PROGRAMMING OF STRESS REGULATION IN 5 - 7 YEAR OLD CHILDREN BY
MATERNAL GESTATIONAL MOOD AND ANTIDEPRESSANT USE

by

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Abstract

Of particular importance to mental health across the life span is our capacity to regulate neuroendocrine responses to stressful events via the hypothalamic pituitary adrenal (HPA) axis. Prenatal exposure to maternal depression and anxiety may be among the earliest adverse experiences shown to influence the developing HPA system, possibly via changes in fetal serotonergic signaling. In addition, the developmental impact of prenatal exposure to serotonin reuptake inhibitor (SRI) antidepressants is often undistinguishable from prenatal maternal mood. The molecular mechanisms underlying how serotonin (5-HT) influences the development of the HPA stress system remain unclear, but may involve epigenetic mechanisms such as DNA methylation.

This thesis explored whether prenatal exposure to SRIs and maternal depressed/anxious mood are associated with altered HPA stress reactivity, characterized by variable basal and stress-induced cortisol concentrations in 5 - 7 year old children. Furthermore, the methylation status in promoter regions of NR3C1 1F (encodes the glucocorticoid receptor) and SLC6A4 (encodes the serotonin transporter) at birth and at 5 - 7 years of age was evaluated and the relationship to children’s cortisol patterns was assessed.

Prenatal exposure to SRIs and higher 3rd trimester maternal depressed/anxious mood were associated with reduced cortisol stress responses at 5 - 7 years. Higher NR3C1 1F methylation at 5 - 7 years was associated with higher diurnal cortisol concentrations and a reduced cortisol stress response. Children exposed to mothers with higher 3rd trimester and concurrent anxious mood exhibited lower SLC6A4 methylation, compared to children exposed to higher 3rd trimester maternal anxious mood alone. Furthermore, children with higher SLC6A4 methylation at birth exhibited a reduced cortisol stress response.
These findings suggest that the relationship between early life experiences and altered stress responses in early childhood may be moderated by epigenetic mechanisms involving the serotonergic and HPA regulatory systems. In addition, an interactive relationship between pre- and postnatal maternal mood with cortisol stress responses and methylation status at 5 - 7 years suggests that an early adverse environment may confer sensitivity toward altered HPA activity and regulation, and that the postnatal environment may shift the HPA stress response towards vulnerability or resilience to stress-related disorders across the lifespan.
Preface

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Reproductive and Developmental Sciences. I composed this thesis in its entirety under the direction and input from Dr. Tim Oberlander and Dr. Angela Devlin. This thesis was revised by Dr. Tim Oberlander, Dr. Angela Devlin, Dr. Joanne Weinberg, and Dr. Wendy Robinson.

This is a prospective study using both archived cord blood samples and children’s saliva samples and buccal swabs collected by members of the Oberlander Laboratory. Members of the Oberlander Laboratory were also responsible for administering maternal mood questionnaires and conducting the study day stress-challenge with the children. I was responsible for organizing all the samples collected from subjects by Oberlander lab members and extracting DNA from cord blood and buccal epithelial cells and conducting all DNA methylation analyses. I prepared the saliva samples to be quantified at the Technical University of Dresden in collaboration with Dr. Clemens Kirschbaum (Dresden, Germany). I conducted statistical analyses for all results.

A manuscript will be prepared for future publication based on the results in Chapters 3-5.

Statement of research ethics approval: This thesis was conducted under ethical approval from the University of British Columbia Research Ethics Board, and the Children’s and Women’s Health Centre of British Columbia Research Review Committee (Certificate numbers: MOP-57837, MOP-54490, MOP-86296).
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<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>5-HTT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>5-HTTLPR</td>
<td>Serotonin transporter linked polymorphic region</td>
</tr>
<tr>
<td>CAR</td>
<td>Cortisol awakening response</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HAM–A</td>
<td>Hamilton rating scale for anxiety</td>
</tr>
<tr>
<td>HAM–D</td>
<td>Hamilton rating scale for depression</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary adrenal axis</td>
</tr>
<tr>
<td>NR3C1 or Nr3c1</td>
<td>Glucocorticoid receptor gene in humans and rodents, respectively</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>Serotonin transporter gene</td>
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<tr>
<td>SRI</td>
<td>Serotonin reuptake inhibitor</td>
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Chapter 1: INTRODUCTION

1.1 Project Overview

Depression is ranked the third leading cause of disease burden worldwide and is a common mental illness across the lifespan.\textsuperscript{1,2,3} Up to 14% of women experience depression during pregnancy, and one third of these women take serotonin reuptake inhibitor (SRI) antidepressants.\textsuperscript{4} Serotonin (5-HT) not only acts as a neurotransmitter regulating mood, attention, and stress responses, but also plays a neurodevelopmental role as a trophic signal in the fetal brain, regulating the growth and development of serotonergic neurons and target tissues.\textsuperscript{5,6} Growth and function of the serotonergic system is highly interrelated with the hypothalamic pituitary adrenal (HPA) system which acts to regulate stress responses through a complex negative feedback mechanism mediated by cortisol binding glucocorticoid receptors.\textsuperscript{7,8,9}

Prenatal exposure to maternal mood disturbances (i.e. anxiety and depression) may be among the earliest adverse experiences that shape the developing stress systems and thereby confer a lifelong risk for mood disorders.\textsuperscript{8,10,11} In addition, prenatal exposure to serotonin reuptake inhibitor (SRI) antidepressants used to treat maternal mood disturbances have been associated with neurobehavioural effects in neonates\textsuperscript{4,12,13}, however, given SRI use occurs in the context of maternal mental illness, the direct effects of prenatal SRI exposure independent of maternal mood is unknown.

Although the molecular mechanisms underlying how 5-HT influences the development of the HPA stress system remain unclear, genetic variations and epigenetic modifications, such as DNA methylation, may explain individual differences in the developmental pathways for risk or resilience of mood disorders later in life.\textsuperscript{11,14} Two candidate genes that may play a role in regulating stress responses in childhood are \textit{NR3C1} (encodes the glucocorticoid receptor) and
SLC6A4 (encodes the serotonin transporter protein). Studies in animal models and humans have shown adverse early life experiences can affect stress responses later in life and may involve changes in the epigenetic regulation of NR3C11\textsubscript{r} and SLC6A4 promoter regions, respectively,\textsuperscript{15,16} and a variant in the SLC6A4 promoter (5-HTTLPR) has been shown to moderate the effect of stressful life events on depression.\textsuperscript{17}

The overall objective of this thesis is to investigate whether prenatal exposure to SRI antidepressants and maternal mood disturbances are associated with altered cortisol stress reactivity, characterized by variable basal and stress-induced salivary cortisol patterns in 5 - 7 year old children. Furthermore, this thesis will evaluate whether diurnal and stress reactivity patterns are accompanied by differential NR3C1 and SLC6A4 promoter methylation status. The following chapters determined the association between prenatal exposure to SRIs and maternal depressed/anxious mood on 5 - 7 year old children’s: (i) basal and stress-challenge salivary cortisol concentrations; (ii) DNA methylation status of three promoter regions (NR3C1 1\textsubscript{D}, NR3C1 1\textsubscript{r}, SLC6A4); and (iii) 5-HTTLPR genotype as a mediator of early prenatal exposures on stress regulation.

The following introduction will cover relevant background information pertaining to stress regulation and certain factors that may contribute to depression or anxiety later in life. I will discuss the critical role of developmental programming on the individual and interrelated serotonergic and HPA regulatory systems. Next, a review of epigenetic modifications, specifically NR3C1 and SLC6A4 promoter DNA methylation, will be outlined as critical factor linking early life experiences and risk for psychopathology. Lastly, I provide a rationale to support my hypothesis for the role of prenatal exposure to SRIs and maternal mood disturbances in the developmental programming of stress regulation in 5 - 7 year old children.
1.2 Depression and Anxiety

1.2.1 Depression and Anxiety Across the Lifespan

Depression is a significant contributor to the global burden of disease and is a common mental illness in children, adolescents, adults, and the elderly. Depression is a clinically and biologically heterogeneous disorder characterized by a state of low or depressed mood, and is often accompanied by other symptoms including low energy or fatigue, loss of interest or pleasure, and disturbances in sleep, energy, appetite, concentration and psychomotor skills. In addition, children and adolescents with major depressive disorders (MDD) may display an irritable mood, rather than sadness. In 2001, the World Health Organization ranked major depression as the third leading cause of disease burden worldwide, and by 2030 it is expected to be the leading cause of disability in high-income countries. Today, more than 350 million people of all ages suffer from depression.

From the National Comorbidity Survey - Adolescence (NCS-A), the median age of onset for MDD was between 11 and 14 years of age. According to the DSM-5, the twelve-month prevalence of MDD in the United States is approximately 7%, with 18 - 29 year olds displaying a 3-fold higher prevalence than individuals 60 years or older. From the 2001 - 2002 NCS - Replication survey including 9090 individuals 18 years or older in the United States, the lifetime prevalence of MDD was found to be 16.2%. While, from 36 984 individuals aged 15 years or older who participated the 2002 Canadian Community Health Survey (CCHS 1.2) the lifetime prevalence of a MDD was found to be 12.2%, with the peak annual prevalence occurring between 15 - 25 years of age. In addition, females are reported to experience a 1.5 to 3-fold higher rate of a MDD than males, beginning in early adolescence. For example, data from the 1990 – 1992 NCS in the United States showed the lifetime prevalence of a MDD in adolescents
and young adults (15 - 24 years) was 20.6% for females and 10.5% for males. Furthermore, approximately 90% of individuals with depression will experience one or more depressive episodes in their lifetime, and the risk of recurrence is higher in younger individuals.

Anxiety disorders are characterized by a number of behavioural and physiological responses including excessive anxiety (anticipation of a future threat), restlessness, being easily fatigued, irritability, and disturbed sleep. Anxiety symptoms tend to be chronic and fluctuate across the lifespan, and the rates of full remission are very low. In addition, females are twice as likely to experience an anxiety disorder, and the age of onset is spread over a broad range. Interestingly, from the NCS survey in the United States, 31.9% of adolescents between 13 - 18 years of age had an anxiety disorder, and the median age of onset for an anxiety disorder was 6 years of age. Among the general community in the United States, the lifetime risk of developing a generalized anxiety disorder is 9.0%, and the 12-month prevalence is 0.9% and 2.9% among adolescents and adults, respectively.

Since the late 1970s, anxiety disorders have been classified separately from mood disorders. However, there is a considerable overlap in symptoms of anxiety and depression, and many rating scales contain common factors between these mood disorders. A long-term and frequent recurrence of depressive symptoms substantially increases the likelihood of developing comorbidities including anxiety disorders. Moreover, individuals with an anxiety disorder are likely to meet criteria for other anxiety and unipolar depressive disorders. In a prospective birth cohort following subjects up to 32 years of age, 48% of subjects with lifetime depression were found to have an anxiety disorder. From the NCS survey, 59.2% of individuals reporting MDD also reported one or more lifetime anxiety disorders. Furthermore, approximately 40% of
adolescents with one class of a mood disorder (i.e., anxiety, behavioural, or mood), meet criteria for another class of mood disorder.²⁹

Individuals’ levels of symptoms of anxiety and depression are relatively stable over time ³²,³³ and may be attributed to genetic influences, environmental experiences, and the complex interplay between these components.³² Romans et al³⁴ analysis of the CCHS 1.2 found an increased risk for depression was associated with female sex, younger age, not being married, unemployment, residing in a rural area, and self-rated fair to poor physical health. Although depression has been studied extensively in recent decades, the underlying molecular mechanisms and biological determinants of depression are currently unknown.⁶ Several hypotheses of psychological and neurological determinants of depression include immune system imbalance, dysregulation of the HPA axis, and neurotransmitter dysfunctions.⁶

Depression is commonly treated with serotonin reuptake inhibitor (SRIs) antidepressants, which are predominately prescribed over tricyclic antidepressants and monoamine oxidase inhibitor antidepressants given the limited side effects of the medication.³⁵ Although studies have established SRIs are safe for treatment of adult depression, the question of safety regarding antenatal use of these antidepressants remains unknown.³⁵ Given the majority of individuals develop MDD by early adulthood, and MDD is common, recurrent, and associated with significant morbidity and mortality in children and adolescents,³⁶,³⁷ the identification of factors contributing to the risk or resilience for depression in children is imperative. Furthermore, maternal depression and anxiety during pregnancy are recognized as key early life experiences that may influence the way a child regulates mood throughout their lifetime and is therefore a crucial component to evaluate when researching the etiology of depression.³⁸,³⁹
1.2.2 Depression During Pregnancy

Women who are pregnant have a considerably increased risk for developing a MDD, particularly women with pre-existing psychiatric illnesses. According to the DSM-5, between 3 - 6% of women will experience the onset of a major depressive episode during pregnancy, or in the weeks following delivery. Additional risk factors for antenatal depression include a family history of depression or bipolar disorder, childhood maltreatment, single motherhood, having more than 3 children, insufficient social support, and domestic violence. Furthermore, antenatal depression is often comorbid with anxiety.

Although the rate of depression in the 1st trimester parallels non-pregnant female populations, the severity increases, and prevalence nearly doubles in the 2nd and 3rd trimester. In a population-based study from British Columbia, 14% of mothers were diagnosed with antenatal depression. These findings are consistent with the Avon cross-sectional study in England reporting 11.8% and 13.5% of mothers were depressed at 18 and 32 weeks gestation, and a systematic review of 21 articles from 13 countries reporting 7.4%, 12.8%, and 12.0% of women were depressed during the 1st, 2nd, and 3rd trimester, respectively. The prevalence of depression varies with geographic location and ethnicity. For instance, Ali Shah et al found a much higher prevalence of antenatal depression in Pakistani women (48.4%), compared to Canadian Aboriginal (31.2%) and European (8.6%) women from Saskatchewan. However, unique factors specific for each group contributed to the antenatal depression and included physical abuse in Pakistani women, sexual abuse in Aboriginal women, and low income in European women. Additionally, many clinical screening questionnaires for depression include questions about fatigue, disturbances in sleep, and altered appetite, which are considerably common variables in unaffected pregnant women. Nonetheless, given these substantial rates of
depression, and noting that many cases go undiagnosed or untreated,\textsuperscript{42} depression during pregnancy has become a major public health concern.\textsuperscript{47}

The treatment interventions for antenatal depression depend on the safety profile of medications, stage of pregnancy, symptom of the patient, and the depression severity.\textsuperscript{42,43} Attempting to minimize risk in the fetus while optimizing maternal benefits makes choice of treatment uniquely challenging.\textsuperscript{48} For mild to moderate depression, nutritional adequacy and weight management, psychosocial approaches, stress reduction therapies, and cognitive behavioural therapy have shown to be effective in reducing depression.\textsuperscript{42,49,50} Treatment with antidepressant medication is considered for women with moderate to severe depression, or upon failure in reducing depressive symptoms with nonpharmacologic options.\textsuperscript{40}

In recent years, SRIs have become increasingly used to treat depression during pregnancy, with paroxetine, fluoxetine, and sertraline being the most common SRI medications prescribed.\textsuperscript{4} Currently, there are no clear clinical guidelines for the treatment of depression during pregnancy. Although the website for The Society of Obstetricians and Gynaecologists of Canada discusses the treatment of depression during pregnancy, in regards to antidepressant use, it states that each case must be evaluated on an individual basis and therefore should be discussed with a physician. However, even in the presence of SRI treatment, prenatal maternal mood disturbances continue for some women, and antenatal mood may have lasting consequences for the cognitive, behavioural, and emotional development of the child.\textsuperscript{51}
1.3 Developmental Programming

1.3.1 Developmental Origins of Health and Disease

Developmental programming suggests that early life experiences can set pathways for adaptability of health and disease later in life.\textsuperscript{38,52} The Developmental Origins of Health and Disease (DOHaD), often called “Barker’s Hypothesis”, was first proposed by David Barker and evolved from a study in 1986 that found a positive correlation between birth weight, geographic location, and infant and adult mortality rates due to cardiovascular disease.\textsuperscript{53} Specifically, Barker et al\textsuperscript{53} reported that birth weight reflected the distribution of ischemic heart disease in England and Wales.

More recently, Gluckman and colleagues state that rather than assuming adverse early life exposures lead to disease, an alternative “predictive-adaptive-response” theory suggests that under certain conditions, \textit{in utero} stress may in fact be advantageous.\textsuperscript{54} In this way, environmental stress during early life may not induce an immediate physiological adaptation, but be beneficial if confronted with stress later in life or possibly increase risk for disease in more favorable conditions.\textsuperscript{54}

Over the years, the concept of developmental programming has grown immensely with countless studies that have established, whether adaptive or maladaptive, that early life adversity has profound effects on shaping both health and illness across the lifespan. Despite this fast growing discipline, the mechanisms underlying such programming have yet to be elucidated; they may involve the interplay between early environments, genetic variations, and epigenetic processes.
1.3.2 Prenatal Exposure to Maternal Mood and Serotonin Reuptake Inhibitors

Prenatal exposure to maternal depression and anxiety may affect fetal neurodevelopment of the HPA stress system and increase the risk of psychopathology later in life.\textsuperscript{10,11} In addition, the use of SRIs during pregnancy has become increasingly common, and the developmental impact of SRIs is often undistinguishable from prenatal maternal mood disturbances.\textsuperscript{55}

Despite a relatively stable incidence of antenatal depression, there has been a substantial rise and approval of SRI use during pregnancy.\textsuperscript{4} For example, in a population based study in British Columbia, Oberlander \textit{et al}\textsuperscript{24} reported the use of SRI medication during pregnancy increased from 2.3\% to 5\% between 1998 and 2001, with 14\% of pregnant women depressed during this time. Similarly, a large cohort study in Tennessee found the proportion of pregnant women using an SRI increased from 2.9\% in 1999 to 10.2\% in 2003,\textsuperscript{56} while a study spanning 7 geographic regions in the United States reported nearly 7\% of pregnant women were prescribed an SRI between 2004 and 2005.\textsuperscript{57} These rates suggest that a substantial number of pregnant women take antidepressants for at least a portion of their pregnancy.

Both maternal mood and SRI use during pregnancy have been associated with negative effects in the developing fetus and adverse neonatal outcomes that may extend well beyond infancy.\textsuperscript{4,12,13} Furthermore, it is both unethical and potentially harmful to study the effects of SRI use during pregnancy in the absence of maternal depression, making it extremely challenging to distinguish between associated risk of fetal SRI exposure independent from maternal mood.\textsuperscript{4} Although the effect of SRI exposure may be evaluated through comparing children with prenatal exposure to SRIs and maternal depression, and with exposure to maternal depression alone, factors such as maternal illness severity may confound the ability to compare these groups.\textsuperscript{55} Thus, the importance of understanding the developmental outcomes in children with prenatal
exposure to SRIs and maternal mood disturbances along with the molecular mechanisms underlying these associations is both urgent and warranted.

1.3.3 Prenatal Stress: Epidemiological Evidence of Developmental Programming

Over 40 years ago, epidemiological research on birth cohorts from the Dutch Hunger Winter of 1944 found early prenatal exposure to the famine was associated with adverse reproductive outcomes in the next generation. \(^{58,59}\) Adult children exposed prenatally to severe maternal dietary energy intakes (500 - 1000 kcal/day) showed an increased risk for congenital anomalies of the central nervous system, schizophrenia, and schizophrenia spectrum personality disorders. \(^{58}\) Additionally, men prenatally exposed to maternal famine during the 1\(^{st}\) and 2\(^{nd}\) trimester showed increased risk for antisocial personality disorder (ASPD), while exposure during the 3\(^{rd}\) trimester was not associated with ASPD. \(^{58}\) These findings suggest that both prenatal exposure to maternal famine and the time during development when exposure occurs impose neurotoxic effects on the developing brain and may be the result of the combined effects of diminished maternal energy intakes and the distress associated with war and famine. \(^{14}\)

Overall, the Dutch Birth Cohort study provided fundamental insights for subsequent research regarding adverse prenatal environments and long-term health outcomes.

