systemic and local GC levels at PND1. Our ability to predict baseline and oxygen-induced local corticosterone levels at PND5, 9 and 13 more frequently than corticosterone levels after isoflurane suggests that another factor is unaccounted for, such as tissue 11β-HSD1 activity. By quantifying enzyme

activity within lymphoid organs, future studies can clarify whether local corticosterone levels are more influenced by synthesis from precursors or regeneration from metabolites.

4.4.3 The SHRP, local glucocorticoid production, and perinatal programming

During the SHRP, some stressors, such as maternal separation and endotoxin, produce slightly larger increases in blood corticosterone levels and have long-lasting effects on the immune system (Bilbo et al. 2005; Hodgson et al. 2001; Shanks et al. 2000; Walker et al. 2010). Other stressors, such as novelty and ether anesthesia, produce small increases in blood corticosterone levels (D'Amato et al. 1992; Tang and Phillips 1977). The present data raise the exciting possibility that stressors that only result in small increases to circulating corticosterone levels may also alter immunity via local production of corticosterone within lymphoid organs. Interestingly, neonatal stressors are commonly administered to mice at or near PND5, when the greatest increase in lymphoid corticosterone was observed in this study and when T- and B-cells are rapidly maturing (Bonneville, et al. 2010; Mittelstadt et al. 2012; Mittelstadt et al. 2018). Increases in local corticosterone levels resulting from various stressors, regardless of whether corticosterone is adrenally or locally produced, can alter the critical process of lymphocyte selection (Mittelstadt et al. 2018). Higher GC levels during lymphocyte development leads to the selection of a more reactive T-cell repertoire and thus, early life stressors and local production may prepare individuals for more harsh conditions later in life (Taves and Ashwell 2020).

The SHRP was characterized many decades ago (Sapolsky and Meaney 1986), but its purpose is still not well understood. Emerging evidence of local GC production during the SHRP suggests that the SHRP facilitates independent organ development. During neonatal development, some brain regions are highly sensitive to GC-induced apoptosis, and GC treatment during early development alters specific regions such as the hippocampus (Franks, et al. 2020). In contrast, lymphoid organs require GCs to modulate development of a fully competent T-cell repertoire (Mittelstadt et al. 2018). The very low blood GC levels during the

SHRP allow organs that require GCs to locally increase levels as needed, while allowing organs where high GC levels are harmful to maintain low levels. To increase our understanding of the purpose of the SHRP, future studies could examine other organ systems and other stressors, both psychological (e.g. isoflurane) and physiological (e.g. endotoxin).

4.5 Conclusions

GCs are produced within lymphoid organs, and local production may be particularly important during the SHRP because of reduced adrenal activity and very low blood corticosterone levels. The present data advance our knowledge of GC physiology in several key ways. First, we demonstrate that the HPA axis develops on a gradient of increasing adrenal responsivity, with low baseline and stress-induced systemic corticosterone levels at PND5. Second, local GC levels do not reflect blood GC levels during the SHRP. Local GC levels may be higher, similar, or lower depending on age and can increase more in organs than in blood. Third, local GC elevation is likely due to the local production of corticosterone. Together, these data provide important insights into the function of the SHRP and suggest a mechanism by which non-immunological stressors can modulate immune system development and have long-lasting implications for immunocyte function.

4.6 Tables and figures

Table 4.1: Summary of p-values from Tukey Post-Hoc tests for Mixed-Effects analysis of steroid levels.

		Progesterone			DOC			Corticosterone			DHC		
		Base vs Oxy	Base vs Iso	Oxy vs Iso	Base vs Oxy	Base vs Iso	Oxy vs Iso	Base vs Oxy	Base vs Iso	Oxy vs Iso	Base vs Oxy	Base vs Iso	Oxy vs Iso
PND1	BL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	BM	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	TH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	SP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PND5	BL	0.99	0.06	0.05	0.89	0.04	0.008	0.009	<0.0001	0.0008	0.02	0.0002	0.40
	BM	0.55	0.0001	<0.0001	0.77	0.02	0.002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	TH	0.99	0.10	0.07	0.93	<0.0001	<0.0001	0.0013	<0.0001	<0.0001	0.0009	<0.0001	<0.0001
	SP	0.33	0.09	0.002	0.19	0.0003	<0.0001	0.009	<0.0001	<0.0001	0.19	<0.0001	<0.0001
PND9	BL	0.42	0.02	0.32	0.008	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	BM	0.54	0.93	0.77	0.004	<0.0001	<0.0001	<0.0001	<0.0001	0.002	0.03	<0.0001	0.0002
	TH	0.28	0.004	0.20	0.001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001
	SP	0.04	<0.0001	0.03	0.006	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	<0.0001	<0.0001
PND13	BL	0.02	<0.0001	0.01	<0.0001	<0.0001	0.25	<0.0001	<0.0001	0.06	<0.0001	<0.0001	0.68
	BM	0.02	<0.0001	0.04	<0.0001	<0.0001	0.02	<0.0001	<0.0001	0.65	<0.0001	<0.0001	0.87
	TH	0.03	<0.0001	0.01	<0.0001	<0.0001	0.001	<0.0001	<0.0001	0.39	<0.0001	<0.0001	0.22
	SP	0.01	<0.0001	0.10	<0.0001	<0.0001	0.10	<0.0001	<0.0001	0.48	<0.0001	<0.0001	0.93

Abbreviations: BL, Blood; BM, Bone Marrow; TH, Thymus; SP, Spleen; Base, Baseline; Oxy, Oxygen; Iso, Isoflurane; n.a., not applicable

Table 4.2: Summary of p-values from Tukey Post-Hoc tests for Two-Way ANOVA of Tissue-Blood corticosterone levels.