*Prenatal exposure to maternal mood disturbances*

Research on early life adversity has expanded to examine the relationship between prenatal stress during sensitive periods of development and behavioural outcomes in children. Antenatal stress and anxiety impacts fetal biobehavioural development, and influences cognitive, behavioural, and emotional outcomes in childhood. \(^{11,60,61}\) Blair *et al* \(^{62}\) reported that higher
maternal antenatal anxiety, between 13 and 17 weeks gestation, was associated with higher negative temperament in children at 2 years of age, independent of sociodemographic factors, obstetrical risk, and postnatal maternal psychological state. Depressed and anxious mood in women during pregnancy has been associated with greater problematic internalizing behaviours at 4 years of age. Maternal anxiety in the 3rd trimester was associated with behavioural/emotional problems at 6 years of age, even after adjusting for postnatal maternal anxiety, suggesting a greater importance of maternal mood during pregnancy on childhood behaviour. In support of this, a study that only evaluated the effects of postpartum depression found no relationship with cognitive performance in children at 5 years of age. On the other hand, maternal anxiety and postnatal depression acted in separate and additive manners for certain risks of behavioural and emotional problems in children at 4 years of age. In contrast, mental development and advanced motor development in 2-year-old children was positively associated with higher levels of maternal anxiety and depressive symptoms during pregnancy, indicating moderate amounts of antenatal distress may in fact be facilitative.

Buss et al. presented the first human evidence that maternal anxiety at 19 weeks of pregnancy was associated with reduced grey matter volume, specifically in brain regions associated with cognitive performance, in their 6 - 9 year old children. This relationship was not observed for maternal anxiety at 25 and 31 weeks of pregnancy, which suggests that the long-term effects of prenatal exposure to maternal anxiety is time-dependent and may be related to the unique timetables of different brain region development illustrating specific periods of vulnerability. Furthermore, maternal anxiety during pregnancy explained 9% and 22% of the variance in self-reported anxiety and ADHD in 8 - 9 year-old children, and was associated with difficulties in performances on computerized cognitive tasks in 14 - 15 year olds. Together,
these studies suggest that high levels of antenatal anxiety may enhance offspring susceptibility for developing a childhood mental health condition.

**Prenatal exposure to SRI antidepressants**

In addition to maternal mood disturbances, SRIs affect fetal serotonergic signaling and have been associated with changes in fetal neurobehavioural development. Using population-level data from Saskatchewan and British Columbia, low birth weight, preterm birth, seizures, and respiratory distress were greater in infants prenatally exposed to SRIs, compared to non-exposed infants. Interestingly, Oberlander et al. found if maternal depressive symptom severity is not accounted for in the analysis, adverse neonatal outcomes may be falsely attributed to SRI exposure rather than maternal depression.

A cluster of symptoms referred to as “poor neonatal adaptation” includes respiratory difficulty, hypoglycemia, irritability, temperature instability, and was reported in 15 - 30% of newborns exposed to SRIs late in pregnancy. These symptoms typically resolve within two weeks. Proposed causes include factors involving gene-SRI interactions, and “withdrawal” or “discontinuation” syndrome to increased SRI levels. Conversely, in a prospective study of 46 women, poor neonatal adaptation was associated with higher maternal anxiety and depression in the 2nd and 3rd trimester, independent of pharmacologic intervention. Whether no effect or a negative effect of SRI exposure was found in neonates, these studies are a few of many that illustrate the difficulty in teasing apart prenatal maternal mood and pharmacologic exposure.

Beyond the infancy period, a study that reported lower APGAR scores in infants with prenatal SRI exposure, compared to no SRI exposure, also found lower Bayley developmental scores and motor quality function in the prenatal SRI-exposed group of children between 6 and
40 months of age. Interestingly, Oberlander et al.\textsuperscript{79} found an association between higher umbilical cord SRI drug levels at birth and higher externalizing behaviours at 4 years of age, however, after accounting for maternal mood at the time of study, the drug levels no longer accounted for these findings. Similarly, \textit{in utero} exposure to tricyclic antidepressant drugs or fluoxetine had no association with global IQ, language development, or behavioural development in preschool children after adjusting for confounders including child’s age, prenatal maternal depression, socioeconomic status, and duration of SRI exposure.\textsuperscript{80}

Overall, it is estimated that approximately 15\% of behavioural problems in childhood can be attributed to maternal antenatal anxiety,\textsuperscript{60} and the molecular mechanisms underlying these effects are likely to be multifaceted in nature, varying in timing of exposure, type of exposure, and the complex interplay between genetic and environmental risk factors.\textsuperscript{14}

\subsection*{1.3.4 The Role of Serotonin}

The neurotransmitter serotonin (5-HT) participates in many physiological and behavioural processes, including mood, emotion, cognition, sexual behaviour, and circadian and neuroendocrine rhythms involving appetite and sleep; all of which are affected by depression.\textsuperscript{6,81,82} While the majority of cell bodies of the serotonergic neurons are localized in the raphe nuclei of the brain stem, long axons of these neurons innervate almost the entire brain.\textsuperscript{6,83} Additionally, high 5-HT concentrations are seen in the gut, lung, kidney, and testis.\textsuperscript{84} The ability of the serotonergic system to dynamically integrate and stabilize central nervous system structure and function relies on processes involving the availability of L-tryptophan (5-HT precursor), 5-HT synthesis, release, re-uptake and metabolism,\textsuperscript{6} and the postsynaptic response mediated by 16
specific 5-HT receptor subtypes.\textsuperscript{82,84,85} Altered serotonergic functioning is implicated in the pathophysiology of many clinical conditions including anxiety, aggression, and depression.\textsuperscript{6}

The transmembrane serotonin transporter (5-HTT) is a key regulator of 5-HT concentrations and governs the intrasynaptic re-uptake of 5-HT into the presynaptic neuron, where it can be degraded or stored for subsequent release.\textsuperscript{6,86} The serotonin transporter determines the magnitude and duration of the 5-HT synaptic signal, and is the initial target for SRI antidepressant medication.\textsuperscript{4,6,86} Through inhibiting the 5-HT re-uptake role of 5-HTT, SRIs work to increase the accumulation of 5-HT in the synapse enabling increased transmission to postsynaptic 5-HT receptors.\textsuperscript{87} The association between 5-HTT abnormalities and depression is widely reported and confirmed through functional imaging\textsuperscript{88} and post mortem brain studies\textsuperscript{89} which have found reduced 5-HTT binding sites in depressed individuals. Additionally, neuroendocrine evidence suggests that depressed patients exhibit decreased function and number in 5-HT\textsubscript{1A} receptors, and increased 5-HT\textsubscript{2} function.\textsuperscript{6,89,90}

Serotonin expression was identified in the developing midbrain before the onset of serotonin signaling and synaptogenesis, illustrating its important role during embryogenesis involving the developmental regulation of cell proliferation, migration, differentiation, and programmed cell death.\textsuperscript{91,92} In fact, 5-HT not only acts as a neurotransmitter in the mature brain, but also plays a neurodevelopmental role in the fetal brain regulating serotonergic neurons and target tissues.\textsuperscript{5}

\textit{Animal models of altered early 5-HT transmission}

Animal models provide a means by which a high level of experimental control enables the evaluation of causal outcomes on behavioural and physiological variables in offspring.
The effects of early changes in 5-HT signaling have been studied in animal models using chronic SRI exposure, transient 5-HTT inhibition, or Slc6a4 -/- mice. In rodents, such variations resulted in profound reductions in 5-HTT that persisted into adulthood, fewer serotonergic neurons, and abnormal emotional behaviours in adult mice, all suggesting that altered 5-HTT during early development can disrupt normal maturation of the serotonergic system and alter 5-HT dependent neuronal processes. Fetal sheep of mothers administered fluoxetine displayed decreased blood oxygen levels, brain blood flow, breathing movements, rapid eye movement during sleep, and altered fetal behaviour. In contrast, Slc6a4 -/- mice and rats have shown increased recall of fear memory and decision making under ambiguity, respectively, and rats exposed to high SRI levels in utero showed a favorable effect on learning and memory in water maze and passive avoidance tests.

Importantly, these studies suggest that a 5-HTT blockade, whether genetically or pharmacologically driven, may be beneficial to postnatal behaviour. Together, these animal models enable examination of altered 5-HTT functions apart from maternal mood, and suggest that an increased serotonergic tone during developmentally sensitive periods alters 5-HT function.

**Human evidence of altered early 5-HT transmission**

In addition to uncertain adverse or beneficial long-term outcomes, the applicability or relevance of animal models to the human case of maternal mood/SRI use during pregnancy and childhood development remains unknown. In humans, maternal prenatal SRI treatment was shown to alter central fetal 5-HT concentrations as SRIs can extensively transfer across the placenta and blood-brain barrier. The placenta is able to convert maternal tryptophan to 5-
HT and release the neurotransmitter into fetal circulation. In addition to maternal SRI dosage and serum concentrations, fetal drug exposure is determined by other factors such as fetal gene variants associated with placental transporter proteins that may influence the rate of placental drug transfer.

In utero SRI exposure has been associated with infant neurobehavioural changes with cord blood 5-HII (metabolite of 5-HT), and lower cord blood S100B protein concentrations, a biomarker of early brain maturation and serotonergic function. Given the vulnerability and sensitivity during fetal neurodevelopment, early life alterations of 5-HT, detected by biomarkers of 5-HT function and concentration, may influence the integral mechanism of 5-HTs role in neural development. In fact, dysfunctions in serotonergic tone during pregnancy have revealed a “serotonergic vulnerability” which may affect emotional regulation in adults, and increase the susceptibility for depression later in life.

1.3.4.1 The 5-HTTLPR Variant

Located on the human chromosome 17q11.2, the SLC6A4 gene encodes for 5-HTT and is widely studied as a genetic factor associated with behavioural and neuropsychiatric disorders. The 5-HTT gene-linked polymorphic region (5-HTTLPR) located in the proximal 5’ promoter region of SLC6A4 has an allelic variation associated with depression and anxiety. A 44 base pair insertion/deletion polymorphism in this region produces a long (l) or short (s) variant, with the long variant transcriptionally more efficient, resulting in higher 5-HTT expression and function. In contrast, the s allele is associated with restricted transcriptional activity of 5-HTT, leading to lower 5-HTT expression and decreased 5-HT reuptake in
presynaptic neurons. Individuals with an s allele also respond less rapidly to SRIs than l/l genotype.

Neurophysiological imaging has shown the s allele is associated with higher activity of the anterior cingulate cortex, and functional magnetic resonance imaging (fMRI) scans reveal greater amygdala activation during an emotion-related task and reduced grey matter and connectivity between these regions, suggesting a relationship between 5-HTT and cognitive function. For instance, Hariri et al found the s allele was associated with reduced 5-HTT expression and increased fear and anxiety and hyperresponsiveness to a fearful stimuli.

In addition, an A-to-G substitution (rs 25531) subdivides the long allele into LA or LG, with the LG allele less transcriptionally efficient and equivalent to reduced 5-HTT expression observed with the s allele. In general, the amount of 5-HT available at postsynaptic sites is substantially affected by the 5-HTTLPR genotype and a combination of other genetic and environmental factors may contribute to disease pathology.

A meta-analysis of 54 studies found the s allele to be associated with an increased risk of developing depression under stress, suggesting the 5-HTTLPR genotype moderates the effect of stressful events on depression. In a prospective birth cohort, Caspi et al found the 5-HTTLPR genotype moderates the effects of life events on depression. Specifically, individuals with greater levels of stressful life events, childhood maltreatment, and the s allele, exhibited more depressive symptoms and rates of depression than individuals with the l/l genotype. In this way, the 5-HTTLPR genotype may function as a key moderator of early life environments and prenatal SRI exposure by influencing additional long-term risk or resilience through altered 5-HT availability.
The 5-HTTLPR genotype was shown to moderate the effects of prenatal exposure to SRIs and maternal mood on neonatal and early life outcomes, respectively.\textsuperscript{117,118} At 6 months of age, an association was found between antenatal anxiety exposure and negative emotionality in infants with an s/s or l/s genotype, compared to the l/l genotype, suggesting the s allele may increase vulnerability to adverse early environmental influences.\textsuperscript{118} Independent of SRI exposure, higher 3rd trimester maternal anxious mood was associated with greater anxious and depressive symptoms in 3-year-old children with the s/s genotype compared to those with one or two l alleles.\textsuperscript{119} Between 8 and 12 years of age, children of mothers with a history of major depression exhibited attentional biases for sad faces (rather than happy or angry), and the bias appeared strongest among children with an s/s genotype.\textsuperscript{120} Overall, the association between 5-HTTLPR and brain endophenotypes is well established, and these studies suggest that the 5-HTTLPR variant may confer a vulnerability to mood disorders associated with adverse early life experiences for some,\textsuperscript{109} but not all individuals,\textsuperscript{121} possibly arising from the trophic role of 5-HT during neurodevelopment.\textsuperscript{122}

1.3.5 The Regulation of the HPA Axis

The hypothalamic pituitary adrenal (HPA) axis is an adaptive system involving many neuronal circuits for maintaining function under changing environmental circumstances.\textsuperscript{123,124} In response to a situation perceived as stressful, a cascade of events involves corticotropin releasing hormone (CRH) release from the hypothalamus, secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, and subsequent cortisol release from the adrenal cortex.\textsuperscript{123} This process is said to take place within a matter of minutes.\textsuperscript{123} Additionally, CRH cell bodies are involved in corticolimbic circuitry of the amygdala and its connections.\textsuperscript{125} Cortisol is
the principal steroid hormone produced by the adrenal cortex that regulates blood pressure, glucose metabolism, and immune competence, and participates in neural survival, neuronal excitability, and neurogenesis. Upon release from the adrenal cortex, cortisol reaches every organ system to achieve ‘allostasis’, the process of integrating the coordination of brain and body function aimed toward re-establishing homeostasis. In addition to mobilizing energy metabolism for coping with a stressor, cortisol shuts down the immune and sympathetic nervous systems fight or flight responses to prevent overshooting and damaging the organisms.

The HPA axis is regulated through a complex negative feedback mechanism, mediated by cortisol binding two intracellular receptor subtypes (mineralcorticoid receptors, MR; and glucocorticoid receptors, GR), which involve inhibiting activity in the hippocampus, hypothalamus, and pituitary. MRs and GRs in the brain play different but complementary actions where the high affinity MRs are believed to play a role in regulation of circadian fluctuations, while low affinity GRs are important in stress response regulation when glucocorticoids are elevated.

Acute overactivation, or prolonged activation, may force a continuous stress response where chronic HPA hyperactivation can be maladaptive, leading to stress-related disorders. Consequently, chronic HPA overactivation and subsequent hypercortisolism can give rise to wear and tear or ‘allostatic load’, which is implicated in a wide variety of illnesses and imposes an increased risk for depression, abdominal obesity, osteoporosis, and cardiovascular problems.

For more than two decades, hyperactivity of the HPA axis is a consistent finding in patients with major depression. A dysregulated HPA axis has been reported by numerous studies with depressed individuals exhibiting higher cortisol concentrations in plasma, urine, and
cerebral spinal fluid; an exaggerated cortisol response to ACTH; enlargement of the pituitary and adrenal glands; and loss of diurnal variation reflected in a flatter daily slope compared to healthy individuals.\textsuperscript{133,134,135,136,137}

\textit{Animal models of early life adversity and dysregulation of the HPA axis}

Research studies in animal models indicate antenatal stress has disruptive effects on stress responses in offspring,\textsuperscript{138,139} and may produce long-term effects on a behavioural and physiological level.\textsuperscript{140,141} Meaney and colleagues have shown that early environmental factors related to maternal-infant behaviour can have profound regulatory effects on HPA axis regulation and stress response that may increase vulnerability to neuropathology in later life.\textsuperscript{142,143} In rodent studies, pups exposed to high maternal handling, compared to pups exposed to low maternal handling, exhibited lower plasma ACTH and corticosterone response to stress, increased hippocampal GR expression, enhanced GC feedback sensitivity, and decreased hypothalamic CRH expression.\textsuperscript{144} Overall, pups that received higher levels of maternal care demonstrated an increased effectiveness of the HPA negative-feedback system and enhanced ability to cope with later life stress.\textsuperscript{144}

\textit{Early life adversity and dysregulation of the HPA axis in humans}

Although there is much less understanding of the mechanisms underlying the developmental effects of exposure to prenatal maternal mood, studies have shown persistent effects of prenatal mood on HPA axis regulation. For example, 2\textsuperscript{nd} and 3\textsuperscript{rd} trimester cortisol from maternal hair samples correlated with 1 and 3 year cortisol levels from children’s hair samples.\textsuperscript{145} Prenatal depression and higher 3\textsuperscript{rd} trimester salivary cortisol concentrations were
associated with increased 2-month infant negative reactivity, and maternal cortisol alone was associated with infant cognitive functioning. At 6 years, maternal prenatal anxiety and heightened morning cortisol predicted higher salivary cortisol concentrations on children’s first day of school; however, concurrent maternal mood wasn’t accounted for in this study. In adolescence, antenatal anxiety was associated with a higher cortisol awakening response at 10 years of age, and both a flattened daytime cortisol profile and diminished cortisol-awakening response at 15 years of age. Interestingly, Bosch et al found an interaction between pre/postnatal adversity and childhood adversity was associated with higher salivary cortisol concentrations between ages 6 - 11, while only adolescent adversity was associated with lower cortisol concentrations at 12 - 13 and 14 - 15 years. Behavioural correlates of cortisol stress reactivity in children also have shown to differ between girls and boys.

Given the increased SRI usage during pregnancy, examining long-term drug effects may provide additional insight into early programming of the HPA system. For instance, after controlling for feeding mode and maternal mood, 3-month-old infants with prenatal SRI exposure exhibited lower early evening basal salivary cortisol concentrations compared to non-SRI-exposed infants.

Furthermore, the placenta is thought to play a critical role in fetal programming as it both incorporates and transduces information from the maternal host environment into the fetal developmental program. In this way, the adapting placental/fetal unit may respond to a maternal stressor through altering the trajectory of fetal brain development and regulation of further stress responses.

Although these studies suggest that environmental factors contribute to calibration of the HPA stress system over the course of early development, discrepancies remain regarding
whether adverse experiences activate or dampen the HPA axis stress response. Boyce et al\textsuperscript{125} proposes such inconsistencies may reveal a bivalent manner in which stress reactivity operates, where adverse early environments may escalate risk in high-stress contexts, but diminish risk and be protective in a supportive and low stress context.

Given HPA axis function is regulated through feedback inhibition upon cortisol binding GRs, studies researching underlying molecular mechanisms in depression have focused on the expression and function of GRs.\textsuperscript{7,155} Pharmacological studies in patients with major depressive disorders have implicated GR with impaired HPA regulation,\textsuperscript{156} and depressed individuals have exhibited reduced GR mRNA expression in the frontal cortex and hippocampus.\textsuperscript{157} Thus, manifestation of allostatic load during the normal course of daily activities, or resulting from stressful events, is thought to be influenced by genetic risk factors and early life events that determine an individual’s susceptibility or resiliency to stressors in adulthood.\textsuperscript{38}

1.3.6 HPA Stress Response and Serotonergic Regulatory Systems

Although the serotonergic and HPA regulatory systems have been described separately thus far, these systems are highly interrelated at a biological level, and interactions of these systems are of particular interest considering the large body of evidence implicating HPA axis dysregulation and altered serotonergic function in depression and anxiety.\textsuperscript{158,159,160} In this way, an interaction between system abnormalities may influence the complex relationship between neurophysiological function and mood, and this link could be an important etiological mechanism in depression.\textsuperscript{161}

Glucocorticoids increase the size of serotonergic neurons, and previous studies have investigated the impact of cortisol on modifying 5-HT turnover and the expression of specific 5-
In turn, 5-HT acts through a negative feedback process and a variety of receptor subtypes to regulate the release of CRH and cortisol. However, sustained exposure to stress induced cortisol may attenuate the serotonergic systems. Tafet et al. demonstrated that prolonged cortisol release increased 5-HTT expression and reduced 5-HT availability in the synaptic cleft, suggesting that a dysregulated HPA axis may be implicated with differential 5-HT uptake rates. Given 5-HT participates in the regulation of cortisol, this finding suggests that a dysregulated axis may be further distanced from a adaptive cortisol response through altering one of its key regulators. Moreover, chronic cortisol secretion may stimulate tryptophan pyrrolase (metabolizing enzyme of tryptophan), which could diminish tryptophan availability and 5-HT production. In animal models, chronic stress induced cortisosterone attenuated the serotonergic system, which was exemplified by down regulation of 5-HT1A expression in the CA1 hippocampus region and dentate gyrus, and decreased 5-HT turnover and release.

Antidepressants have shown not only to improve depressive symptoms but directly modulate GRs. As a result, SRIs not only target the serotonergic system, but increased 5-HT turnover has shown to increase GR expression in hypothalamic and hippocampal rat neurons, illustrating a restored GR mediated inhibition of the HPA axis. Whether the effects of antidepressants depend directly on GR, or on mechanisms involving GR remains unclear, but these effects are thought to include second-messenger signaling mechanisms mediated by cycling AMP (cAMP) and protein kinase A (PKA) cascades, differential phosphorylation of GR serine residues, and inhibition of cell membrane pumps.
The HPA and serotonergic system in animal models

In a rodent model, the effect of early maternal care on hippocampal GR expression has shown to be mediated by 5-HT turnover at 5-HT7 receptors.\textsuperscript{172,173,174} In this way, 5-HT is a critical link between early environmental stimulation and the development of central GR binding capacity.\textsuperscript{172} Early postnatal SRI treatment (week 1 - 3) in prenatally stressed mice was shown to reduce plasma corticosterone concentrations in response to a stressor, and lower 5-HT turnover rate.\textsuperscript{175} However, no behavioural or structural benefits resulted from late postnatal treatment with an SRI, suggesting a critical window for neurodevelopmental plasticity and therapeutic intervention.\textsuperscript{175}

The HPA and serotonergic system in humans

Recent findings have suggested that 5-HT may play a role in early HPA stress regulation in humans. During a standard heel prick procedure, a significantly higher salivary cortisol response was exhibited in newborns with the s/s 5-HTTLPR genotype, compared to l/s or l/l individuals, independent of pre- or perinatal environmental factors.\textsuperscript{176} Similarly, Gotlib \textit{et al}\textsuperscript{177} found girls with the s/s genotype exhibited a higher and more prolonged salivary cortisol concentrations in response to a laboratory stressor, compared to individuals with the l allele, further indicating an association between the 5-HTTLPR genotype and biological stress reactivity. In young adults, an interaction between 5-HTTLPR genotype and stressful life events on a laboratory endocrine stress response was found only when the life event occurred during the first 5 years of life.\textsuperscript{176} Thus, a differential threshold in cortisol stress responses, as a function of 5-HTTLPR genotype, may increase susceptibility for depression in the face of stressful life events for certain individuals.
The pathogenesis of depression was once thought to result from simple imbalances of fast acting neurotransmitters, but given substantial evidence involving the collective long-term effects of stress, cortisol, 5-HT, antidepressants and many other factors, the multifaceted nature of stress related disorders is quite apparent. In addition to the interrelated development and function of the serotonergic and HPA systems, these systems appear to be extremely sensitive to the effects of early life experience.

1.4 Epigenetic Regulation as a Programming Mechanism

During pre and postnatal periods, the developing brain is highly sensitive to environmental influences that have the capacity to shape neural circuits and determine the structural and functional aspects of brain and behaviour. Originally, epigenetic regulation was once thought to be limited to processes involving cellular differentiation, genomic imprinting, and disease, however, recent findings now indicate a diverse range of environmental exposures can influence epigenetic variations across the lifespan. Thus, beyond allelic variations, gene expression can be influenced through epigenetic mechanisms including DNA methylation, chromatin modifications, and miRNAs. In this way, epigenetic changes are now being increasingly recognized as critical factors linking the dynamic interaction between early life experiences, genetic variations and activity, and risk for psychopathology.

Epigenetics, literally meaning ‘above’ or ‘on top of’ genetics, are molecular mechanisms that influence gene expression without altering the underlying nucleotide sequence. DNA methylation involves the addition of a methyl group to the 5’ position of cytosine to modify the accessibility of DNA to the process of gene transcription. Methylation of cytosines that lie within transcription promoter regions are often associated with gene silencing. Such effects
occur through direct interference of transcription factor binding, or through proteins that recognize methylated cytosines (Methyl-CpG binding proteins) and use co-repressor molecules to mediate transcription factor binding.\textsuperscript{180,181}

The investigation of epigenetic pathways in context of prenatal exposure to maternal mood and antidepressants has become an invaluable tool that may explain individual differences in developmental risk or resilience.\textsuperscript{11,14}

1.4.1 \textit{SLC6A4} Methylation

As previously mentioned, decades of research has focused on the role of 5-HT and related genetic variations that alter early levels of 5-HT and modify risk for mood disorders in the face of early life adversity.\textsuperscript{17,182} Now recent attention has shifted to understanding how epigenetic modifications, such as methylation of the \textit{SLC6A4} promoter, influences the risk of major depression that may reflect a mechanism that alters 5-HT signaling during development\textsuperscript{183}. DNA methylation of the \textit{SLC6A4} was shown to influence mRNA expression and subsequent 5-HT levels.\textsuperscript{183,184} For instance, mice overexpressing 5-HTT have exhibited lower extracellular 5-HT, and lower 5-HT concentrations in different brain regions.\textsuperscript{185} Interestingly, Philibert \textit{et al}\textsuperscript{186} found an association between \textit{SLC6A4} methylation and mRNA expression only when 5-HTTLPR genotype was controlled for in their analyses, suggesting that both genotype and methylation influence gene expression.

Adults that have experienced abuse during childhood have shown greater \textit{SLC6A4} promoter methylation in lymphoblasts, suggesting that adverse early experiences may have lasting effects on gene regulation through epigenetic modification.\textsuperscript{16} In a study of patients with major depression, higher \textit{SLC6A4} methylation status in leukocytes was significantly correlated
with a range of childhood adversities, as well as family history of depression, higher perceived stress, and more severe psychopathology at presentation. Conversely, depressive symptoms during adolescence were found not to be associated with SLC6A4 methylation in buccal cells; however, depressive symptoms were more common in individuals with 5-HTTLPR s allele and higher SLC6A4 methylation, compared to individuals with no depressive symptoms. Higher maternal depressed mood during the 2nd trimester of pregnancy was associated with lower methylation of the SLC6A4 promoter in newborn cord blood leukocytes. Interestingly, higher 2nd trimester antenatal depressed mood was associated with lower maternal SLC6A4 methylation status, while no associations were found with 3rd trimester maternal mood or were related to maternal SRI treatment.

For the most part, these findings support the association between SLC6A4 methylation and mood disorders, and further illustrate the importance of a critical role of variations in 5-HT signaling that might provide a link between early life experience and depression. In this way, early environmental factors influencing SLC6A4 methylation may contribute to an underlying molecular mechanism associated with development of mood disorders across the lifespan, possibly through altered 5-HT signaling in the developing fetus. Furthermore, increasing use of SRI antidepressants during pregnancy has introduced a key prenatal factor that alters 5-HT signaling during developmentally critical periods, thereby contributing another early life influence affecting the developing serotonergic system.

1.4.2 NR3C1 Methylation

Located on chromosome 5q31.3, NR3C1 encodes the GR and has a complex untranslated 5’ region with alternative first exons, expressed in a tissue- and cell-specific fashion.
contains eight translated exons (2 - 9) and nine alternative first exons (1A - 1I), each with its own promoter that can be regulated by DNA methylation. In human tissue, Exon 1E and 1F are highly expressed in the hippocampus as well as the immune system, and exon 1B and 1C are broadly expressed in many tissues. Although Turner et al. found expression of 1D exclusive to adult hippocampal tissue, a recent study indicated exon 1D is expressed in human placental tissue.

After binding cortisol, the GR translocates to the nucleus where it regulates transcription of glucocorticoid-responsive genes. GR expression is controlled by a variety of mechanisms including diverse NR3C1 mRNA splice variants and miRNAs, differential expression of GR-chaperones, transcriptional co-regulators, genetic variants, and other epigenetic modifications. In particular, the activity of transcription factors binding various NR3C1 promoters is regulated by binding site methylation. In this way, chronic exposure to early life stressors can challenge the capacity of allostatis, and may influence the regulation of GR expression through epigenetic pathways involving NR3C1 methylation.

*Early life adversities and Nr3c1 methylation in animal models*

In rodent models, differences in early postnatal maternal care affect the development of individual variations in HPA responses to stress. Within the first week of life, offspring of mothers exposed to high levels of maternal care (increased licking and grooming (LG) and arched-back nursing (ABN)) show differences in hippocampal Nr3c1 exon 1F promoter methylation that persist into adulthood, compared to offspring from low maternal care. Furthermore, Nr3c1 mRNA expression was inversely correlated with methylation of the binding site for nerve growth factor-inducible protein A (NGFI-A), a regulatory transcription factor
controlling hippocampal \textit{Nr3c1} expression. Thus, higher methylation levels may result in lower GR expression, fewer GRs, and reduced feedback control of the HPA axis activity.\textsuperscript{60}

Daniels et al\textsuperscript{196} were unable to show differential methylation status of the \textit{Nr3c1} exon 1\textsubscript{7} promoter in response to an early adverse event (maternal separation), however, the use of different rat strains and maternal study paradigms suggest that previous findings may be species-specific and occur only under certain patterns of maternal care. Overall, findings from studies in rodent models have provided evidence of an underlying mechanism for the long-term effect of maternal care on offspring gene expression,\textsuperscript{15} and they continue to be an efficient approach for elucidating the role of epigenetic mechanisms in the pathophysiology of mental illness.\textsuperscript{194}

\textit{Human evidence of early life adversity and NR3C1 methylation}

Human evidence for the role of epigenetic modifications in development programming is beginning to emerge, and a number of studies have shown similar findings between humans and animal models.\textsuperscript{194} Given the sequence homology between the promoter regions of \textit{Nr3c1} exon 1\textsubscript{7} in rodents, and \textit{NR3C1} exon 1\textsubscript{F} in humans, the NGFI-A transcription factor is thought to regulate human \textit{NR3C1} transcription.\textsuperscript{197} Analysis of the \textit{NR3C1} exon 1\textsubscript{F} promoter region has revealed higher site-specific methylation and lower GR expression in postmortem hippocampal samples from suicide victims that experienced abuse during childhood, compared to suicide victims who were not abused during childhood and controls (victims of sudden death with no child abuse).\textsuperscript{198} Given that \textit{NR3C1} 1\textsubscript{F} methylation and hippocampal GR expression in samples from suicide victims without childhood history of abuse was not different than controls, McGowan et al\textsuperscript{198} proposed the altered \textit{NR3C1} 1\textsubscript{F} methylation may be a function of childhood adversity that has left epigenetic marks well into adulthood. It is important to note that human brain tissue is rarely
accessible to study so these findings provide important evidence for the role of $NR3C1_{1F}$ methylation in human brain circuitry following early adversity. In peripheral blood of adults with borderline personality disorder, depression, and bipolar disorder, greater $NR3C1$ exon 1$_F$ promoter methylation was associated with childhood abuse, and the severity of abuse was positively correlated with methylation status.\textsuperscript{199,200} In healthy adults, higher leukocyte methylation was associated with a history of child abuse and attenuated cortisol responses to a pharmacological HPA stress test.\textsuperscript{201} In general, very low levels of methylation in the $NR3C1$ 1$_F$ promoter are reported (< 10%), and the biological significance of small methylation changes within this range is unclear.

Although the majority of DNA methylation findings have been in adults that have endured childhood adversity, the perinatal period and early childhood years mark a time of brain development sensitivity that may be particularly susceptible to epigenetic modifications. For instance, in DNA from cord blood samples, infants have shown greater $NR3C1$ 1$_F$ methylation at the potential NGFI-A consensus binding site in response to higher 3\textsuperscript{rd} trimester maternal depressed mood, compared to infants exposed to lower 3\textsuperscript{rd} trimester maternal mood.\textsuperscript{202} Additionally, greater $NR3C1$ 1$_F$ methylation in the infants at birth was associated with a higher salivary cortisol stress responses at 3 months of age, independent of neonatal characteristics, 3\textsuperscript{rd} trimester maternal mood, and maternal mood at 3 months.\textsuperscript{152,202} Similarly, higher prenatal maternal mood scores and salivary cortisol concentrations were associated with higher $NR3C1$ 1$_F$ methylation in infant cord blood leukocytes.\textsuperscript{203} Beyond infancy, 9 - 10 year old children whose mothers reported intimate partner violence during their pregnancy exhibited higher $NR3C1$ 1$_F$ promoter methylation in peripheral blood, compared to children whose mothers reported no violence during pregnancy.\textsuperscript{204} These findings in older children suggest that antenatal stress may
induce persistent alterations in DNA methylation. In contrast, one study found experience of stressful life events (SLEs) in adolescence was associated with higher NR3C1 1F methylation in peripheral blood of adolescents, compared to adolescents with no SLEs, and these findings were independent of exposure to perinatal stress and SLEs in childhood.205

Studies have started to investigate a broader region of the NR3C1 promoter. In placenta tissue from normotensive and hypertensive pregnancies, Hogg et al193 used microarray analysis to identify candidate genes involved in cortisol signaling and bioavailability. Although NR3C1 appeared largely unmethylated, significantly different methylation patterns were identified in the region proximal to the exon 1D promoter.193 Interestingly, a study in pigs which share physiological and first exon similarities with humans,206 found 1D expression was most abundant in hippocampal tissue, and above average in all tissues related to the neuroendocrine system.207 Although these findings cannot be generalized to expression profiles in human tissues, Hompes et al203 analyzed site specific methylation in the promoter region of NR3C1 1D and found infant methylation status in cord blood leukocytes was positively correlated with certain prenatal maternal anxiety scores in all three trimesters, and negatively correlated with 1st trimester maternal cortisol. Thus, given the role of cortisol in the developmental programming of disease, this finding may reveal a target region of NR3C1 to analyze in addition to the widely studied exon 1F promoter.

Overall, these studies provide evidence that both epigenetic NR3C1 modifications and early environmental factors may contribute to the calibration of HPA stress regulation during early development.
1.5 Rationale and Hypothesis

Developmental programming suggests that our environment during gestation sets pathways for adaptability of health and disease later in life.\textsuperscript{38,52} Mechanisms underlying such programming are poorly understood, but may involve the interplay between gestational environment, genetic variations, and epigenetic processes.\textsuperscript{143,152} Individuals who show altered HPA responses to stress are at an increased risk for a variety of disorders, including anxiety and depression.\textsuperscript{143,149}

Maternal depression and anxiety during pregnancy are recognized as key early life influences that affect the developing HPA stress systems, possibly via changes in fetal serotonergic signaling.\textsuperscript{39,189} Antenatal maternal mood and SRI antidepressant use during pregnancy affect central fetal 5-HT concentrations involved in regulating the development of serotonergic neurons and target tissues.\textsuperscript{5} Although the developmental impact of prenatal exposure to SRIs is often undistinguishable from maternal mood disturbances,\textsuperscript{55} these early life factors may alter early 5-HT concentrations and ultimately influence the developing HPA axis. This ‘altered’ programming of critical pathways, possibly via genetic or epigenetic processes, may have the potential to influence an individual’s HPA stress regulation throughout a lifetime.

Infants exposed to higher 3\textsuperscript{rd} trimester maternal depressed mood have displayed higher \textit{NR3C1} \textsubscript{1F} promoter methylation in newborn cord blood,\textsuperscript{202} and greater \textit{NR3C1} \textsubscript{1F} methylation in the infants was associated with higher salivary cortisol stress responses at 3 months of age.\textsuperscript{152, 202} In addition, higher 2\textsuperscript{nd} trimester maternal depressed mood was associated with lower methylation of \textit{SLC6A4} in newborn cord blood,\textsuperscript{52} and a variant in the promoter of \textit{SLC6A4} (5-\textit{HTTLPR}) moderates the effect of stressful life events on depression.\textsuperscript{17} The influence of \textit{NR3C1} and \textit{SLC6A4} on regulating stress responses highlights the importance of investigating both their
individual and interactive roles in HPA axis programming that might have arisen from adverse early life environments. However, few studies have assessed the effect of epigenetic factors and genetic variants on the relationship between prenatal exposure to SRI antidepressants and maternal mood disturbances (depression and anxiety) on stress regulation in early childhood.

Based on these findings, I hypothesize that **prenatal exposure to SRI antidepressants and 3rd trimester maternal mood disturbances will be associated with altered cortisol patterns in 5 - 7 year old children, characterized by variable basal and stress-induced cortisol concentrations. Furthermore, NR3C1 and SLC6A4 promoter methylation at birth and at 5 – 7 years will be associated with cortisol patterns at 5 - 7 years of age.** This hypothesis will be addressed by the following aims (located in Chapters 3, 4, and 5):

**Aim 1:** To determine the relationship between prenatal exposure to SRIs and maternal mood disturbances with the diurnal rhythm and cortisol stress response at 5 - 7 years of age.

**Aim 2:** To determine the relationship between prenatal exposure to SRIs and maternal mood disturbances on methylation status of the NR3C1 1F, NR3C1 1D, and SLC6A4 promoter regions at 5 - 7 years, and the association between methylation status at birth and 5 - 7 years on children’s diurnal rhythm and cortisol stress response.

**Aim 3:** To determine the relationship between 5-HTTLPR genotype, SLC6A4 methylation at 5 – 7 years, and the diurnal rhythm and cortisol stress response in children with prenatal exposure to SRIs and 3rd trimester maternal mood disturbances.
Chapter 2: MATERIALS AND METHODS

2.1 Study Participants

With approval from the University of British Columbia Research Ethics Board, Children’s and Women’s Health Centre of British Columbia Research Review Committee, and informed parent consent, two cohorts [cohort A (n=99), cohort B (n=92)] of mothers were recruited in their early second trimester. The study evaluating the impact of prenatal SRI and maternal depressed mood exposure on neonatal health took place between February 2002 and April 2005 for cohort A, and between January 2007 and March 2010 for cohort B. Of the original 191 mothers who completed 2nd trimester data collection, samples from 23 mothers and infants at delivery were not available for analysis (i.e. mothers withdrew for personal reasons prior to delivery, maternal blood was not obtained in the 3rd trimester, infant cord blood was not obtained at birth, inadequate DNA yield). The SRI antidepressants taken by mothers included paroxetine, fluoxetine, sertraline, venlafaxine, and citalopram. Mothers were only included in the study if they took no other serotonergic medications or other psychotropic medications during their pregnancy.