		PND5 vs PND9	PND5 vs PND13	PND9 vs PND13
Bone Marrow	Baseline	0.99	0.90	0.91
	Oxygen	0.99	0.57	0.51
	Isoflurane	<0.0001	<0.0001	0.007
Thymus	Baseline	0.99	0.71	0.69
	Oxygen	0.99	0.01	0.01
	Isoflurane	0.08	<0.0001	<0.0001
Spleen	Baseline	0.99	0.94	0.93
	Oxygen	0.92	0.17	0.08
	Isoflurane	0.01	<0.0001	0.0002

Table 4.3: Mean and 95% CI for the isoflurane group in Tissue-Blood at PND5, PND9, and PND13.

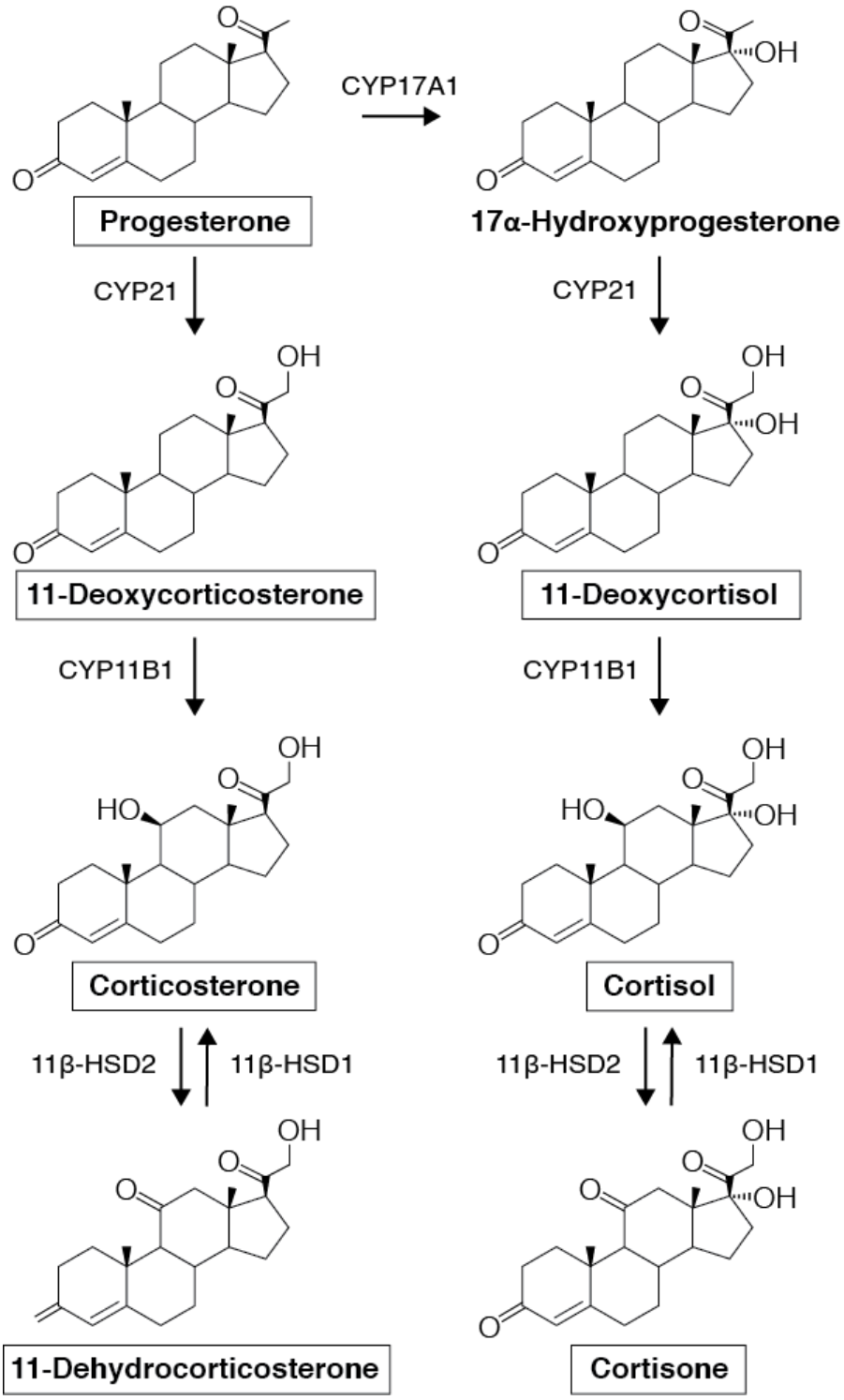
	PND5			PND9			PND13		
	Mean	Upper Limit	Lower Limit	Mean	Upper Limit	Lower Limit	Mean	Upper Limit	Lower Limit
BM	47.74	77.54	17.95	-0.50	3.01	-4.01	-22.57	-9.31	-35.83
TH	10.75	18.58	2.92	2.71	5.42	-0.01	-26.45	-12.88	-40.02
SPL	12.80	20.60	5.01	0.12	6.12	-5.89	-18.17	-5.85	-30.48

Abbreviations: BM, Bone Marrow; TH, Thymus; SPL, Spleen; PND, Postnatal Day

Table 4.4: Multiple linear regression analysis examining the factors that predict local corticosterone levels

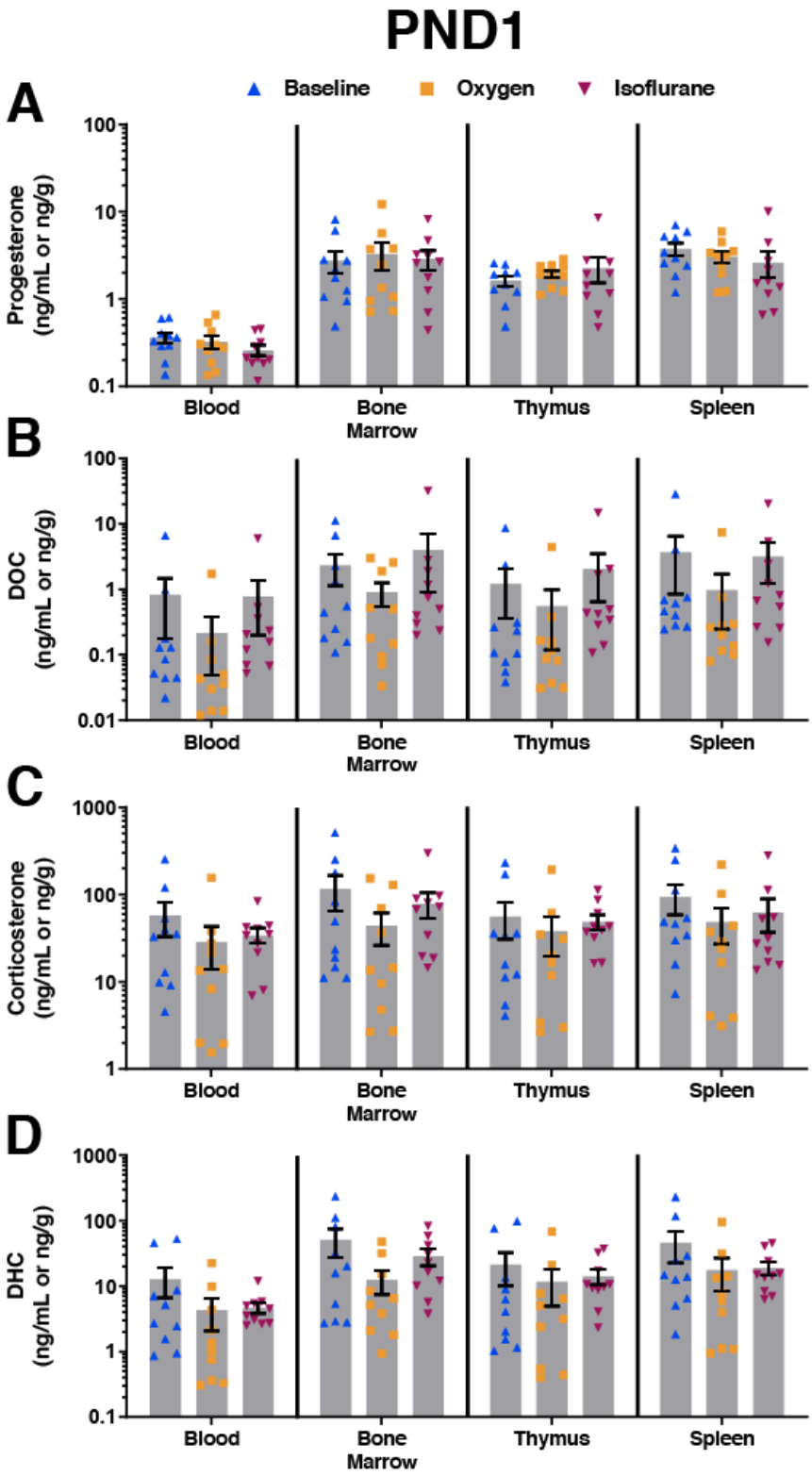
Age	Treatment	Tissue	r^2	$F_{(5,4)}$	p	Predictor	$t_{(4)}$	p
PND1	Baseline	BM	0.25	1.61	0.33			
		TH	0.99	354.36	<0.0001	TH DHC	4.11	0.01
		SPL	0.97	52.07	0.001	SPL DOC	2.87	0.05
	Oxygen	BM	0.9	17.20	0.008	BM DHC	6.12	0.004
		TH	1	540.06	<0.0001	TH DHC	6.87	0.002
						WB DHC	-3.09	0.04
		SPL	0.97	53.53	0.0009			
	Isoflurane	BM	0.96	41.50	0.002			
		TH	0.75	6.49	0.047	WB DOC	2.82	0.05
		SPL	0.93	24.51	0.004			
PND5	Baseline	BM	0.94	29.66	0.0029	BM DOC	8.29	0.001
		TH	0.95	38.25	0.002	TH DOC	-8.65	0.001
						TH DHC	6.22	0.003
						WB DOC	9.31	0.0007
						WB CORT	5.86	0.004
						WB DHC	6.03	0.004
		SPL	0.77	7.16	0.04	WB CORT	4.28	0.01
	Oxygen	BM	0.35	1.98	0.26			
		TH	0.51	2.84	0.17			
		SPL	0.83	9.91	0.03			
	Isoflurane	BM	0.27	1.66	0.32			
		TH	0.55	3.16	0.14			
		SPL	0.53	3.01	0.15			
PND9	Baseline	BM	0.63	4.05	0.10			
		TH	0.99	142.42	0.0001	WB DHC	7.12	0.002
		SPL	0.97	52.87	0.001	WB DHC	4.58	0.01
	Oxygen	BM	0.9	17.25	0.008	WB DOC	-2.87	0.05
						WB DHC	3.68	0.02
		TH	0.98	72.94	0.0005	WB DHC	6.15	0.004
		SPL	0.87	12.84	0.01			
	Isoflurane	BM	-0.14	0.78	0.61			
		TH	0.08	1.15	0.46			
		SPL	-0.26	0.63	0.69			
PND13	Baseline	BM	0.74	6.09	0.05			
		TH	0.78	7.38	0.04			
		SPL	0.9	16.77	0.009			
	Oxygen	BM	0.59	3.62	0.12			
		TH	0.87	12.93	0.01			
		SPL	0.68	4.89	0.07	SPL DOC	-3.82	0.02
						WB DOC	4.71	0.009
	Isoflurane	BM	0.38	2.12	0.24			
		TH	-0.21	0.69	0.66			
		SPL	0.82	9.31	0.03	SPL DOC	4.18	0.01

Figure 4.1: Simplified glucocorticoid synthetic pathway.