A 6 year follow up study to examine the long term effects of prenatal exposure to maternal depression/anxiety and SRIs was conducted between October 2009 and February 2011 for cohort A (n=75), and began in October 2012 for cohort B (n=47). Of the 122 mothers and children included in the study to date, samples from 11 mothers and children were not available for analysis, and 14 children did not participate in the laboratory stress challenge day (i.e. mothers had relocated out of British Columbia, mothers could not be tracked down or contacted, changes in family dynamics, mothers were too busy to participate). In addition, one subject was removed from the study after extremely elevated cortisol concentrations were observed in the
child, who later was identified to have a seizure disorder. To enable time for laboratory experiments and data analysis, sample collection for this project was cut off in September 2013, leaving 111 women and children. Descriptive maternal information can be found in Table 2.1.

2.2 Maternal Mood Assessments of Anxiety and Depression

Maternal mood was assessed using clinician (blinded to medication group status) measures in the 3rd trimester and at 5 - 7 years (concurrent measure) using two instruments. Measures included The Hamilton Rating Scale for Depression (HAM-D), a 21-item clinician administered scale designed to assess depressive mood. Scores on this scale have a range from 0 - 63, with higher scores indicating a higher level of depression in the patient. A score ranging from 0 - 7 suggest no or minimal levels of depression, 8 - 13 indicate mild depression, 14 - 18 suggest moderate depression, 19 - 22 indicate severe depression, and scores of 23 or higher are associated with very severe depression. The Hamilton Rating Scale for Anxiety (HAM-A) is a 14-item clinician administered scale designed to assess severity of anxiety. Scores on this scale have a range between 0 - 56, with higher scores indicating higher levels of anxiety in the patient. A score ranging from 0 - 13 suggest no or minimal levels of anxiety, 14 - 17 indicate mild anxiety, 18 - 24 indicate moderate anxiety, and 25 - 30 are associated with severe anxiety in the patient. The term “maternal mood disturbances” is used to describe both anxiety and depression scores in the following chapters.
Table 2.1. Descriptive maternal information

<table>
<thead>
<tr>
<th>Maternal Characteristics</th>
<th>SRI Treated (n = 45)</th>
<th>Non SRI Treated (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age of at birth (years)</td>
<td>32.76 ± 5.00</td>
<td>33.73 ± 4.60</td>
</tr>
<tr>
<td>Caesarian-section rate, n (%)</td>
<td>18 (40.0)</td>
<td>20 (30.3)</td>
</tr>
<tr>
<td>Maternal education (years)</td>
<td>16.38 ± 3.30</td>
<td>17.76 ± 2.67*</td>
</tr>
<tr>
<td>Prenatal smoking, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Reported prenatal alcohol use (drinks/pregnancy), n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25 (55.6)</td>
<td>37 (57.8)</td>
</tr>
<tr>
<td>1 - 5</td>
<td>15 (33.3)</td>
<td>16 (25.0)</td>
</tr>
<tr>
<td>6 - 10</td>
<td>1 (2.2)</td>
<td>6 (9.4)</td>
</tr>
<tr>
<td>11 - 22</td>
<td>3 (6.7)</td>
<td>5 (7.8)</td>
</tr>
<tr>
<td>&gt; 23</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Prenatal SRI use (days)</td>
<td>263 ± 70.62</td>
<td>n/a</td>
</tr>
<tr>
<td>Prenatal SRI medication use, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paroxetine</td>
<td>15 (33.3)</td>
<td>n/a</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>5 (11.1)</td>
<td>n/a</td>
</tr>
<tr>
<td>Sertraline</td>
<td>7 (15.6)</td>
<td>n/a</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>12 (26.7)</td>
<td>n/a</td>
</tr>
<tr>
<td>Citalopram</td>
<td>6 (13.3)</td>
<td>n/a</td>
</tr>
<tr>
<td>Maternal anxiety scores (HAM A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>11.34 ± 7.84</td>
<td>6.33 ± 4.94*</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>10.83 ± 6.50</td>
<td>6.36 ± 5.73*</td>
</tr>
<tr>
<td>Maternal depression scores (HAM-D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>9.84 ± 6.62</td>
<td>4.17 ± 4.22*</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>11.68 ± 7.10</td>
<td>6.52 ± 5.82*</td>
</tr>
</tbody>
</table>

Note: N=111 Mothers. Continuous values are displayed as mean ± standard deviation (SD). *p<0.05 for differences between SRI treated and non SRI treated groups. HAM-A: Hamilton rating scale for anxiety; HAM - D: Hamilton rating scale for depression; n/a: not applicable. N’s vary slightly across measures because of missing data.
2.3 Sample Collection for DNA Isolation

2.3.1 Cord Blood Collection and DNA Isolation

Samples of newborn cord blood were collected in EDTA-coated tubes (BD Vacutainer, NJ) and stored at -80°C for subsequent analysis. Genomic DNA was extracted from whole blood using the Flexigene DNA Blood Kit (Qiagen Inc., Valencia, CA), following the Isolation of DNA from Whole Blood protocol. Sample DNA yield and purity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

2.3.2 Buccal Cell Collection and DNA Isolation at 5 - 7 Years of Age

A buccal epithelial sample was collected from children using Isohelix SK-1 buccal swabs (Cell Projects, UK) during the laboratory study day, or at home if the child did not participate in the study day. Buccal swabs were stored at room temperature with Isohelix Dri-Capsules (Cell Project, UK). Genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen Inc., Mississauga, ON) following the Buccal Swab Spin Protocol. DNA yield and purity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

2.4 Salivary Cortisol Collection and Quantification at 5 - 7 Years of Age

Saliva sampling was carried out using eye sponges (bvi. Beaver Visitec). Upon collection, saliva samples were centrifuged for 7 minutes at 3000 rpm and stored at -20°C until analysis. Salivary cortisol concentrations were analyzed via a commercially available chemiluminescence immunoassay (CLIA, IBL-Hamburg, Germany).
2.4.1 Salivary Cortisol Basal Conditions

A week before the lab challenge study, saliva was collected 3 times (waking, +20 minutes, between 5:00 -7:00 pm) on four consecutive weekdays from children to allow the assessment of a diurnal cortisol profile. For the waking and + 20 minute sample collection, parents were instructed to take children’s saliva samples before the child had anything to eat or drink, and prior to brushing their teeth. For the evening sample, parents were informed to collect children’s samples at least 1 hour after a eating a meal, 30 minutes after eating a snack, and 3 hours after children brushed their teeth. Parents also kept a diary noting the exact collection time point for each saliva sample, the child’s daily activities, and any additional information that may influence the cortisol concentration (i.e. child was running around between waking and +20 minutes, child was upset or acting out around dinner time).

2.4.2 Salivary Cortisol Laboratory (Stress-Challenge) Conditions

To determine the children’s HPA cortisol response to lab based challenge stressors, eight saliva samples were collected over the course of a study day (Table 2.2). Parents were instructed to collect two saliva samples from their child before the laboratory visit (waking and +20 minutes), and one sample between 5:00 and 7:00 pm after the laboratory visit. Five saliva samples were collected while children participated in an executive functioning task, the Kaufman Brief Intelligence Test, and the McArthur Battery Test. Parents were instructed to follow the same guidelines as mentioned in section 2.4.1, and the sample collected upon arrival to the lab was taken at least 15 minutes after the child had anything to eat or drink.

The hypothalamic activity in response to a stressor can be observed in salivary cortisol concentrations approximately 20 - 30 minutes after the onset of the stressor.\textsuperscript{129,210}
Therefore, saliva samples were collected after the executive functioning task and the Kaufman Brief Intelligence Test to reflect the stress response at the beginning of these tasks. Similarly, saliva samples were collected 20 and 40 minutes after the start of McArthur Battery Test, to reflect the cortisol stress response when the test started and approximately 20 minutes into the test. Table 2.2 outlines the saliva samples collected on the study day and the name that each study day task is referred to in chapters 3 - 5.

<table>
<thead>
<tr>
<th>Saliva Sample</th>
<th>Time point during study day</th>
<th>Name of time point in following analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Waking</td>
<td>Waking</td>
</tr>
<tr>
<td>2</td>
<td>+ 20 minutes</td>
<td>Wake + 20 minutes</td>
</tr>
<tr>
<td>3</td>
<td>Arrival to lab (Study day begins)</td>
<td>Arrival</td>
</tr>
<tr>
<td>4</td>
<td>After Executive Functioning Tasks</td>
<td>Task 1</td>
</tr>
<tr>
<td>5</td>
<td>After Kaufman Brief Intelligence Test</td>
<td>Task 2</td>
</tr>
<tr>
<td>6</td>
<td>20 minutes after start of MacArthur Battery</td>
<td>Task 3</td>
</tr>
<tr>
<td>7</td>
<td>40 minutes after start of MacArthur Battery</td>
<td>Task 4</td>
</tr>
<tr>
<td>8</td>
<td>Between 5:00 and 7:00pm</td>
<td>Evening</td>
</tr>
</tbody>
</table>
2.5 5-HTTLPR Genotyping Assay

Genomic DNA from cord blood leukocytes was used to genotype the 5-HTTLPR variant (rs25531). The s and l alleles were identified as previously described.\textsuperscript{211} PCR was carried out with primers flanking the variant region [corresponding to the nucleotide positions -1416 to -1397 (stpr5, GGCGTTGCGTCCTGAAT GC) and -910 to -889 (stpr3, GAGGGACTGAGCTGGACAACCAC), relative to the transcriptional start site] to generate a 484- (s short allele) or 528-base pair (l long allele) PCR product. PCR amplification was carried out in a final volume of 30 µl with 50 ng of genomic DNA, 2.5 mM dNTPs (dGTP/7-deaza-2’-dGTP=1/1), 0.1 µg of sense and antisense primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, and 1 U of Taq DNA polymerase. Amplification conditions were: 95°C for 30 seconds, 61°C for 20 seconds, 72°C for 1 minute for 35 cycles. PCR products representing the respective alleles were distinguished by agarose gel electrophoresis. As a quality control, 5% of samples were randomly chosen and retested and their genotypes were consistent with previous results.

2.6 DNA Methylation Assays at Birth and at 5 - 7 Years of Age

2.6.1 Bisulphite Pyrosequencing

The methylation status of the \textit{NR3C1} exon 1\textsubscript{F}, \textit{NR3C1} exon 1\textsubscript{D}, and \textit{SLC6A4} promoters were quantified by bisulphite pyrosequencing.\textsuperscript{212} Genomic DNA from cord blood leukocytes (1 µg) and buccal cell DNA (200ng) were bisulphite-treated using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) following the manufacturer’s protocol, and stored at -20°C until further analysis. Briefly, bisulfite conversion involves a deamination process, whereby
unmethylated cytosines in single-stranded DNA are converted to uracil residues that are recognized as thymine in subsequent PCR amplifications and pyrosequencing, whereas methylated cytosines are protected from deamination and remain as cytosines enabling methylated and unmethylated cytosines to be distinguished from one another.\textsuperscript{213}

The methylation status of NR3C1 1\textsubscript{F}, NR3C1 1\textsubscript{D}, and SLC6A4 promoter regions was determined by pyrosequencing of fragments representing each region amplified (by PCR) from the bisulfite converted DNA. NR3C1 1\textsubscript{F} and SLC6A4 PCR and sequencing primers were designed using the PyroMark Assay Design software (version 2.0) and obtained from Integrated DNA Technologies (IDT, Coralville, IA). Assay 1 and 2 PCR and sequencing primers for NR3C1 1\textsubscript{D} were determined in a study involving an Infinium HumanMethylation 450 BeadChip array.\textsuperscript{193} The promoter regions of NR3C1 1\textsubscript{F}, NR3C1 1\textsubscript{D}, and SLC6A4 were amplified by PCR using HotStar Taq DNA Polymerase (Qiagen) and forward and reverse primers (Table 2.3). PCR products including a negative control were run on an agarose gel to visualize optimized amplification conditions and to ensure contamination did not occur. PCR products were purified and sequenced using a PyroMark Q96 MD System (Biotage, Foxboro, MA) following the manufacturer’s suggested protocol, and sequencing primers (Table 2.3). The percent methylation at each CpG site was quantified using the Pyro Q-CpG software, version 1.0.9 (Biotage).

\textbf{2.6.2 SLC6A4 Bisulphite Pyrosequencing}

A 130 bp CpG-rich region of the SLC6A4 promoter was amplified by PCR. Amplification conditions were: 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 50 cycles. A 99 bp region of the PCR fragment was sequenced and contained 10 CpG sites (Figure 2.1).
2.6.3  *NR3C1 Exon 1* F Bisulphite Pyrosequencing

A 346 bp CpG-rich region of the *NR3C1* 1_F promoter was amplified by PCR. Amplification conditions were: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. Two sequencing primers were used to sequence a 112 bp region containing 13 CpG sites (Figure 2.2).

2.6.4  *NR3C1 Exon 1* D Bisulphite Pyrosequencing

A 168 bp (Assay 1) and 225 bp (Assay 2) CpG-rich region of the *NR3C1* 1_D promoter was amplified by PCR. Amplification conditions for Assay 1 were: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. Amplification conditions for Assay 2 were: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. The sequenced region of Assay 1 was 23 bp and contained 4 CpG sites, and the sequenced region of Assay 2 was 21 bp and contained 6 CpG sites (Figure 2.3).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR3C1 F</td>
<td>F</td>
<td>GTT TTT TTA GAG GGR GTG TTA GGT</td>
<td>393 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>Bi/AAC TCC CCA AAA AAA AAA ATA AC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>GAG TGG GTT TGG AGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>AGA AAA AGA AAA TTG GAG AAA TT</td>
<td></td>
</tr>
<tr>
<td>NR3C1 D</td>
<td>F</td>
<td>TTA TTT TTA AGA ATT AAG GAA GG</td>
<td>168 bp</td>
</tr>
<tr>
<td>(Assay 1)</td>
<td>R</td>
<td>Bi/CCC CCT ACT CTA ACA TCT TAA AA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>ATA TTG TAT TTT ATT AAG ATG G</td>
<td></td>
</tr>
<tr>
<td>NR3C1 D</td>
<td>F</td>
<td>AGT TTG TTT TTT GGG TTT AGA AGG</td>
<td>225 bp</td>
</tr>
<tr>
<td>(Assay 2)</td>
<td>R</td>
<td>Bi/AAA TAA ACT TTC AAC AAA CCT CTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>GGT TTT AGA ATT TTT TGG AG</td>
<td></td>
</tr>
<tr>
<td>SLC6A4</td>
<td>F</td>
<td>Bi/GTA TTG TTA GGT TTT AGG AAG AAA GAG AGA</td>
<td>203 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AAA AAT CCT AAC TCT TCT ACT CTT TAA CTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>AAA CTA CAC AAA AAA ACA AAT</td>
<td></td>
</tr>
</tbody>
</table>

F: forward, R: reverse, S: sequence, Bi: biotin, R: G/A. NR3C1 F/D: nuclear receptor subfamily 3, group C, member 1 (Exon 1F/Exon 1D), SLC6A4: solute carrier family 6, member 4.
Figure 2.1 Schematic representation of the SLC6A4 promoter region analyzed for methylation status
The portion analyzed by bisulfite sequencing is shown in bold. The CpGs are underlined and numbered. Non-coding and coding exons are indicated by white and black boxes, respectively. Adapted from Devlin et al.52

Figure 2.2 Schematic representation of the NR3C1 1F promoter region analyzed for methylation status
The portion analyzed by bisulfite sequencing is shown in bold. The CpGs are underlined and numbered. Non-coding and coding exons are indicated by white and black boxes, respectively. Capital letters represent exon 1F. Adapted from Oberlander et al.202 and Turner et al.191
Figure 2.3 Schematic representation of the NR3C1 1D promoter regions analyzed for methylation status

The portion analyzed by bisulfite sequencing is shown in bold. The CpGs are underlined and numbered. Non-coding and coding exons are indicated by white and black boxes, respectively. Assay 1 and 2 indicated promoter regions in exon 1D as previously described (Hogg et al. 193). Adapted from Hogg et al. 193 and Turner et al. 191.
2.7 Statistical Analysis

The measures of cortisol used in the diurnal analysis were the average cortisol concentrations for three time points across four days. Cortisol data were log transformed to attain normality and examined for outliers defined as any value more than ±3 SD from the mean. Six outliers were identified and ‘winsorized’ following the method of Tukey (1977)\(^{214}\), which involves replacing the outlier value with the closest value within the 3 standard deviation range. Based on the trapezoid method by Pruessner et al\(^{215}\) the area under the curve with respect to ground (AUC\(_G\)) was calculated for the diurnal pattern and cortisol reactivity over the study day. All cortisol analyses were performed with log-transformed values, and figures are displayed using non-transformed values. All analyses were conducted using SPSS, Version 20.0 (SPSS Inc., Chicago, IL).

In the following analyses, both maternal depressed and anxious mood in the 3\(^{rd}\) trimester were evaluated given their association with neonatal and behavioural outcomes later in life.\(^{148}\) Given prenatal exposure to mood disturbances (depression and anxiety) may be associated with adverse postnatal experiences such as maternal mood, maternal depressed and anxious mood in early childhood were included as covariates in all analyses. Third trimester and 5 - 7 year maternal mood scores were normally distributed and included in all statistical models as continuous variables. Maternal depressed and anxious mood were evaluated separately to assess individual relationships of depressed and anxious mood with children’s cortisol patterns.

Maternal Depression Score and Maternal Anxiety Score subheadings located in the results section indicates which maternal mood score was included as a covariate in the analysis. In addition, children’s age and sex were included as covariates in all analyses. Statistical significance was set at alpha=0.05.
Given the data for this thesis was collected in a follow-up study from a longitudinal prospective cohort, data collection is dependent on the number of subjects that return, and not the number of subjects recruited, and therefore a power calculation was not performed. Given this thesis focused on exploring trends between prenatal SRI exposure and maternal mood on NR3C1 and SLC6A4 methylation status, and associations between NR3C1 and SLC6A4 methylation status and cortisol patterns, multiple comparisons were not applied due to heightened risk of Type II errors. Instead, effect sizes (partial eta squared; \( \eta^2 \)) were reported to allow for examination of the magnitude of predictive relationships, explanations were provided describing which analysis were performed and why, and interpretations were provided for each result. The statistical analyses performed in Aim 1, 2, and 3 are discussed below and outlined in Table 2.4. A methods section within each aim provides an overview of the analyses performed in that aim.

*Aim 1: Cortisol Patterns at 5 - 7 Years of Age*

Repeated-measures analysis of variance (ANOVA) models were used to determine the association between prenatal SRI exposure and maternal mood in the 3rd trimester and at 5 - 7 years on child cortisol patterns across the diurnal and stress-challenge day. The Greenhouse-Geissner correction of degrees of freedom was applied where appropriate. In addition, analysis of covariance (ANCOVA) models were used to explore individual components of the diurnal rhythm and stress-challenge patterns. Investigated outcome variables were cortisol concentrations at each time point, cortisol change scores between time points, and area under the curve.
**Aim 2: DNA Methylation as a Programming Mechanism**

The association between prenatal exposure to SRIs, 3rd trimester and 5 - 7 year maternal mood, and NR3C1 1F, NR3C1 1D, and SLC6A4 promoter methylation status at specific CpG sites in early childhood was assessed using multiple analyses of covariance (MANCOVA) models. To determine the association between DNA methylation status at birth and 5 - 7 years on children’s cortisol patterns, the methylation status at specific CpG sites were included as covariates in repeated measures and analysis of covariance models.