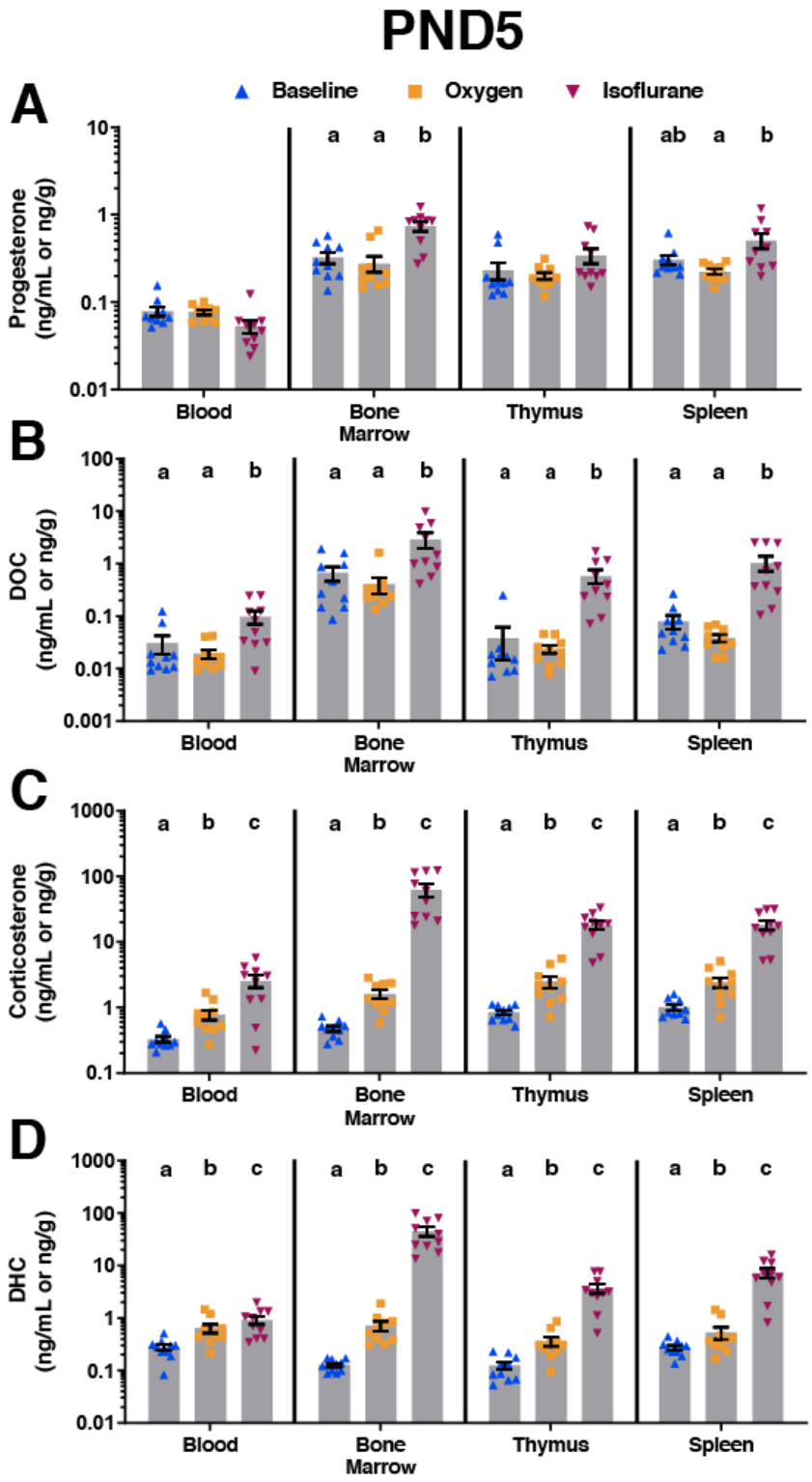
Boxes indicate steroids measured in this study.

Figure 4.2: Steroid concentrations in whole blood, bone marrow, thymus, and spleen of mice at PND1.



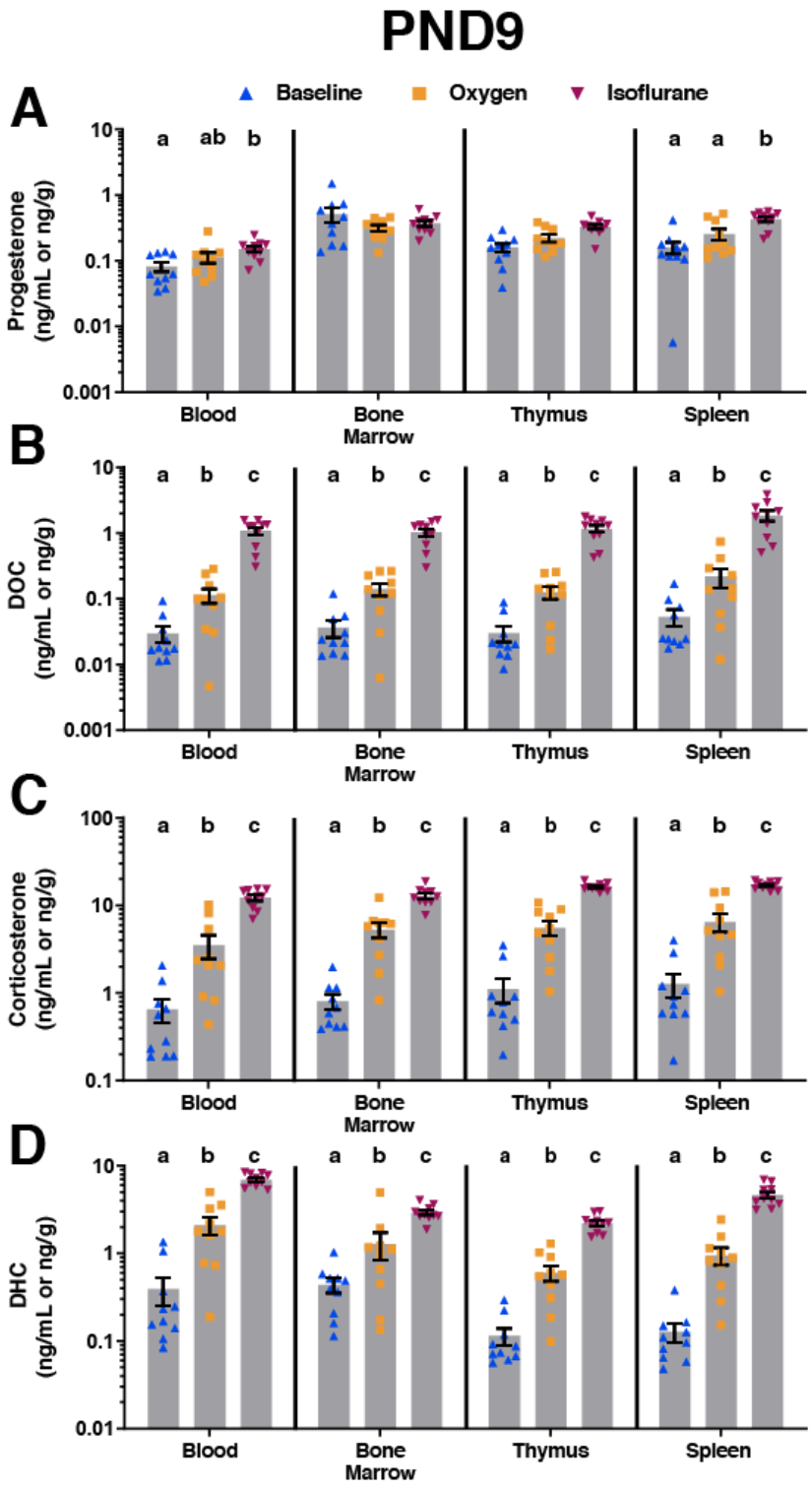
In post-natal day 1 (PND1) mice, concentrations of (A) progesterone, (B) deoxycorticosterone, (C) corticosterone, (D) dehydrocorticosterone in whole blood, bone marrow, thymus, spleen. Data are shown as mean ± SEM. Tukey's post-hoc test was used to determine differences in baseline, oxygen, and isoflurane groups within each tissue, significant differences are denoted by letters. n=10 for all steroids and tissues.