**Aim 3: 5-HTTLPR Genotype and Cortisol Patterns at 5 – 7 Years of Age**

MANCOVA models were used to evaluate the association between 5-HTTLPR genotype and SLC6A4 promoter methylation status at 5 - 7 years. Additionally, children’s 5-HTTLPR genotype and methylation status were included as covariates in repeated measures and analysis of covariance models to determine the association between genotype, methylation status, and cortisol patterns.
<table>
<thead>
<tr>
<th>Aim 1</th>
<th>Analysis</th>
<th>Dependent Variables</th>
<th>Independent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goal 1A and 1B</td>
<td>Repeated measures ANOVA</td>
<td>Diurnal cortisol rhythm, Cortisol stress reactivity</td>
<td>Prenatal SRI exposure, *Maternal mood scores (3rd trimester and 5 - 7 year)</td>
</tr>
<tr>
<td></td>
<td>ANCOVA</td>
<td>Cortisol concentrations, Cortisol change scores, AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Aim 2</td>
<td>MANOVA</td>
<td>DNA methylation status in early childhood (NR3C1&lt;sub&gt;1F&lt;/sub&gt;, NR3C1&lt;sub&gt;1D&lt;/sub&gt;, SLC6A4)</td>
<td>Prenatal SRI exposure, *Maternal mood scores (3rd trimester and 5 - 7 year)</td>
</tr>
<tr>
<td>Goal 2B and 2C</td>
<td>Repeated measures ANOVA</td>
<td>Diurnal cortisol rhythm, Cortisol stress reactivity</td>
<td>Methylation status at birth and at 5 - 7 Years</td>
</tr>
<tr>
<td></td>
<td>ANCOVA</td>
<td>Cortisol concentrations, Cortisol change scores, AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>Prenatal SRI exposure, *Maternal mood scores (3rd trimester and 5 - 7 year)</td>
</tr>
<tr>
<td>Aim 3</td>
<td>Repeated measures ANOVA</td>
<td>Diurnal cortisol rhythm, Cortisol stress reactivity</td>
<td>Children’s 5-HTTLPR genotype, Prenatal SRI exposure</td>
</tr>
<tr>
<td></td>
<td>ANCOVA</td>
<td>Cortisol concentrations, Cortisol change scores, AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>*Maternal mood scores (3rd trimester and 5 - 7 year)</td>
</tr>
<tr>
<td>Goal 3B</td>
<td>MANOVA</td>
<td>DNA methylation status in early childhood (NR3C1&lt;sub&gt;1F&lt;/sub&gt;, NR3C1&lt;sub&gt;1D&lt;/sub&gt;, SLC6A4)</td>
<td>Children’s 5-HTTLPR genotype, Prenatal SRI exposure, *Maternal mood scores (3rd trimester and 5 - 7 year)</td>
</tr>
<tr>
<td></td>
<td>Repeated measures ANOVA</td>
<td>Diurnal cortisol rhythm, Cortisol stress reactivity</td>
<td>Children’s 5-HTTLPR genotype, Methylation status at birth and 5 - 7 Years</td>
</tr>
<tr>
<td></td>
<td>ANCOVA</td>
<td>Cortisol concentrations, Cortisol change scores, AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>Prenatal SRI exposure, *Maternal mood scores (3rd trimester and 5 - 7 year)</td>
</tr>
</tbody>
</table>

*Maternal mood scores (Hamilton Anxiety Rating Scale (HAM A) or the Hamilton Depression Rating Scale (HAM D)). Children’s age and sex were controlled for in all analyses. ANOVA: Analysis of variance; MANOVA: Multivariate analysis of variance; ANCOVA: Analysis of covariance; AUC<sub>G</sub>: Area under the curve with respect to ground
Chapter 3: AIM 1: CORTISOL PATTERNS AT 5 - 7 YEARS OF AGE

Altered cortisol responses are a vulnerability marker for a variety of stress-related diseases and psychiatric disorders.\textsuperscript{151} Although a substantial amount of evidence supports the concept that early life adversities influence HPA-axis function,\textsuperscript{151} there is considerable variability in the degree and direction in which cortisol regulation is altered.\textsuperscript{217,218} Therefore, for Aim 1 I sought to determine the relationship between prenatal exposure to SRIs and maternal depressed and anxious mood on the diurnal rhythm and cortisol stress response as an indicator of HPA-axis function in early childhood (5 - 7 years of age). Aim 1 was addressed in the following sub-aims (See Figure 3.1 for schematic representation):

\textbf{Aim 1A}: To determine the relationship between prenatal exposure to SRIs and 3\textsuperscript{rd} trimester maternal depressed/anxious mood on the cortisol diurnal rhythm in early childhood.

\textbf{Aim 1B}: To determine the relationship between prenatal exposure to SRIs and 3\textsuperscript{rd} trimester maternal depressed/anxious mood on the cortisol stress response in early childhood.
Figure 3.1 Sub-aims addressed in Aim 1.
3.1 Aim 1 Methods

3.1.1 Aim 1A: The Diurnal Cortisol Rhythm at 5 - 7 Years

Aim 1A included 99 children (No exposure = 60, SRI exposure = 39) with complete data from the three diurnal time points. Children were between 5.11 to 7.72 years of age, with a mean age of 6.03 years. Descriptive maternal information can be found in Table 3.1. Table 3.2 displays descriptive information and salivary cortisol collection times for children included in Aim 1A, separated by prenatal SRI exposure.

To capture cortisol’s diurnal rhythm multiple salivary collections were obtained from three sample time points over four days at home: waking (immediately after wake-up), 20 minutes later [to assess the cortisol awakening response (CAR)], and evening. Given cortisol rhythms may vary between days due to differences in factors including time of waking and duration of sleep\(^{219}\), a mean was taken from each of the three time points over four days to establish a reliable measurement of daytime cortisol rhythm in each child.

The diurnal rhythm was analyzed using repeated-measures ANOVA and general linear models with prenatal SRI exposure as a between-subjects factor and age, sex, and both 3\(^{rd}\) trimester and 5 - 7 year maternal depression (HAM - D) and anxiety (HAM - A) mood scores as covariates. Maternal depression and anxiety mood scores were separately adjusted for as a continuous variable to assess individual relationships between anxious maternal mood and the diurnal rhythm, and depressed maternal mood and the diurnal rhythm. Given the collection time of the Wake + 20 minute saliva sample was significantly later in the SRI exposed group (8:16am), compared to the non-exposed group (7:51am)(p = 0.041), the time between Waking and Wake + 20 minutes was adjusted for in the model.
### Table 3.1 Descriptive maternal information defined by SRI use during pregnancy

<table>
<thead>
<tr>
<th></th>
<th>SRI use during pregnancy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=39)</td>
<td>No (n=60)</td>
<td>p-value</td>
</tr>
<tr>
<td>Maternal age at birth (years)</td>
<td>32.38 ± 4.90</td>
<td>33.60 ± 4.52</td>
<td>0.163</td>
</tr>
<tr>
<td>Maternal years of education</td>
<td>16.55 ± 3.41</td>
<td>17.71 ± 2.72</td>
<td>0.058</td>
</tr>
<tr>
<td>Prenatal smoking</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>*Prenatal alcohol use</td>
<td>3.33 ± 7.35</td>
<td>2.55 ± 5.06</td>
<td>0.531</td>
</tr>
<tr>
<td>*Maternal anxiety scores (HAM-A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>10.32 ± 6.34</td>
<td>6.18 ± 4.77</td>
<td>0.000</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>11.22 ± 6.47</td>
<td>6.10 ± 5.43</td>
<td>0.000</td>
</tr>
<tr>
<td>*Maternal depression scores (HAM-D)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>9.34 ± 5.87</td>
<td>4.05 ± 4.03</td>
<td>0.001</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>11.92 ± 7.04</td>
<td>6.37 ± 5.73</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*Note:* N = 99 Mothers. Continuous values are displayed as mean ± standard deviation (SD). Groups were compared using t-tests. N’s vary slightly across measures because of missing data. *number of unit drinks during pregnancy.

### Table 3.2 Descriptive information for children with diurnal cortisol data, defined by prenatal SRI exposure

<table>
<thead>
<tr>
<th></th>
<th>Prenatal SRI exposure</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=39)</td>
<td>No (n=60)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age (years)</td>
<td>6.17 ± 0.63</td>
<td>5.99 ± 0.58</td>
<td>0.157</td>
</tr>
<tr>
<td>Sex % M/F</td>
<td>32.5/67.5</td>
<td>44.4/55.6</td>
<td>0.159</td>
</tr>
<tr>
<td>Cortisol collection time (hh:mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>7:48 ± 1:00</td>
<td>7:31 ± 0:55</td>
<td>0.148</td>
</tr>
<tr>
<td>Wake + 20 min</td>
<td>8:16 ± 1:03</td>
<td>7:51 ± 0:55</td>
<td>0.041</td>
</tr>
<tr>
<td>Evening</td>
<td>18:46 ± 1:01</td>
<td>18:44 ± 0:55</td>
<td>0.837</td>
</tr>
</tbody>
</table>

*Note:* N=99 Children. Continuous values are displayed as mean ± standard deviation (SD). Groups were compared using t-tests for continuous variables and Chi-Square tests for categorical variables. Collection times were analyzed using decimal time and converted to hh:mm in the table.
3.1.2 **Aim 1B: Cortisol Stress Reactivity at 5 - 7 Years**

Overall, the study day consisted of eight cortisol measures across the day, including three diurnal measures (Waking, Wake + 20 min, Evening), as in Aim 1A, and five samples during the lab visit (Arrival to lab, Task 1, Task 2, Task 3, Task 4). Aim 1B focused on evaluating cortisol stress responses during the tasks administered in the lab, setting stress reactivity in the context of the diurnal rhythm.

Cortisol concentrations differed between time points over course of the lab visit, indicated by a main effect of time, suggesting a stress response in the children \((F_{4,208} = 13.987, p = 0.000)\). In particular, a decrease in salivary cortisol concentrations was shown following the cortisol sample taken upon arrival and throughout Task 1 and Task 2. These findings indicate Task 1 and 2 did not provoke a stress response and the observed decline may be a component of the natural fall in cortisol across the day and acclimatization to the lab conditions. Higher cortisol concentrations were displayed at Task 3 and Task 4, compared to cortisol concentrations at Arrival, Task 1, and Task 2, indicating a stress response was successfully elicited by Task 3 and Task 4. Thus, Task 3 and Task 4 are referred to as Stress 1 and Stress 2, respectively, in the following results and discussion. A mean of the cortisol concentrations from Arrival, Task 1, and Task 2 was assigned as the Baseline in the following analyses. Calculating a mean baseline measure enabled a reliable representation of the children’s baseline cortisol concentrations before the McArthur Task, and in some cases, enabled children with missing data from one of these measures to be included in the analysis.

Corresponding to the analysis performed in Aim 1A, cortisol reactivity was analyzed using repeated-measures ANOVA and general linear models with prenatal SRI exposure as a between-subjects factor and age, sex, and maternal mood in the 3rd trimester and at 5 - 7 years as
covariates. Separate models were analyzed with maternal depressed and anxious mood, as previously described in Aim 1A methods.

Sixty-four children had complete data for measures of Baseline, Stress 1, Stress 2, and Evening. This group is referred to as “Completed lab visit and corresponding evening cortisol measures”. Table 3.3 displays descriptive data from the 64 children, separated by prenatal SRI exposure (SRI exposed = 37, non-exposed = 27). The time of the waking sample collection was significantly later in the SRI exposed group (7:52 am), compared to the non-exposed group (7:27 am) (p = 0.029). Therefore, the time between waking and collection of the baseline sample was controlled for in the following analyses.

Thirty-three children had complete data for all eight cortisol measures across the study day (Waking, Wake + 20 minutes, Arrival to lab, Task 1, Task 2, Stress 1, Stress 2, and Evening). This group is referred to as “Completed lab visit and diurnal cortisol measures”. Table 3.4 displays descriptive data from the 33 children, separated by prenatal SRI exposure (SRI exposed = 14, non-exposed = 19). In addition to the analyses conducted in the “Completed lab visit and corresponding evening cortisol measures” group, the cortisol reactivity patterns evaluated in this subset of children incorporated the morning cortisol measures before arrival to the lab (Waking, Wake + 20 minutes), and individual cortisol concentrations from Arrival, Task 1 and Task 2.
Table 3.3 Maternal mood scores and descriptive information for children with completed lab visit and corresponding evening cortisol data, defined by prenatal SRI exposure

<table>
<thead>
<tr>
<th></th>
<th>Prenatal SRI exposure</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=27)</td>
<td>No (n=37)</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal anxiety scores (HAM-A)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>9.77 ± 6.15</td>
<td>5.29 ± 4.18</td>
<td><strong>0.001</strong></td>
<td></td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>9.30 ± 5.73</td>
<td>6.06 ± 5.72</td>
<td><strong>0.030</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Maternal depression scores (HAM-D)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>8.69 ± 5.64</td>
<td>3.91 ± 4.18</td>
<td><strong>0.000</strong></td>
<td></td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>10.11 ± 6.12</td>
<td>6.28 ± 6.30</td>
<td><strong>0.018</strong></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>6.00 ± 0.59</td>
<td>5.90 ± 0.51</td>
<td>0.472</td>
<td></td>
</tr>
<tr>
<td>Sex % M/F</td>
<td>33.3/66.7</td>
<td>51.4/48.6</td>
<td>0.119</td>
<td></td>
</tr>
<tr>
<td><strong>Cortisol collection time (hh:mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>7:52 ± 0:52</td>
<td>7:27 ± 0:43</td>
<td><strong>0.029</strong></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10:56 ± 0:58</td>
<td>10:43 ± 1:13</td>
<td>0.484</td>
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<tr>
<td>Stress 1</td>
<td>12:16 ± 1:00</td>
<td>12:09 ± 1:18</td>
<td>0.689</td>
<td></td>
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<tr>
<td>Stress 2</td>
<td>12:42 ± 1:16</td>
<td>12:28 ± 1:18</td>
<td>0.502</td>
<td></td>
</tr>
<tr>
<td>Evening</td>
<td>18:31 ± 1:07</td>
<td>18:55 ± 1:17</td>
<td>0.201</td>
<td></td>
</tr>
</tbody>
</table>

*Note:* Continuous values are displayed as mean ± standard deviation (SD). Categorical variables are displayed as n (%). Groups were compared using t-tests for continuous variables, and Chi-Square tests for categorical variables. Collection times were analyzed using decimal time and converted to hh:mm in table. N’s vary slightly across maternal mood scores because of missing data.
Table 3.4 Maternal mood scores and descriptive information for children with completed study day and diurnal cortisol data defined by prenatal SRI exposure

<table>
<thead>
<tr>
<th>Prenatal SRI Exposure</th>
<th>Yes (n= 19)</th>
<th>No (n= 14)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal anxiety scores (HAM-A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>9.84 ± 6.67</td>
<td>6.14 ± 4.38</td>
<td>0.081</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>8.74 ± 4.89</td>
<td>6.07 ± 5.28</td>
<td>0.145</td>
</tr>
<tr>
<td>Maternal depression scores (HAM-D)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>8.74 ± 5.92</td>
<td>4.71 ± 8.74</td>
<td>0.039</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>9.95 ± 5.82</td>
<td>6.57 ± 5.71</td>
<td>0.107</td>
</tr>
<tr>
<td>Age (years)</td>
<td>6.01 ± 0.58</td>
<td>5.79 ± 0.39</td>
<td>0.222</td>
</tr>
<tr>
<td>Sex % M/F</td>
<td>36.8/63.2</td>
<td>50.0/50.0</td>
<td>0.344</td>
</tr>
<tr>
<td>Cortisol collection time (hh:mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>7:48 ± 0:55</td>
<td>7:25 ± 0:48</td>
<td>0.242</td>
</tr>
<tr>
<td>Wake + 20 min</td>
<td>8:15 ± 0:47</td>
<td>7:49 ± 0:51</td>
<td>0.146</td>
</tr>
<tr>
<td>Arrival</td>
<td>10:16 ± 0:56</td>
<td>10:00 ± 0:56</td>
<td>0.489</td>
</tr>
<tr>
<td>Task 1</td>
<td>11:50 ± 0:58</td>
<td>10:36 ± 1:12</td>
<td>0.529</td>
</tr>
<tr>
<td>Task 2</td>
<td>11:16 ± 1:01</td>
<td>11:01 ± 1:13</td>
<td>0.496</td>
</tr>
<tr>
<td>Stress 1</td>
<td>12:10 ± 1:01</td>
<td>11:58 ± 1:18</td>
<td>0.639</td>
</tr>
<tr>
<td>Stress 2</td>
<td>12:31 ± 1:01</td>
<td>12:18 ± 1:18</td>
<td>0.606</td>
</tr>
<tr>
<td>Evening</td>
<td>18:40 ± 1:12</td>
<td>18:55 ± 1:24</td>
<td>0.587</td>
</tr>
</tbody>
</table>

Note: Continuous values are displayed as mean ± standard deviation (SD). Categorical variables are displayed as n (%). Groups were compared using t-tests for continuous variables, and Chi-Square tests for categorical variables. Collection times were analyzed using decimal time and converted to hh:mm in table. N’s vary slightly across maternal mood scores because of missing data.


### 3.2  Aim 1 Results

#### 3.2.1  Aim 1A: The Diurnal Cortisol Rhythm at 5 - 7 Years

Cortisol concentrations averaged over four sample collection days displayed a diurnal pattern across the day (Figure 3.2 A). Prenatal exposure to SRIs and maternal depressed and anxious mood were not associated with the diurnal cortisol rhythm at 5 - 7 years of age (p > 0.05).

Younger age was associated with a different diurnal cortisol pattern across the day compared to older age, whereby, younger children showed higher morning and lower evening cortisol concentrations ($F_{2,156} = 6.864$ and $7.102$, $p = 0.004$ and 0.004, adjusting for maternal anxious and depressed mood, respectively).

In particular, older age was associated with lower Waking cortisol concentrations and a flatter slope from Waking to Evening ($\beta = -0.292$, $p = 0.011$, $\eta^2 = 0.082$; $\beta = -0.349$, $p = 0.002$, $\eta^2 = 0.118$). These relationships remained significant when maternal depressed mood was adjusted for in the analysis. Maternal mood scores did not vary between children’s age (p > 0.05). Overall, older children displayed a more pronounced CAR, and flatter diurnal slope from Waking to Evening (Figure 3.2 B).
Figure 3.2 Diurnal salivary cortisol concentrations at 5 - 7 years of age. (A) Cortisol concentrations averaged over four days for all children included in diurnal cortisol analyses (N = 99 Children). (B) Age was associated with different diurnal cortisol patterns across the day ($F_{2,156} = 6.864, p=0.004$), whereby older age was associated with lower Waking cortisol concentrations and a flatter diurnal slope (Waking minus Evening) compared to younger age, adjusting for sex, SRI exposure, time between Waking and Wake + 20 minutes, and 3rd trimester/5 - 7 year maternal anxiety scores (HAM A) ($\beta = -0.292, p = 0.011, \eta^2 = 0.082; \beta = -0.349, p = 0.002, \eta^2 = 0.118$). Age was evaluated as continuous variable in general linear models and defined by a median age split (5.87 years) in Figure 3.2 B. Values shown as mean ± SEM.
3.2.2 Aim 1B: Cortisol Stress Reactivity at 5 - 7 Years

3.2.2.1 Stress Challenge: Completed Lab Visit and Corresponding Evening Cortisol

*Table 3.5* displays the cortisol concentrations from 64 children evaluated, defined by prenatal SRI exposure (Baseline, Stress 1, Stress 2, Evening) (SRI exposed = 27, Non-exposed = 37). T-tests showed children with prenatal SRI exposure displayed significantly higher evening cortisol concentrations, compared to children with no prenatal SRI exposure (p = 0.004).