Figure 4.3: Steroid concentrations in whole blood, bone marrow, thymus, and spleen of mice at PND5.



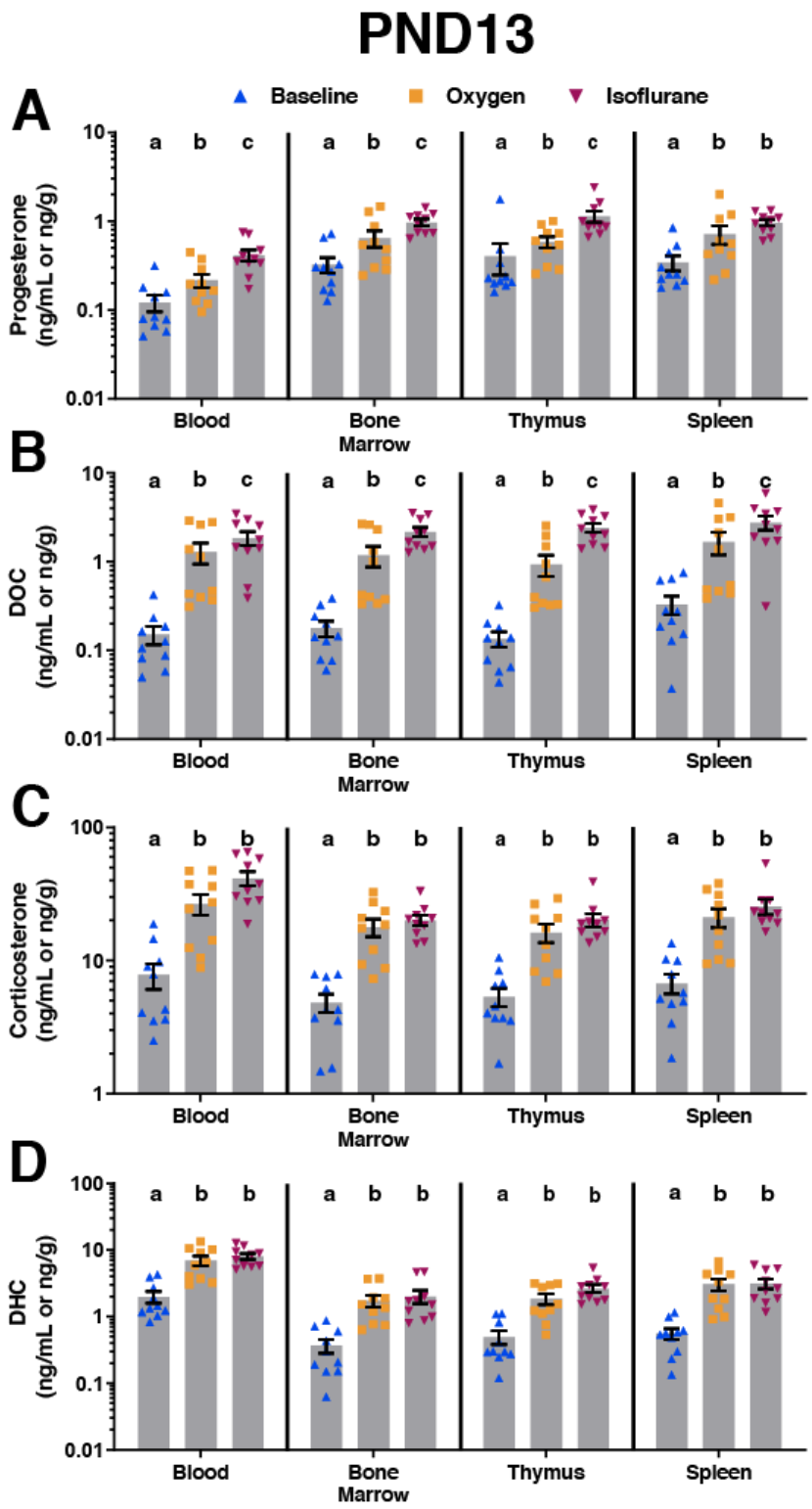
In post-natal day 5 (PND5) mice, concentrations of (A) progesterone, (B) deoxycorticosterone, (C) corticosterone, (D) dehydrocorticosterone in whole blood, bone marrow, thymus, spleen. Data are shown as mean ± SEM. Tukey's post-hoc test was used to determine differences in baseline, oxygen, and isoflurane groups within each tissue, significant differences are denoted by letters. n=10 for all steroids and tissues.

Figure 4.4: Steroid concentrations in whole blood, bone marrow, thymus, and spleen of mice at PND9.



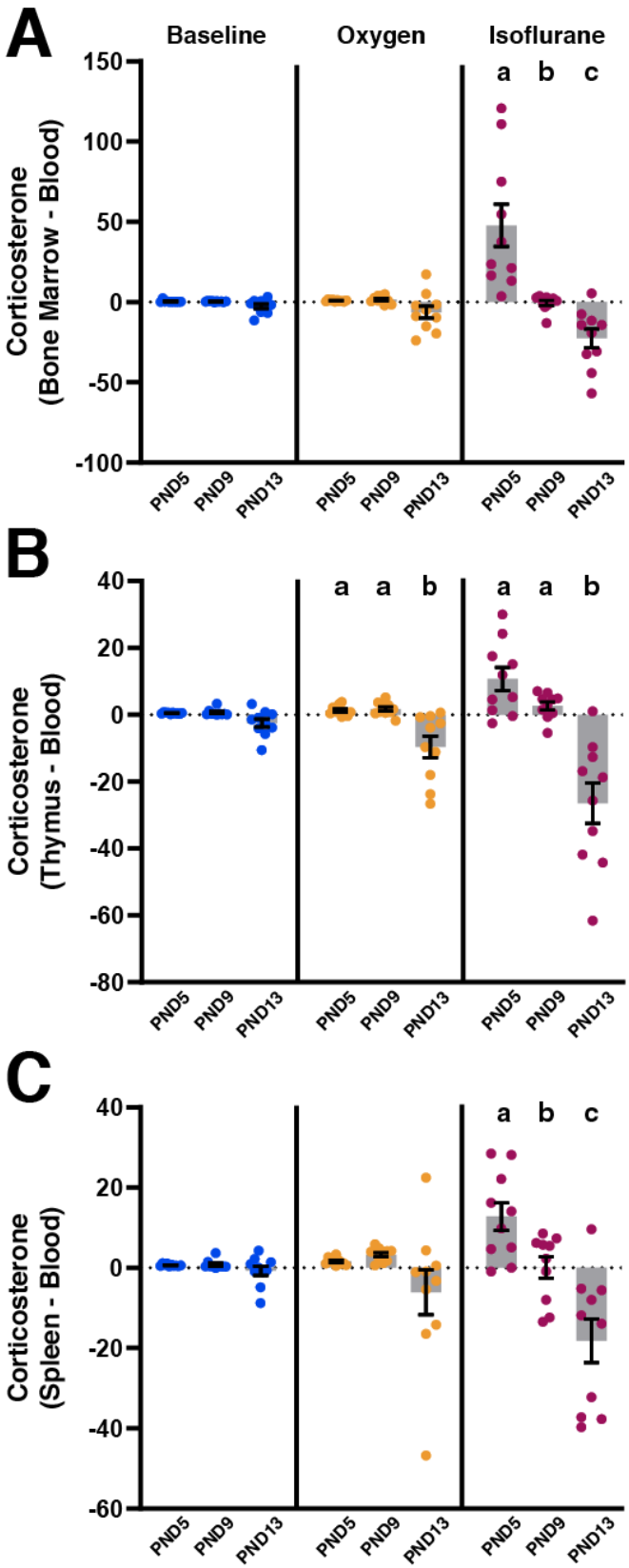
In post-natal day 9 (PND9) mice, concentrations of (A) progesterone, (B) deoxycorticosterone, (C) corticosterone, (D) dehydrocorticosterone in whole blood, bone marrow, thymus, spleen. Data are shown as mean ± SEM. Tukey's post-hoc test was used to determine differences in baseline, oxygen, and isoflurane groups within each tissue, significant differences are denoted by letters. n=10 for all steroids and tissues.

Figure 4.5: Steroid concentrations in whole blood, bone marrow, thymus, and spleen of mice at PND13.



In post-natal day 13 (PND13) mice, concentrations of (A) progesterone, (B) deoxycorticosterone, (C) corticosterone, (D) dehydrocorticosterone in whole blood, bone marrow, thymus, spleen. Data are shown as mean ± SEM. Tukey's post-hoc test was used to determine differences in baseline, oxygen, and isoflurane groups within each tissue, significant differences are denoted by letters. n=10 for all steroids and tissues.

Figure 4.6: Differences in tissue – blood corticosterone levels.



(A) bone marrow, (B) thymus, (C) spleen. Data are show as mean ± SEM**.** Tukey's post-hoc test was used to determine differences between PND5, PND9, and PND13 corticosterone levels within each treatment group, significant differences are denoted by letters. n=10 for all ages and tissues.

Chapter 5 General discussion

Tissues are traditionally thought of as passive recipients of adrenally-produced glucocorticoids. However, a growing body of evidence indicates that some tissues take an active role in modulating local glucocorticoid levels, either by production or metabolism/inactivation (Hamden et al. 2019; Hamden et al. 2021; Taves et al. 2011a; Taves et al. 2016a; Taves et al. 2015). As a result, glucocorticoids produced within tissues can act in a paracrine and/or autocrine fashion (Mittelstadt et al. 2018), complementary to their classical role as endocrine signals.