**Maternal Anxiety Scores**

Children with prenatal SRI exposure showed a significantly different cortisol pattern across the lab visit and into the evening, whereby, SRI exposed children displayed a delayed increase in cortisol with stress and an attenuated decrease to lower evening cortisol concentrations, compared to non-exposed children. \(F_{3,159} = 5.744, p = 0.004, \eta^2 = 0.099\), *(Figure 3.3 A)*. To examine where the differences in cortisol concentrations arose across the stress challenge and evening cortisol in association with prenatal SRI exposure, general linear models were used to assess specific time points and the change in cortisol between time points. SRI exposed children displayed a flatter cortisol slope between Baseline and Evening, an attenuated decrease in cortisol between Stress 2 and Evening, and higher Evening cortisol concentrations \(\beta = -0.388, p = 0.008, \eta^2 = 0.128; \beta = 0.392, p = 0.004, \eta^2 = 0.128; \beta = 0.368, p = 0.007, \eta^2 = 0.128, \text{ Figure 3.3 B})*.

Interestingly, an interaction between SRI exposure and 5 - 7 year maternal anxious mood scores was associated with Stress 2 cortisol concentrations, suggesting that the pre- and postnatal environment may influence cortisol reactivity in early childhood. \(\beta = 0.567, p = 0.022, \eta^2 = 0.097\). Therefore, to evaluate this interaction subjects were separated by SRI exposure group in
the following analyses and general linear models assessed the cortisol concentration at Stress 2, as well as the change in cortisol to arrive at Stress 2 (between Stress 1 and Stress 2) (SRI exposure = 27 children, no exposure = 37 children). To examine the influence of pre- and postnatal environments on children's cortisol reactivity, an additional model included an interaction term between 3rd trimester and 5 - 7 year maternal mood scores.

In the SRI exposed group, higher 5 - 7 year maternal anxious mood was associated with higher Stress 2 cortisol concentrations, compared to lower 5 - 7 year maternal anxious mood ($\beta = 0.454$, $p = 0.032$, $\eta^2 = 0.200$). Furthermore, an interaction between 3rd trimester and 5 - 7 year maternal mood in the SRI exposed group was associated with the change in cortisol between Stress 1 and Stress 2, whereby, children exposed to higher 3rd trimester and 5 - 7 year maternal anxious mood displayed a blunted change in cortisol between Stress 1 and Stress 2, compared to children exposed to lower 3rd trimester and higher 5 - 7 year maternal anxious mood, who showed an increase in cortisol following Stress 1 ($\beta = 1.933$, $p = 0.002$, $\eta^2 = 0.393$, Figure 3.4).

This finding suggests that if SRI use successfully lowers 3rd trimester maternal mood, children may prompt a typical stress response, however, if children are exposed to higher 3rd trimester and 5 - 7 year maternal anxious mood they display a blunted stress response. This result highlights the impact of both pre- and postnatal maternal mood with cortisol reactivity in early childhood.

Maternal Depression Scores

Similarly, children with prenatal SRI exposure showed a delayed increase in cortisol with stress and elevated evening cortisol concentrations, compared to non-exposed children ($F_{3,156} = 5.526$, $p = 0.006$, $\eta^2 = 0.096$). General linear models were used to assess which cortisol concentrations were associated with prenatal SRI exposure on the study day. Children with SRI
exposure displayed a flatter slope between Baseline and Evening, higher evening cortisol concentrations, and an attenuated decrease in cortisol between Stress 2 and Evening ($\beta = -0.367$, $p = 0.012$, $\eta^2 = 0.113$; $\beta = 0.372$, $p = 0.010$, $\eta^2 = 0.118$; $\beta = -0.396$, $p = 0.004$, $\eta^2 = 0.146$)

To characterize the main effect of prenatal SRI exposure on different cortisol reactivity patterns, subjects were separated by exposure group in the following analyses (SRI exposure = 27 children, no exposure = 37 children). Using this model, an interaction between 3rd trimester and 5 - 7 year maternal mood scores in the SRI exposed group was associated with the change in cortisol between Stress 1 and Stress 2, whereby children of mothers with higher 3rd trimester and 5 - 7 year maternal depressed mood scores displayed a blunted change in cortisol between Stress 1 and Stress 2, compared to children exposed to lower 3rd trimester and higher 5 - 7 year maternal depressed mood, who showed an increase in cortisol following Stress 1 ($\beta = 2.190$, $p = 0.000$, $\eta^2 = 0.483$, Figure 3.4 B). No associations between maternal mood scores and cortisol reactivity patterns were observed in the non-exposed children ($p > 0.05$).

### 3.2.2.2 Stress Challenge: Completed Lab Visit and Diurnal Cortisol Measures

Table 3.6 displays the cortisol concentrations from 33 children evaluated, defined by prenatal SRI exposure (Waking, Wake + 20 minutes, Arrival, Task 1, Task 2, Stress 1, Stress 2, Evening) (SRI exposed = 14, Non exposed = 19). Compared to the analyses conducted in the “Completed lab visit and corresponding evening cortisol measures”, the cortisol reactivity patterns evaluated in this subset of children incorporated the morning diurnal cortisol measures before arrival to the lab and individual cortisol concentrations from Arrival, Task 1 and Task 2.
**Maternal Anxiety Scores**

SRI exposed children exhibited a different cortisol pattern across the study day, compared to non-exposed children, although this association failed to reach significance ($F_{7,189} = 2.477$, $p = 0.053$, $\eta^2 = 0.084$). To examine where the differences in cortisol concentrations arose across the stress challenge day, general linear models were used to assess specific time points and the change between time points. In particular, a steeper decline in cortisol between Arrival and Task 1 was found in child exposed to an SRI and higher 3rd trimester maternal anxious mood, compared to children with no exposure to SRIs and lower 3rd trimester maternal anxious mood (SRI: $\beta = 0.380$, $p = 0.036$, $\eta^2 = 0.152$; 3rd tri: $\beta = -0.554$, $p = 0.003$, $\eta^2 = 0.277$).

Interestingly, children of mothers with higher anxious mood at 5 - 7 years displayed a higher Wake + 20 minute cortisol concentration, compared to children of mothers with lower anxious mood at 5 - 7 years ($\beta = 0.484$, $p=0.009$, $\eta^2 = 0.228$, Figure 3.5). This finding suggests that mothers with higher anxious mood at 5 - 7 years may influence their children’s HPA cortisol reactivity, reflected by higher morning cortisol concentrations.

**Maternal Depression Scores**

Similarly, SRI exposed and non-exposed children showed a significantly different cortisol stress response across the study day, whereby SRI exposed children appear to have a less reactive cortisol response with stress and elevated evening cortisol concentrations, compared to non-exposed children ($F_{7,189} = 2.599$, $p = 0.045$, $\eta^2 = 0.088$, Figure 3.6). A significantly greater decline in cortisol between Arrival and Task 1 was displayed in children exposed to an SRI and higher 3rd trimester maternal depressed mood, compared to children with no prenatal SRI exposure and lower 3rd trimester maternal depressed mood (SRI: $\beta = 0.399$, $p = 0.034$, $\eta^2 =$
0.156; 3rd Tri: $\beta = -0.584$, $p = 0.008$, $\eta^2 = 0.231$). An interaction between maternal depressed mood in the 3rd trimester and at 5 - 7 years was associated with the change in cortisol between Task 2 and Stress 2 ($\beta = 1.651$, $p = 0.003$, $\eta^2 = 0.292$), whereby children exposed to higher 3rd trimester and 5 - 7 year maternal depressed mood showed a smaller increase in cortisol between these two time points, compared the children of mothers with lower 3rd trimester maternal depressed mood who displayed a greater stress response following Task 2 (Figure 3.7 A).

Further analyses showed a reduced change in cortisol between Stress 1 and Stress 2 was associated with this observed interaction ($\beta = 1.627$, $p = 0.004$, $\eta^2 = 0.277$, Figure 3.7 B).

Together these findings suggest children with chronic (3rd trimester and 5 - 7 year) exposure to higher maternal depressed mood display a blunted cortisol stress response.
Table 3.5 Cortisol concentrations for children with completed lab visit and corresponding evening data, defined by prenatal SRI exposure

<table>
<thead>
<tr>
<th>Cortisol concentrations (nmol/L)</th>
<th>Yes (n= 27)</th>
<th>No (n= 37)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Baseline</td>
<td>4.64 ± 2.41</td>
<td>4.94 ± 2.95</td>
<td>0.677</td>
</tr>
<tr>
<td>Stress 1</td>
<td>4.93 ± 2.65</td>
<td>5.87 ± 4.15</td>
<td>0.529</td>
</tr>
<tr>
<td>Stress 2</td>
<td>5.25 ± 5.81</td>
<td>5.81 ± 3.14</td>
<td>0.347</td>
</tr>
<tr>
<td>Evening</td>
<td>3.92 ± 5.07</td>
<td>1.63 ± 1.04</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>AUCG</td>
<td>14.47 ± 6.06</td>
<td>14.98 ± 7.02</td>
<td>0.355</td>
</tr>
</tbody>
</table>

Note: Values are displayed as mean ± standard deviation (SD). Groups were compared using t-tests and log transformed cortisol concentrations.
* Baseline is calculated from a mean of Baseline, Task 1, and Task 2.

Figure 3.3 Association between prenatal SRI exposure and children’s stress reactivity and evening cortisol concentrations at 5 - 7 years. (A) Children with prenatal SRI exposure displayed a delayed cortisol response with stress and elevated evening cortisol concentrations compared to non-exposed children (F = 5.744, p = 0.004, η² = 0.099). (B) Children with prenatal SRI exposure displayed higher Evening cortisol concentrations compared to non-exposed children (β = 0.368, p = 0.007, η² = 0.128). Models were adjusted for age, sex, time between Waking and Baseline, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years. N = 64 Children (SRI exposed = 27, Non exposed = 37). Values are displayed as mean ± SEM.
Figure 3.4 Interaction between maternal mood in the 3rd trimester and at 5 - 7 years with the change in cortisol (Stress 2 minus Stress 1) in children with prenatal SRI exposure. Higher 3rd trimester and 5 - 7 year maternal anxious mood (A) and maternal depressed mood (B) was associated with a blunted cortisol stress response in 5 - 7 year old children, adjusting for age, sex, and time between Waking and Baseline ($\beta = 1.933, p = 0.002, \eta^2 = 0.393; \beta = 2.190, p = 0.000, \eta^2 = 0.483$). Maternal mood scores (HAM A and HAM D) at 5 - 7 years were evaluated as continuous variables in general linear models and defined by a median split in Figure 3.4. N = 27 children with prenatal SRI exposure.

Table 3.6 Cortisol concentrations for children with completed lab visit and diurnal data defined by prenatal SRI exposure

<table>
<thead>
<tr>
<th>Cortisol concentrations (nmol/L)</th>
<th>Prenatal SRI Exposure</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n= 19)</td>
<td>No (n= 14)</td>
</tr>
<tr>
<td>Waking</td>
<td>14.17 ± 5.52</td>
<td>14.00 ± 5.03</td>
</tr>
<tr>
<td>Wake + 20 min</td>
<td>15.43 ± 6.39</td>
<td>16.59 ± 7.86</td>
</tr>
<tr>
<td>Baseline</td>
<td>6.14 ± 4.32</td>
<td>5.15 ± 3.31</td>
</tr>
<tr>
<td>Task 1</td>
<td>4.57 ± 3.44</td>
<td>4.13 ± 1.91</td>
</tr>
<tr>
<td>Task 2</td>
<td>2.90 ± 1.69</td>
<td>3.31 ± 1.24</td>
</tr>
<tr>
<td>Stress 1</td>
<td>4.69 ± 2.64</td>
<td>6.39 ± 4.57</td>
</tr>
<tr>
<td>Stress 2</td>
<td>5.44 ± 2.80</td>
<td>5.37 ± 1.52</td>
</tr>
<tr>
<td>Evening</td>
<td>2.99 ± 1.49</td>
<td>3.06 ± 0.95</td>
</tr>
<tr>
<td>AUCG</td>
<td>62.68 ± 24.96</td>
<td>71.85 ± 30.15</td>
</tr>
</tbody>
</table>

Note: Values are displayed as mean ± standard deviation (SD). Groups were compared using t-tests and log transformed cortisol concentrations.
Figure 3.5 Association between maternal anxiety mood scores (HAM A) at 5 - 7 years and children’s Wake + 20 minute cortisol concentrations. Children exposed to higher 5 - 7 year maternal depressed mood displayed higher Wake + 20 minute cortisol concentrations, compared to lower 5 - 7 year maternal depressed mood, adjusting for age, sex, prenatal SRI exposure, and 3rd trimester maternal depression scores ($\beta = 0.484$, $p=0.009$, $\eta^2 = 0.228$). N = 33 children (SRI exposure = 14, No exposure = 19).

Figure 3.6 Association between prenatal SRI exposure and cortisol patterns in 5 - 7 year old children across the stress-challenge study day. Children with prenatal SRI exposure show a less reactive cortisol stress response and flatter decline in cortisol into the evening, compared to children with no prenatal SRI exposure, adjusting for age, sex, and maternal depression scores (HAM D) in the 3rd trimester and at 5 - 7 years ($F_{7,189} = 2.599$, $p = 0.045$, $\eta^2 = 0.088$). N = 33 children (SRI exposure = 14, No exposure = 19). Values are displayed as mean ± SEM.
Figure 3.7 Interaction between maternal depressed mood in the 3rd trimester and at 5 - 7 years with children’s cortisol reactivity change scores. Children exposed to higher 3rd trimester and 5 - 7 year maternal depressed mood displayed a smaller increase in cortisol with stress between Task 2 and Stress 2 (A) and Stress 1 and Stress 2 (B) compared to children exposed to higher 3rd trimester and lower 5 - 7 year maternal depressed mood, adjusting for sex, age, and prenatal SRI exposure ($\beta = 1.651$, $p = 0.003$, $\eta^2 = 0.292$; $\beta = 1.627$, $p = 0.004$, $\eta^2 = 0.277$). N = 33 children. Maternal depression scores (HAM D) at 5 - 7 years was evaluated as a continuous variable in general linear models and defined by a median split in Figure 3.7. Values are displayed as mean ± SEM.
3.3 Aim 1 Discussion

3.3.1 Aim 1A: The Diurnal Rhythm at 5 - 7 Years

In Aim 1A, cortisol patterns were evaluated under resting conditions in early childhood. Examining everyday patterns of cortisol rhythm is an informative tool in child development studies.\(^{210}\) The diurnal cortisol rhythm is typically characterized by high cortisol concentrations upon waking with a substantial increase in cortisol 35 – 40 minutes after waking (CAR), and a subsequent decline over the remainder of the day.\(^{220}\) The circadian rhythm of cortisol production is well established in adults, and there is evidence that this pattern can be observed as early as 2 months of age.\(^{221}\)

More recently, the magnitude of the CAR is becoming increasingly recognized as a reliable measure of HPA activity, and both a heightened and diminished CAR is associated with a range of psychosocial factors.\(^{222}\) Although measures of waking cortisol, average cortisol, and slope across the day have been linked to stress exposure, it is not clear which indicators are reliably altered by early exposure.\(^{150}\) For this reason, all the above measures were included in my analyses.

A typical diurnal cortisol pattern across the day was exhibited in children at 5 - 7 years, but 52.0% of the children did not display a CAR. However, given the second saliva samples were collected approximately 20 minutes post-waking, it is possible I did not capture the peak CAR. Similarly, two studies collecting a sample 30 minutes after waking found a minimal,\(^{223}\) and no\(^{148}\) CAR in children approximately 11 years of age. Furthermore, the cortisol rise upon awakening may be smaller in children than adults,\(^{224}\) making it more difficult to detect in this age group.
Prenatal SRI exposure and 3rd trimester depressed/anxious maternal mood were not associated with differences in diurnal patterns across the day. Older age was associated with lower waking cortisol concentrations, and a subsequently more defined CAR, as well as a flatter slope across the day, compared to younger age. Studies have reported children’s age as a distinguishing factor in cortisol patterns across the day, or suggest that age may be associated with variability in the diurnal rhythm.150,224,225 In 3 - 6 year old children, age was negatively correlated with morning and afternoon salivary cortisol, but not reactivity throughout the day,225 and older adolescents of parents with and without bipolar disorder, have displayed higher salivary cortisol concentrations 60 minutes after waking and at 3:00pm, compared to younger adolescents.226 Furthermore, in a sample of 10 - 12 year olds, age was positively associated with salivary evening cortisol.224 Developmentally, HPA function changes over time, with an initial reactive period after birth, followed by a hypo-responsive period by the end of the first year of life and during childhood, and hyper responsiveness throughout puberty and into adulthood.227 Specifically, both pubertal and adolescent stages are marked by unique responses to stress, significant alterations in HPA activity, and a rise in susceptibility to various mood disorders.228 In this way, these findings may reflect a transition period observed in early childhood, and it would therefore be of interest to evaluate these children within the adolescent window.

While literature suggests that sex may be a potential confounder when studying the diurnal rhythm,150,224 such that females exhibit higher cortisol values than males, I did not observe different cortisol patterns between boys and girls at 5 - 7 years of age. Given studies have shown a higher CAR224 and diurnal curve219 in females 11 and 17 years of age, respectively, this may suggest that sex differences result from various stages of pubertal development, possible not detectable in early childhood. Furthermore, although experimental
work in animal models suggest that the effects of prenatal stress may be sex specific,\textsuperscript{229} human studies show inconsistent findings.\textsuperscript{150,217} Sex differences in patterns of social behaviour in relation to cortisol activity have been identified previously,\textsuperscript{225} however, I did not assess social patterns in this thesis. Nonetheless, women are twice as likely to suffer from a stress-related psychiatric disorder, and sex differences in stress responses and HPA functioning may account for these findings.\textsuperscript{230}

In contrast to my findings, prenatal anxiety was associated with higher awakening salivary cortisol in 10-year-old children,\textsuperscript{148} a modest reduction in the CAR\textsuperscript{150} and a flatter slope in 15 year old children.\textsuperscript{149,150} Although these studies suggest that a programming effect of antenatal anxiety on diurnal patterns in children, not all findings are consistent. For instance, young adults exposed to prenatal stress displayed no differences in basal diurnal cortisol concentrations compared to controls.\textsuperscript{217} Given these studies were conducted in older children and adolescents, compared to 5 - 7 year old children, these findings suggest that age may be a distinguishing factor in variable diurnal cortisol patterns.

Overall, the diurnal pattern displayed a strong variation with children’s age, and given the small age range of children in this study, it is particularly interesting that such a large effect was found. These findings further question what child-related characteristics (e.g. age, sex, temperament) affect cortisol patterns, and questions if these children were at a critical transition period in HPA function.