In this dissertation, I have presented three studies that examine the hypothesis that in early life when blood glucocorticoid levels are low, tissues can upregulate local glucocorticoid levels, either by synthesis or regeneration, and local glucocorticoid production increases in response to stressors. I found that 1) 11-dehydrocorticosterone is present in the blood and lymphoid tissues of neonatal and adult mice and is locally elevated in the bone marrow and spleen of neonatal mice, 2) corticosterone is locally elevated in the hippocampus and hypothalamus, but not the cerebral cortex, of neonatal mice, and 3) across early development the relationship between blood and tissue glucocorticoid levels varies and tissue levels can be either higher, similar to, or lower than blood levels depending on age and context. Together, these studies add to our knowledge of local glucocorticoid production and further our understanding of the stress hyporesponsive period. I demonstrated that both corticosterone and 11-dehydrocorticosterone are locally elevated in the lymphoid organs of neonatal mice, that discrete brain regions produce glucocorticoids, and that local glucocorticoid levels can increase more in response to an acute stressor than blood levels. These data provide a possible mechanism for how neonatal stressors may produce lymphoid organ- or brain region-specific effects.

5.1 Major findings- local glucocorticoid production in lymphoid organs and brain

5.1.1 Local glucocorticoid production in mouse lymphoid organs

There is now a large body of evidence that mouse lymphoid organs produce glucocorticoids, particularly during the neonatal period. In vitro and ex vivo experiments have demonstrated that bone marrow, thymus, and spleen can produce glucocorticoids. Lymphoid glucocorticoids can be synthesized from precursors (e.g. 11-deoxycorticosterone) and regenerated from metabolites (e.g. 11-dehydrocorticosterone), with the latter being the greater contributor to local levels (Taves et al. 2016a). Additionally, in vivo studies have demonstrated that tissue levels are greater than blood levels during neonatal development (Taves et al. 2015). Locally elevated glucocorticoids can be due to local production and/or sequestration, but evidence has shown that local production is more likely (Taves and Ashwell 2020; Taves et al. 2011a). Finally, studies utilizing mouse knockout models lacking CYP11B1 in thymic epithelial cells have shown altered T cell development and demonstrated that the thymus produces biologically relevant corticosterone, even in adrenal-intact mice (Mittelstadt et al. 2018).

In Chapter 1, I provided evidence for local glucocorticoid production by confirming that neonatal mice upregulate lymphoid corticosterone levels, and I provided the first evidence that 11-dehydrocorticosterone is present in all lymphoid organs across mouse development. In Chapter 3, I demonstrated that neonatal lymphoid organs increase glucocorticoid production in response to isoflurane stress and that local glucocorticoid levels increase far more than circulating levels at PND5. Together, these studies add to the body of evidence that lymphoid organs locally produce glucocorticoids, as 11-dehydrocorticosterone is available within lymphoid organs to be regenerated into corticosterone, and stress increases lymphoid glucocorticoid production. Future studies should seek to determine if synthesis from precursors or regeneration from 11-dehydrocorticosterone is a greater contributor to local glucocorticoid

production in vivo and if different stressors (i.e., psychological vs physiological) have different effects on local glucocorticoid production.

5.1.2 Local glucocorticoid production in mouse discrete brain regions

In addition to lymphoid organs, the brain also produces glucocorticoids. Evidence for local glucocorticoid production in the brain includes: the presence of all necessary steroidogenic enzymes within discrete brain regions such as the hippocampus (Holmes and Seckl 2006; Mellon and Deschepper 1993; Moisan et al. 1990; Taves et al. 2015), administration of adrenocorticotropic hormone increases CYP11B1 mRNA in the hypothalamus and cerebral cortex (Ye et al. 2008), brain tissue produces corticosterone from precursors in vitro (Higo et al. 2011; MacKenzie et al. 2000), corticosterone is detectable in the hippocampus of adrenalectomized animals (Croft et al. 2008; Hojo et al. 2011), and finally, mice deficient for 11β-HSD1 have lower corticosterone levels and higher 11-dehydrocorticosterone levels in the hippocampus and cerebral cortex than wildtype mice (Cobice et al. 2013).

Here, I provide further evidence of local glucocorticoid production in discrete brain regions. Specifically, I confirm the presence of key steroidogenic enzyme transcripts in three glucocorticoid-sensitive regions across development, demonstrate that corticosterone is locally elevated in a region-specific manner, that local glucocorticoid levels within discrete brain regions are more tightly correlated than with blood glucocorticoids, and that local corticosterone levels are better predicted by local 11-deoxycorticosterone and 11-dehydrocorticosterone levels than by any glucocorticoid in the blood. These data support the hypothesis that the brain can selectively increase glucocorticoid signaling within discrete regions. The finding that corticosterone is locally elevated within the neonatal hippocampus and hypothalamus, but not the cerebral cortex, is particularly interesting. Behavioral effects of neonatal stressors are often attributed to the hippocampus and hypothalamus, and local increases in glucocorticoid levels in these regions provide a possible mechanism for neonatal programming. Future studies should

examine the effects of stress on glucocorticoid production in the neonatal and adult brain, as well as the biological relevance of brain glucocorticoid production.

5.1.3 The pattern between systemic and local glucocorticoid levels changes across development

There is growing appreciation that local glucocorticoid levels do not reflect systemic glucocorticoid levels (Taves et al. 2015). In Chapters 2, 3, and 4, I demonstrated that, in both the immune and nervous systems, the relationship between blood and tissue glucocorticoid levels is dependent on age, tissue/region, and physiological context. For example, in the brain, at the same age, baseline glucocorticoid levels were either greater or equal to blood levels depending on the brain region. Further, in Chapter 4, I showed that at postnatal day 5 when blood glucocorticoid levels are lowest, lymphoid glucocorticoid levels are similar to blood levels but local levels increase far more than blood levels after a stressor. While just eight days later, lymphoid glucocorticoid levels are lower than blood levels at baseline and have a lesser increase after a stressor. These studies indicate that glucocorticoid signaling is modulated within tissues either by local production or inactivation. Therefore, these studies further support the idea that blood measurements are not enough to elucidate the effects of stress on glucocorticoid signaling within tissues. Future studies should seek to understand the mechanism by which tissues modulate local increases or decreases in glucocorticoid levels.

5.2 Additional contributions and future directions

5.2.1 Development of a novel 11-dehydrocorticosterone assay

It is now widely accepted that tissues can intracellularly regenerate corticosterone from 11-dehydrocorticosterone in the blood. As such, numerous studies have focused on intracellular 11β-HSD1 mRNA levels, protein expression, and enzyme activity (Gathercole et al. 2013; Pradhan, et al. 2019; Rensel et al. 2018). However, there are virtually no studies on endogenous 11-dehydrocorticosterone levels in any species, and there is no commercially

available method to do so. This is likely because we have viewed 11-dehydrocorticosterone solely as an inactive glucocorticoid metabolite, rather than a source for local corticosterone production. Here, we established an accurate, precise, sensitive, specific, and easy-to-use immunosorbent assay to measure endogenous 11-dehydrocorticosterone in mice, rats, and birds. Further studies of 11-dehydrocorticosterone levels in the blood and tissues will advance our understanding of local corticosterone production and provide insight into if local corticosterone levels are more reliant on synthesis from precursors or regeneration from inactive metabolites in vivo (Taves et al. 2016a).

5.2.2 11-dehydrocorticosterone levels increase in response to stress in rats and songbirds

Of the few studies that have measured 11-dehydrocorticosterone, only 3 have measured it in rats, and none have measured it in any bird species (Hundertmark et al. 2002a; Obut et al. 2004; Tagawa et al. 2007). Despite this, rats and songbirds have been widely used to investigate intracellular regeneration of 11-dehydrocorticosterone, such as to study adipose tissue regulation in rats, and in pre-migratory muscle preparation in birds (Hughes, et al. 2008; Nixon, et al. 2012; Pradhan et al. 2019). Here, I confirmed that 11-dehydrocorticosterone is present in the blood of rats. Additionally, I was the first to report that 11-dehydrocorticosterone is present in songbirds and that levels increase in response to stress in both rats and birds. These data are vital in furthering our knowledge of corticosterone production in the body. Future studies focused on 11β-HSD1 and 11β-HSD2 should measure 11-dehydrocorticosterone, in addition to corticosterone, to provide a more complete picture of local glucocorticoid production.

5.2.3 Liquid chromatography tandem mass spectrometry in steroid analysis

Immunoassays revolutionized the field of endocrinology, by providing an easy-to-use method to measure steroid hormones. Today, liquid chromatography tandem mass spectrometry has created a new “gold standard” in steroid biology and facilitated unprecedented

advancements in the field (Hamden et al. 2019; Handelsman 2017). Relative to liquid chromatography tandem mass spectrometry, immunoassays are very limited as they can typically measure a single analyte at a time, lack sensitivity, and most importantly, lack specificity, all of which can result in overestimating (Fischer, et al. 2021). Here, we developed a novel liquid chromatography mass spectrometry method to measure of panel of 7 steroids in a single small sample. We used our assay to measure active glucocorticoids, their precursors, and metabolites to better understand if local glucocorticoid levels are a result of production or regeneration. Further, this method can be used to better understand the interconnectedness between various endocrine systems in the body. For example, when glucocorticoid levels increase in the blood, glucocorticoid receptor signaling increases in the hypothalamic-pituitary-gonadal axis to provide negative feedback on sex steroid production. By measuring steroids of different classes in a single sample, we can gain a more we can better understand the dynamic relationship between systemic steroid signaling.

5.2.4 Rapid local glucocorticoid production

The pituitary rapidly secretes adrenocorticotropic hormone (< 1 min) from the time of perceiving a stressor, and adrenal glucocorticoid production and blood glucocorticoid levels increase after ~ 3 min (Romero and Reed 2005b). In Chapters 2 and 3, I report that glucocorticoid levels within lymphoid organs, hippocampus, and hypothalamus are higher than blood glucocorticoid levels at baseline. In these studies, animals were euthanized < 3 min from disturbing their home cage with the brief use of isoflurane as an anesthetic, and thus, should represent baseline glucocorticoid levels in the blood and tissues. However, in study 4, when no isoflurane was administered, I did not report local elevation of glucocorticoids within lymphoid organs at baseline. With our understanding that lymphoid organs produce glucocorticoids, and isoflurane increases glucocorticoid production, these data suggest that tissue levels rapidly increase < 3 min from the perception of stressor. There is evidence that, similar to the adrenals,

lymphoid organs express adrenocorticotropic hormone receptor and can increase glucocorticoid production in response to pituitary-derived adrenocorticotropic hormone (Talaber, et al. 2015; Vacchio et al. 1994). Thus, lymphoid organs could be responding to pituitary adrenocorticotropic hormone to rapidly increase local levels. These data warrant further investigation and hold important implications for studies interested in preventing the effects of stress on tissues prior to experimental manipulation.

5.3 Conclusions

In this dissertation, I present evidence for glucocorticoid production in the immune and nervous systems. To our knowledge, these are the first data on 11-dehydrocorticosterone levels in the blood of neonatal mice, in lymphoid tissues of any species at any age, in rats after stress, and in songbirds. This is the first report of local elevation of glucocorticoids within discrete brain regions of mice, and finally the first data on local glucocorticoid levels in response to a stressor also in mice. Together, these studies demonstrate that local glucocorticoid regulation and production is dependent on age, tissue, and physiological context, that local glucocorticoid levels can far exceed blood levels, and provide important insight into the purpose of the stress hyporesponsive period. More generally, this work highlights that steroids are locally-produced, and thus, local steroid levels do not reflect blood steroid levels. These data are broadly applicable to researchers studying all aspects of endocrinology, immunology, and neuroscience and highlight the importance of measuring local steroid levels to gain an accurate understanding of local steroid signaling.

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