### 3.3.2 Aim 1B: Cortisol Stress Reactivity at 5 - 7 Years

In Aim 1B, cortisol patterns at 5 - 7 years were evaluated in response to a stressor. There is mounting evidence indicating that early life adverse events may shape stress regulation across
the lifespan. Altered HPA activity may mediate the increased vulnerability to stress related disorders later in life. Optimal functioning of the HPA system is characterized by the ability to mount a physiological response to a cognitive/social stressor, and consequently both hypoactivation and hyperactivation of a stress response may reflect pathophysiological processes. Studies in children demonstrated that early environmental and postnatal conditions may influence stress-responsive biological systems later in life. At 5 - 7 years of age, prenatal SRI exposure and maternal depressed and anxious mood in the 3rd trimester and at 5 - 7 years were associated with variable cortisol reactivity measures.

Taking all these factors into account, three main findings were found from my analyses. First, I found that prenatal exposure to SRIs and higher 3rd trimester maternal depressed and anxious mood has the ability to leave a persistent influence on stress regulation in childhood. Children with prenatal SRI exposure exhibited a blunted cortisol response throughout the lab stressor, an attenuated fall in cortisol into the evening, and a sustained elevation in evening cortisol concentrations compared to non-exposed children, suggesting either a delay or inability to regulate cortisol following a stressor.

The implications of HPA dysregulation remain unclear; however, several groups have provided evidence for the association between maternal anxiety during pregnancy and behavioural, emotional, and cognitive problems in infancy and childhood. For instance, prenatal anxiety was associated with behavioural/emotional problems at 7 years of age, and ADHD symptoms and externalizing problems in 8 - 9 year old children. Higher and more variable salivary cortisol at 13 years have been associated with postnatal depression, and predicted elevated depressive symptoms at 16 years of age. Interestingly, shy or introverted children have shown diminished cortisol reactivity in response to a normative stressor (beginning...
of first grade), compared to children characterized as not shy or introverted, suggesting that a blunted stress response may be associated with certain behavioural correlates in young children. However, the extent to which altered cortisol affects children’s vulnerability to illness, and if such HPA alterations will endure beyond childhood and adolescence is unknown.

Second, although prenatal mood scores and SRIs were found to be associated with cortisol regulation at 5 - 7 years, concurrent maternal mood scores displayed an interactive relationship with early exposures on cortisol reactivity patterns in children. Specifically, children exposed to higher 3rd trimester and 5 - 7 year maternal depressed/anxious mood displayed a blunted cortisol response to a lab challenge stressor. In contrast, children exposed to higher 5 - 7 year maternal depressed/anxious mood alone, displayed similar reactivity responses to children only exposed to higher 3rd trimester maternal mood scores or lower pre- and postnatal maternal mood scores. Namely, chronic pre- and postnatal exposure to maternal mood disturbances may be associated with a diminished adaptive stress response, reflected by blunted cortisol reactivity.

There is substantial literature illustrating the impact of early life and concurrent maternal mood on childhood stress measures. For example, 4.5 year olds exposed to maternal stress during infancy and concurrently displayed higher afternoon salivary cortisol concentrations compared to children exposed only to higher concurrent maternal stress. Furthermore, compared to children with lower cortisol concentrations, children with higher cortisol concentrations exhibited greater mental health conditions, determined through interviews with mothers, teachers, and the children. Chronic exposure to a stressor can also lead to blunted cortisol concentrations and responses to stress. For instance, a blunted salivary cortisol response to a psychosocial stress task was displayed in otherwise healthy women exposed to physical abuse in childhood compared to women with no physical abuse in childhood, and
healthy adults showed significantly lower salivary cortisol responses when exposed to a high number of adverse childhood events, compared to no or minimal adverse childhood events. Additionally, young children with depression exhibited a reduced percent change in salivary cortisol to a laboratory stressor compared to healthy controls. Accumulating evidence suggests that cortisol hyporeactivity resulting from chronic stress exposure may reflect a pathophysiological process as clinically important as hyperactivation of the HPA axis. However, the extent to which deviations from the ‘normal range’ influence disease is currently undefined.

Lastly, prenatal maternal anxious mood did not have separate effects on children’s cortisol reactivity than prenatal maternal depressed mood, as similar interactions were observed with 3rd trimester and 5 - 7 year maternal anxious mood, and 3rd trimester and 5 - 7 year maternal depressed mood. However, given the high comorbidity of depression and anxiety, the ability to find differential relationships of cortisol patterns may be unlikely. In addition, I was unable to establish whether SRI exposure has direct effects on cortisol stress regulation in children, or if SRIs indirectly influence cortisol reactivity through moderating maternal mood. Given serotonergic signaling may be altered in response to antenatal mood and SRI antidepressants, the ability to distinguish between these early life experiences presents a challenging task. Thus, distinguishing the effects of prenatal SRI exposure and maternal mood remains a key challenge, as they both appear to influence cortisol reactivity in early childhood.

Overall, Aim 1 provided a detailed evaluation of both resting cortisol patterns of the diurnal rhythm, and the cortisol response to a stress challenge at 5 - 7 years of age. The main findings from the stress challenge suggest that prenatal exposure to SRIs and 3rd trimester
maternal depressed/anxious mood may have lasting effects on HPA regulation, and maternal mood at 5 - 7 years appears to have a moderating influence on this association. In this way, early life adversities may elicit a sensitization to subsequent life stressors, but the current environmental status may further provoke or repress an altered stress response. Furthermore, the mechanisms by which the early environment influences fetal neurodevelopment is currently unknown, but may involve the interplay between gestational environment, genetic variations, and epigenetic processes.\textsuperscript{143,202}
Chapter 4: AIM 2: DNA METHYLATION AS A PROGRAMMING MECHANISM

Epigenetic mechanisms, such as DNA methylation, have been implicated as a means by which environmental factors such as maternal behavior (in rodent models) can influence gene expression to produce long-term health consequences. The perinatal period and early childhood years mark a time of brain development sensitivity that may be particularly susceptible to epigenetic modifications, and altered HPA activity has been associated with differential methylation in key regulatory genes associated with the negative feedback mechanism. Therefore, for Aim 2 I investigated the association between prenatal exposure to SRIs and maternal depressed/anxious mood on the methylation status of the NR3C1 1F, NR3C1 1D, and SLC6A4 promoter, and the association between methylation status and cortisol patterns in early childhood (5 - 7 years of age). The following sub-aims were addressed in Aim 2 (See Figure 4.1 for schematic representation):

Aim 2A: To determine the relationship between prenatal SRI exposure and maternal depressed/anxious mood on DNA methylation status of NR3C1 1F, NR3C1 1D, and SLC6A4 promoter region in early childhood.

Aim 2B: To determine the relationship between DNA methylation status of NR3C1 1F, NR3C1 1D, and SLC6A4 promoter region and diurnal rhythm and cortisol stress response in early childhood.

Aim 2C: To determine the relationship between DNA methylation status of the NR3C1 1F and SLC6A4 promoter region at birth and children’s diurnal rhythm and cortisol stress response.
Figure 4.1 Sub-aims addressed in Aim 2.
4.1 Aim 2 Methods

4.1.1 Aim 2A: Gene-specific Methylation Status at 5 - 7 Years

The methylation status at each of the 13 CpGs in \( NR3C1 \) exon 1\(_F\), 10 CpGs in \( NR3C1 \) exon 1\(_D\), and 10 CpGs \( SLC6A4 \) promoters were quantified by bisulphite pyrosequencing\(^{212}\) in buccal epithelial cells from 111 children (SRI exposure = 45, No exposure = 66).

\textit{Methylation status of the \( NR3C1 \) 1\(_F\) Promoter Region:} Given that CpG sites 1, 2, 3, and 4 in the promoter region of \( NR3C1 \) 1\(_F\) have been associated with prenatal mood disturbances and cortisol reactivity at 3 months,\(^{202}\) and CpG sites 3 and 4 may contain an NGFI-A binding site,\(^{198,191}\) the following analyses focused on the methylation status of these specific CpG sites (Figure 4.2). Following previous studies,\(^{201,202}\) additional CpG sites in the \( NR3C1 \) 1\(_F\) promoter were evaluated in an exploratory manner.

\textit{Methylation status of the \( NR3C1 \) 1\(_D\) Promoter Region:} The promoter region of \( NR3C1 \) exon 1\(_D\) has not been characterized to the same extent as exon 1\(_F\), and therefore all 10 CpG sites and then mean of the sites were evaluated in the following analyses.

\textit{Methylation status of the \( SLC6A4 \) Promoter Region:} The methylation status of \( SLC6A4 \) in newborn cord blood was reported to be sensitive to prenatal maternal mood.\(^{52}\) However, given specific CpG sites in the promoter region have not been characterized as more vulnerable than others, all 10 CpG sites and the mean of these sites were evaluated in the following analyses.

Multivariate regression models were used to examine the relationships between prenatal SRI exposure and 3\(^{rd}\) trimester maternal anxiety and depression mood scores on methylation status of the \( NR3C1 \) 1\(_F\), \( NR3C1 \) 1\(_D\), and \( SLC6A4 \) promoter in 5 - 7 year old children. These analyses included prenatal SRI exposure as a fixed factor, and age, sex, and maternal mood scores in 3\(^{rd}\) trimester and at 5 - 7 years as covariates. Separate models were included using 3\(^{rd}\)
trimester and 5 - 7 year maternal anxiety or depression mood scores as previously described in Aim 1. Statistical analyses were not adjusted for multiple comparisons; instead, effect sizes ($\eta^2$) were computed to display the magnitude of relationships, explanations were provided describing which CpG sites were evaluated and why, and interpretations were provided for results.

4.1.2 Aim 2B: Methylation Status and Cortisol Patterns at 5 - 7 Years

Analyses of diurnal rhythm included 99 children (No exposure = 60, SRI exposure = 39) with complete methylation data and diurnal cortisol measures (Waking, Wake + 20 minutes, Evening). Cortisol stress reactivity was evaluated in 64 children (No Exposure = 37, SRI Exposure = 27) with complete methylation data and both lab visit and corresponding evening cortisol measures (Baseline, Stress 1, Stress 2, Evening). These are the same subjects evaluated in Aim 1A and 1B, respectively.

Repeated measures and general linear models were used to examine the relationships between $NR3C1_{1F}$, $NR3C1_{1D}$ and $SLC6A4$ methylation status and children’s diurnal cortisol rhythm and cortisol stress reactivity. CpG sites were evaluated as indicated in Aim 2A, and age, sex, prenatal SRI exposure, and maternal mood scores in the 3rd trimester and at 5 - 7 years were included as covariates in all analyses. Given the diurnal collection time of the Wake + 20 minute saliva sample was significantly later in the SRI exposed group (8:16am), compared to the non-exposed group (7:51am)(p = 0.041), the time between Waking and Wake + 20 minutes was controlled for in the diurnal pattern analyses. On the stress challenge day, the collection time of the Waking saliva sample was significantly later in the SRI exposed group (7:52am), compared to the non-exposed group (7:27am)(p = 0.029), and therefore the time between Waking and Baseline samples was controlled in the cortisol reactivity analyses.
4.1.3 Aim 2C: Methylation Status at Birth and Cortisol Patterns at 5 - 7 Years

The methylation status of the 13 CpGs and 10 CpGs in the promoter region of NR3C1 \textsubscript{1F} and SLC6A4, respectively, were quantified in cord blood leukocytes. Analyses of the diurnal rhythm included 80 children (No Exposure = 49, SRI Exposed = 31) with complete methylation data at birth and 5 – 7 year diurnal cortisol data (Waking, Wake + 20 minutes, Evening). These are the same subjects evaluated in Aim 1A. Analyses of cortisol stress reactivity included 52 children (No Exposure = 31, SRI Exposure = 21) with complete methylation data at birth and stress challenge cortisol data (Baseline, Stress 1, Stress 2, Evening). These are the same subjects evaluated in Aim 1B.

![Figure 4.2 Schematic representation of the CpG sites analyzed in the NR3C1 \textsubscript{1F} promoter at 5 - 7 years of age.](image)

The CpGs are underlined and numbered. The potential NGFI-A consensus binding site is located in grey boxes. The mean of CpG sites 6 - 13 (blue font) was used as a single variable in analyses. Non-coding and coding exons are indicated by white and black boxes, respectively. Capital letters represent exon \textsubscript{1F}. The portion analyzed by bisulfite sequencing is shown in bold. Adapted from Oberlander et al\textsuperscript{202} and Turner et al\textsuperscript{191}.
4.2 Aim 2 Results

4.2.1 Aim 2A: Gene-specific Methylation Status at 5 - 7 Years of Age

4.2.1.1 Buccal Epithelial Cell NR3C1 1F Methylation Status at 5 - 7 Years

Figure 4.3 A displays the methylation status of 13 CpG sites in the NR3C1 1F promoter, separated by prenatal SRI exposure. Overall levels of NR3C1 exon 1F promoter methylation were similar to those previously observed in buccal epithelial DNA from young adults (Table 4.1). The mean methylation status (± SD) across 13 CpG sites was 4.68 % (± 2.98 %). Similar to Tyrka et al.201 methylation levels at CpG 5 showed weak to moderate associations with the other 12 CpG sites evaluated (range r = 0.091 – 0.318), and CpGs 6 - 13 showed mainly weak and moderate associations with CpGs 1 - 5 (r = 0.067 – 0.423) (Table 4.2). Thus, CpGs 1 - 5 were evaluated individually. Given CpG sites 6 - 13 showed moderate to very strong intercorrelations (r = 0.342 – 0.822), a mean percent methylation of CpGs 6 - 13 was used as a single variable in the following analyses.

T-tests performed revealed significantly higher methylation levels of NR3C1 1F CpG 5 in females, compared to males (p=0.006). Albeit a low level of CpG 2 methylation (mean 2.98%, range 0.00 - 10.91%), an interaction between 3rd trimester and concurrent maternal anxious mood was associated with CpG 2 methylation (F = 4.336, p = 0.040, η² = 0.048, Figure 4.4). Specifically, children exposed to higher 3rd trimester and concurrent maternal anxious mood displayed lower CpG 2 methylation levels, compared to children exposed to higher 3rd trimester maternal anxious mood alone who exhibited higher methylation levels. However, given the small percent levels of CpG 2 methylation, the biological significance of this findings is unclear.

Younger age was associated with a higher methylation status at CpG 5 and the mean of CpG sites 6-13 (F = 5.541, p = 0.021, η² = 0.061; F = 15.785, p = 0.000, η² = 0.155). Similarly,
low methylation percentages were observed at CpG 5 and the mean of CpGs 6 - 13 (Table 4.1). The relationship between age and methylation status remained significant when adjusting for maternal depression scores in the 3\textsuperscript{rd} trimester and at 5 - 7 years.

4.2.1.2 Buccal Epithelial Cell \textit{NR3C1} 1\textsubscript{D} Methylation Status at 5 - 7 Years

Figure 4.3 B displays the methylation status of 10 CpG sites in \textit{NR3C1} 1\textsubscript{D} promoter, separated by prenatal SRI exposure. The mean methylation status (± SD) across 10 CpG sites was 4.53 % (± 3.21%). The methylation status in buccal epithelial cells observed at CpGs 6, 7, and 8 were within the same range previously reported in cord blood (Table 4.1).\textsuperscript{203} However, compared to the mean methylation status observed at CpGs 5 and 6 (3.04% and 3.36%, respectively), Hompes \textit{et al}\textsuperscript{203} showed considerably higher methylation levels at these sites in cord blood (~32.0%).

Prenatal SRI exposure and 3\textsuperscript{rd} trimester maternal mood scores were not associated with methylation status in the \textit{NR3C1} 1\textsubscript{D} promoter (\(p > 0.05\)). Corresponding to results observed in the \textit{NR3C1} 1\textsubscript{F} promoter region, younger age was associated with higher \textit{NR3C1} 1\textsubscript{D} methylation at CpGs 1, 5, 10, and mean methylation (\(F = 7.552, p = 0.007, \eta^2 = 0.080; F = 7.042, p = 0.009, \eta^2 = 0.075; F = 3.980, p = 0.049, \eta^2 = 0.044; F = 9.034, p = 0.003, \eta^2 = 0.094\)). Moreover, low methylation percentages were observed at CpGs 1, 5, 10 and the mean (Table 4.1). The significant associations observed between age and methylation status remained when 3\textsuperscript{rd} trimester and 5 - 7 year maternal depression scores were adjusted for in the model.
4.2.1.3 Buccal Epithelial Cell SLC6A4 Methylation Status at 5 - 7 Years

Figure 4.5 displays the methylation status of 10 CpG sites in SLC6A4 promoter, separated by prenatal SRI exposure. Overall levels of SLC6A4 promoter methylation were within the same range previously reported in buccal epithelial cells from adolescents (Table 2.1). The mean methylation status (± SD) across 10 CpG sites was 3.00 % (± 1.23 %). Interestingly, moderate to strong intercorrelations were observed with all 10 CpG sites evaluated (range, r = 0.149 – 0.701), except with CpG 8. In particular, CpG 8 had a modest association with CpG 5 (r = 0.229), a moderate association with CpG 10 (r = 0.328), and no association with the other CpG sites evaluated (r = -0.086 to – 0.017).

Children exposed to higher 3rd trimester and concurrent maternal anxious mood exhibited lower methylation at SLC6A4 CpG 6, 8, and 9, compared to children exposed to higher 3rd trimester maternal anxious mood alone (F = 5.250, p = 0.024, η² = 0.044; F = 8.737, p = 0.004, η² = 0.092; F = 6.368, p = 0.013, η² = 0.069, Figure 4.6). Figure 4.6 displays the observed interaction between 3rd trimester and 5 - 7 year maternal anxious mood on SLC6A4 CpG 8 methylation. Children also displayed low methylation percentages at CpGs 6, 8, and 9 (mean 1.10%, range 0.00 - 3.17%; mean 3.78%, range 2.28 - 6.40%; mean 2.26%, range, 0.00 - 7.68%, respectively), and therefore these findings may not be biologically meaningful as 1) CpGs 6 and 8 do not exceed the technical reliability of the pyrosequencing assay (5%), and 2) this degree of change would not be expected to result in a detectable alteration in gene expression.

Younger age was associated with lower SLC6A4 methylation at CpGs 3, 4, 5, and the mean (F = 102.10, p = 0.000, η² = 0.540; F = 27.225, p = 0.000, η² = 0.238; F = 9.227, p = 0.003, η² = 0.096; F = 34.912, p = 0.000, η² = 0.286). The association between age and
methylated remained significant when adjusting for maternal depression scores in the regression model.
Figure 4.3 Methylation status at each CpG site analyzed in the exon 1_F (A) and exon 1_D (B) promoter region of NR3C1 at 5 - 7 years of age. Median methylation percentages are presented and whiskers refer to the interquartile range. No significant differences were observed between exposure groups (p > 0.05). N = 111 Children (No Exposure = 66, SRI Exposure = 45).
Table 4.1 Mean methylation, standard deviation, and range of individual CpG sites within the NR3C1 F, NR3C1 D, and SLC6A4 promoter regions in buccal epithelial DNA at 5 - 7 years of age

<table>
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<td>0.00 - 10.91</td>
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Note: N = 111 children.
Table 4.2 Association between CpG methylation status in the NR3C1 promoter at 5 - 7 years of age

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*Note*: N = 111 children. Pearson correlations were used to evaluate the association between CpG methylation. **p<0.01, *p<0.05.
Figure 4.4 Interaction between maternal mood scores in the 3rd trimester and at 5 - 7 years with methylation status of CpG 2 in the NR3C1 1F promoter at 5 – 7 years. Children exposed to higher 3rd trimester and 5 - 7 year maternal anxious mood displayed lower CpG 2 methylation, compared to children exposed to higher 3rd trimester maternal anxious mood alone, adjusting for age, sex, and prenatal SRI exposure (F = 4.336, p = 0.040, η² = 0.048). Maternal anxiety scores (HAM A) at 5 - 7 years was evaluated as continuous variable in general linear models and defined by a median split in Figure 4.4. N = 111 children.

Figure 4.5 Methylation status at each CpG site analyzed in the promoter region of SLC6A4 at 5 - 7 years of age. Median methylation percentages are presented and whiskers refer to the interquartile range. No significant differences were observed between exposure groups (p >0.05). N = 111 Children (No Exposure = 66, SRI Exposure = 45).
**Figure 4.6 Interaction between maternal anxious mood in the 3rd trimester and at 5 - 7 years with methylation status of CpG 8 in the SLC6A4 promoter at 5 – 7 years.** Children exposed to higher 3rd trimester and 5 - 7 year maternal anxious mood displayed lower CpG 8 methylation, compared to children exposed to higher 3rd trimester maternal anxious mood alone, adjusting for age, sex, and prenatal SRI exposure ($F = 8.737, p = 0.004, \eta^2 = 0.092$). Maternal anxiety scores (HAM A) at 5 - 7 years was evaluated as continuous variable in general linear models and defined by a median split in Figure 4.6. N = 111 children.
4.2.2 Aim 2B: Methylation Status and Cortisol Patterns at 5 - 7 Years of Age

4.2.2.1 Methylation Status and the Diurnal Cortisol Rhythm at 5 – 7 Years

NR3CI 1F Methylation Status and the Diurnal Rhythm at 5 - 7 Years

Maternal Anxiety Scores

The mean methylation status of NR3CI 1F CpGs 6 - 13 was positively associated with Waking and Wake + 20 minute cortisol concentrations ($\beta = 0.231, p = 0.044, \eta^2 = 0.053; \beta = 0.247, p = 0.022, \eta^2 = 0.068, \textbf{Figure 4.7 A and B}$). In addition, a higher mean methylation status of CpGs 6 - 13 was associated with a higher AUC$_G$ ($\beta = 0.305, F = 7.864 p = 0.006, \eta^2 = 0.095, \textbf{Figure 4.7 C}$).

Maternal Depression Scores

Similarly, the mean methylation of CpGs 6 - 13 was positively associated with Waking, Wake + 20 minutes, and AUC$_G$ cortisol concentrations ($\beta = 0.227, p = 0.048, \eta^2 = 0.051; \beta = 0.250, p = 0.022, \eta^2 = 0.067; \beta = 0.313, p = 0.006, \eta^2 = 0.095$).

NR3CI 1D Methylation Status and the Diurnal Rhythm at 5 - 7 Years

Maternal Anxiety Scores

A higher methylation status of CpG 4 was associated with lower Waking cortisol concentrations, and a greater increase in cortisol between Waking and Wake + 20 minutes, compared to lower CpG 4 methylation ($\beta = -0.264, p = 0.017, \eta^2 = 0.073; \beta = -0.265, p = 0.016, \eta^2 = 0.074$). Higher CpG 2 and mean methylation status were associated with flatter decline in cortisol between Waking and Evening ($\beta = -0.239, p = 0.023, \eta^2 = 0.066; \beta = -0.252, p = 0.022$).
\[ \eta^2 = 0.067 \text{, Figure 4.8}. \] However, given the small variability in methylation levels of CpG 2 and 4 \textit{(Table 2.1)}, the biological significance of this finding is unclear.

\textit{Maternal Depression Scores}

Likewise, lower Waking cortisol concentrations and a greater increase in cortisol between Waking and Wake + 20 minutes was associated with higher CpG 4 methylation \((\beta = -0.250, p = 0.021, \eta^2 = 0.068; \beta = -0.278, p = 0.014, \eta^2 = 0.077). \) Moreover, higher CpG 2 and mean methylation levels were associated with a flatter decline in cortisol between Waking and Evening \((\beta = -0.235, p = 0.024, \eta^2 = 0.065; \beta = -0.251, p = 0.022, \eta^2 = 0.067). \)

\textbf{SLC6A4 Methylation Status and the Diurnal Rhythm at 5 - 7 Years}

\textit{Maternal Anxiety Scores}

Using repeated measures analyses, methylation levels at CpG 2 and 10 were associated with diurnal cortisol concentrations across the day \((F_{4,156} = 12.427, p = 0.000, \eta^2 = 0.138; F_{4,156} = 4.956, p=0.016, \eta^2 =0.060). \) Although lower CpG 2 and 10 methylation levels were associated with a more typical diurnal pattern, reflected by a defined CAR, followed by a greater fall in cortisol across the day and lower evening cortisol concentrations, the percent methylation at each CpG 2 and 10 was considerably low \(\text{(mean 1.10%, range 0.00 - 3.34%; mean 3.38, range 1.65 - 6.53%, respectively)}. \)

These characteristics of the diurnal rhythm were also observed when evaluating individual components of diurnal cortisol measures. In particular, CpG 2, 7, 9, and 10 methylation levels were negatively associated with Wake + 20 minute cortisol concentrations \((\beta = -0.287, p = 0.010, \eta^2 = 0.084; \beta = -0.242, p = 0.027, \eta^2 = 0.062; \beta = -0.259, p = 0.017, \eta^2 = \)
0.071; $\beta = -0.283$, $p = 0.011$, $\eta^2 = 0.081$). Additionally, lower CpG 2 methylation was associated with lower evening cortisol concentrations ($\beta = 0.322$, $p = 0.002$, $\eta^2 = 0.120$) and a greater decline in cortisol between Waking and Evening ($\beta = -0.316$, $p = 0.003$, $\eta^2 = 0.111$), compared to higher CpG 2 methylation.

**Maternal Depression Scores**

CpG 2 and 10 methylation levels were associated with the diurnal cortisol rhythm across the day ($F_{4,156} = 11.769$, $p = 0.000$, $\eta^2 = 0.133$; $F_{4,156} = 5.050$, $p = 0.015$, $\eta^2 = 0.062$). CpG 2, 9, and 10 methylation levels were negatively associated with Wake + 20 minute cortisol concentrations ($\beta = -0.277$, $p = 0.013$, $\eta^2 = 0.078$; $\beta = -0.263$, $p = 0.015$, $\eta^2 = 0.074$; $\beta = -0.292$, $p = 0.004$, $\eta^2 = 0.088$). Moreover, lower CpG 2 methylation was associated with lower Evening cortisol concentrations, and greater decline in cortisol between Waking to Evening ($\beta = 0.338$, $p = 0.002$, $\eta^2 = 0.117$; $\beta = -0.305$, $p = 0.004$, $\eta^2 = 0.104$).

**4.2.2.2 Methylation Status and the Cortisol Stress Response at 5 - 7 Years**

**NR3C1** 1f Methylation Status and the Cortisol Stress Response at 5 - 7 Years

**Maternal Anxiety Scores**

Higher CpG 3 methylation was associated with lower Baseline cortisol concentrations, and a greater increase in cortisol between Baseline and Stress 2 ($\beta = -0.399$, $p = 0.009$, $\eta^2 = 0.121$; $\beta = -0.382$, $p = 0.006$, $\eta^2 = 0.138$, Figure 4.9 A and B). Additionally, higher CpG 1 methylation was associated with lower cortisol concentrations at Stress 1, and a greater increase in cortisol
between Stress 1 and Stress 2 ($\beta = -0.384, p = 0.006, \eta^2 = 0.141; \beta = -0.346, p = 0.011, \eta^2 = 0.119$).

*Maternal Depression Scores*

Similarly, an association was found between higher CpG 3 methylation and both lower Baseline cortisol concentrations and a greater increase in cortisol between Baseline and Stress 2 ($\beta = -0.403, p = 0.003, \eta^2 = 0.161; \beta = -0.376, p = 0.005, \eta^2 = 0.149$). Lower cortisol concentrations at Stress 1 and a greater increase in cortisol between Stress 1 and Stress 2, were associated with higher CpG 1 methylation ($\beta = -0.381, p = 0.005, \eta^2 = 0.145; \beta = -0.333, p = 0.014, \eta^2 = 0.113$).

*NR3CI 1D Methylation Status and the Cortisol Stress Response at 5 - 7 Years*

*Maternal Anxiety Scores*

The following results are outlined in Table 4.3. Higher NR3CI 1D methylation levels at CpG 5, 6, 7, 8 and the mean were associated with higher cortisol concentrations at Baseline ($p < 0.05$). Higher cortisol concentrations at Stress 1 was associated with higher methylation levels at CpG sites 5, 6, 7, and the mean, and higher methylation at CpGs 6 and 7 were associated with higher cortisol concentrations at Stress 2 ($p < 0.05$). Moreover, the AUC$_G$ between Baseline, Stress 1, Stress 2, and Evening, was positively associated with the methylation status of CpG 5, 6, 7, and the mean ($p < 0.05$). Figure 4.10 displays the relationship between CpG 5 methylation status and cortisol concentrations at Baseline, Stress 1, and the AUC$_G$. 

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Maternal Depression Scores

The association observed between exon 1D methylation status and cortisol reactivity at 5 - 7 years remained significant in the same direction and magnitude when adjusting for 3rd trimester and concurrent maternal depression scores. Additionally, higher CpG 8 methylation status was associated with higher Baseline cortisol concentrations and AUCG (β = 0.416, p = 0.036, η² = 0.083; β = 0.321, p = 0.040, η² = 0.080).

SLC6A4 Methylation Status and the Cortisol Stress Response at 5 - 7 Years

Maternal Anxiety Scores

Higher methylation levels at CpG 4 and 10 were associated with a smaller increase in cortisol between Baseline and Stress 1 (β = 0.329, p = 0.037, η² = 0.082; β = 0.328, p = 0.039, η² = 0.092). A smaller increase in cortisol between Baseline and Stress 2 was associated with higher methylation at CpGs 4, 7, and 10 (β = 0.324, p = 0.039, η² = 0.081; β = 0.231, p = 0.028, η² = 0.091; β = 0.346, p = 0.028, η² = 0.092).

Maternal Depression Scores

No associations were observed between SLC6A4 methylation and cortisol reactivity measures at 5 - 7 years when adjusting for 3rd trimester and 5 - 7 year maternal depression scores.
Figure 4.7 Relationship between the mean methylation status of NR3C1 1F CpGs 6 - 13 and diurnal cortisol measures at 5 - 7 years of age. Children with higher mean methylation of CpGs 6 – 13 displayed higher Waking (A), Wake + 20 minute (B), and AUC$_G$ (C) cortisol concentrations compared to children with lower methylation of CpGs 6 – 13, adjusting for age, sex, time between Waking and Wake + 20 minutes, prenatal SRI exposure, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years ($\beta = 0.231, p = 0.044, \eta^2 = 0.053; \beta = 0.247, p = 0.022, \eta^2 = 0.068; \beta = 0.305, F = 7.864 p = 0.006, \eta^2 = 0.095$). N = 99 children (No exposure = 60, SRI exposure = 39).
Figure 4.8 Relationship between NR3C1 1D CpG mean methylation status and the cortisol diurnal slope (Evening minus Waking) at 5 - 7 years of age. Children with higher CpG mean methylation displayed a flatter decline in cortisol across the day, compared to children with higher CpG mean methylation, adjusting for age, sex, SRI exposure, time between Waking and Wake + 20 minutes, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years ($\beta = -0.252, p = 0.022, \eta^2 = 0.067$). N = 99 children (No exposure = 60, SRI exposure = 39).

Figure 4.9 Relationship between NR3C1 1F CpG 3 methylation status and cortisol stress reactivity measures at 5 - 7 years of age. Higher CpG 3 methylation levels were associated with lower Baseline cortisol concentrations (A), and a greater increase in cortisol between Baseline and Stress 2 (B) compared to lower CpG 3 methylation, adjusting for age, sex, SRI exposure, time between Waking and Baseline, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years. ($\beta = -0.399, p = 0.009, \eta^2 = 0.121; \beta = -0.382, p=0.006, \eta^2 = 0.138$). N = 64 children.
### Table 4.3 The association between methylation status of the NR3C1 1D promoter region and cortisol stress measures at 5 - 7 years of age

<table>
<thead>
<tr>
<th>CpG Sites</th>
<th>Baseline</th>
<th>Stress 1</th>
<th>Stress 2</th>
<th>AUC&lt;sub&gt;G&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.010</td>
<td>0.023</td>
<td>ns</td>
<td>0.003</td>
</tr>
<tr>
<td>β</td>
<td>0.364</td>
<td>0.317</td>
<td></td>
<td>0.404</td>
</tr>
<tr>
<td>η²</td>
<td>0.122</td>
<td>0.097</td>
<td></td>
<td>0.158</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.003</td>
<td>0.008</td>
<td>0.020</td>
<td>0.003</td>
</tr>
<tr>
<td>β</td>
<td>0.480</td>
<td>0.361</td>
<td>0.310</td>
<td>0.396</td>
</tr>
<tr>
<td>η²</td>
<td>0.157</td>
<td>0.129</td>
<td>0.101</td>
<td>0.156</td>
</tr>
<tr>
<td>7</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>p</td>
<td>0.002</td>
<td>0.004</td>
<td>0.008</td>
<td>0.003</td>
</tr>
<tr>
<td>β</td>
<td>0.424</td>
<td>0.390</td>
<td>0.352</td>
<td>0.402</td>
</tr>
<tr>
<td>η²</td>
<td>0.170</td>
<td>0.152</td>
<td>0.131</td>
<td>0.161</td>
</tr>
<tr>
<td>8</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.032</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>β</td>
<td>0.306</td>
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<td></td>
</tr>
<tr>
<td>η²</td>
<td>0.087</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.024</td>
<td>0.020</td>
<td>0.020</td>
<td>0.022</td>
</tr>
<tr>
<td>β</td>
<td>0.326</td>
<td>0.325</td>
<td>0.325</td>
<td>0.322</td>
</tr>
<tr>
<td>η²</td>
<td>0.096</td>
<td>0.101</td>
<td>0.101</td>
<td>0.099</td>
</tr>
</tbody>
</table>

*Note:* General linear models adjusted for age, sex, prenatal SRI exposure, time between Waking and Baseline, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years. CpG sites 1, 2, 3, 4, 9, and 10 were not associated with cortisol stress reactivity. AUC<sub>G</sub>: Area under the curve with respect to ground. p: P-value; β: Standardized Beta; η²: Partial eta squared.
Figure 4.10 Relationship between *NR3C1* 1D CpG 5 methylation status and children’s cortisol stress reactivity. Higher CpG 5 methylation levels were associated with higher Baseline (A), Stress 1 (B), and AUC_50 (C) cortisol concentrations, compared to lower CpG 5 methylation, adjusting for age, sex, SRI exposure, time between Waking and Baseline, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years (β=-0.364, p = 0.010, η² = 0.122; β=-0.317, p=0.023, η² = 0.097; β=-0.404, p = 0.003, η² = 0.158). N = 64 Children (No Exposure = 37, SRI Exposure = 27).
4.2.3 Aim 2C: Methylation Status at Birth and Cortisol Patterns at 5 - 7 Years of Age

NR3CI F Methylation Status at Birth and Cortisol Patterns at 5 - 7 Years

**Figure 4.11 A** displays the methylation status in cord blood at 13 CpG sites in the NR3CI F promoter, separated by prenatal SRI exposure. The mean methylation status (± SD) across 13 CpG sites was 3.72% (± 1.51%). Similar to the methylation status observed at 5 - 7 years, children’s CpG 5 methylation levels at birth were either not associated, or weakly associated with CpG sites 6 - 13 (range r = -0.085 - 0.197). The methylation status of CpGs 6 - 13 showed mainly moderate to strong intercorrelations (r = 0.111 – 0.806), except between CpG 6 and 10 (r = -0.043), and CpG 8 and 13 (r = -0.040) (**Table 4.4**). Thus, a mean methylation of CpG sites 6 - 13 was used in the following analyses, and CpGs 1 - 5 were evaluated individually.

Children’s diurnal cortisol rhythm was not associated with their methylation status at birth (p > 0.05). No associations were found between neonatal NR3CI F methylation status and cortisol reactivity measures (p > 0.05).

SLC6A4 Methylation Status at Birth and Cortisol Patterns at 5 - 7 Years

**Figure 4.11 B** displays the methylation status in cord blood at 10 CpG sites in the SLC6A4 promoter, separated by prenatal SRI exposure. The mean methylation status (± SD) across 10 CpG sites was 6.42 % (± 1.91 %). T-tests revealed SLC6A4 methylation levels were significantly lower in infants with prenatal SRI exposure at CpGs 4, 6, and 7 (p = 0.050, 0.022, 0.047, respectively). However, given the mean percent difference between exposure groups was approximately 1%, this small variation likely has no biological significance.
Maternal Anxiety Scores

Children’s diurnal cortisol rhythm at 5 - 7 years was not associated with SLC6A4 methylation status at birth (p > 0.05). Using a repeated measures analysis, the methylation status of CpGs 1, 4, 6, 7, 8, and the mean were found to have a main effect on cortisol reactivity over the stress challenge and corresponding evening (F3,123 = 3.822, p = 0.026, η²=0.085; F = 3.159, p = 0.049, η²=0.0772, F= 3.567, p = 0.034, η²=0.080; F= 4.219, p = 0.018, η²=0.093; F= 3.411, p = 0.029, η²=0.077; F= 4.177, p = 0.019, η²=0.092). Figure 4.12 displays the main effect of CpG mean methylation status on cortisol reactivity over the lab visit and evening, whereby higher mean SLC6A4 CpG methylation at birth was associated with a blunted stress response at 5 - 7 years of age, compared to lower mean CpG methylation. Given that interindividual variation in methylation levels were observed (Table 4.5), these findings suggest that SLC6A4 promoter methylation in early life may have a biological effect on longer-term stress regulation. In all repeated measures analyses, a main effect of SRI exposure was observed (p < 0.05).

As shown in Table 4.6, higher neonatal methylation levels in the SLC6A4 promoter were associated with lower cortisol concentrations at Stress 1 and 2, a flatter cortisol slope between the stress tasks and evening measures, and a reduced AUCG.

Maternal Depression Scores

The associations observed between neonatal SLC6A4 methylation and cortisol reactivity at 5 - 7 years remained significant in the same direction and magnitude when adjusting for maternal depression scores.
Figure 4.11 Methylation status at each CpG site analyzed in the NR3C1 1F (A) and SLC6A4 (B) promoter regions in cord blood. Median methylation percentages are presented and whiskers refer to the interquartile range. N = 80 infants (No exposure = 49, SRI exposure = 31).
Table 4.4 Association between CpG methylation status in the \( NR3C1 \) \( 1_F \) promoter at birth

<table>
<thead>
<tr>
<th>( NR3C1 ) ( 1_F ) CpG sites</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.054</td>
<td>0.155</td>
<td>-0.032</td>
<td>0.195</td>
<td>0.269*</td>
<td>-0.004</td>
<td>0.242*</td>
<td>-0.052</td>
<td>0.038</td>
<td>0.053</td>
<td>0.000</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-0.051</td>
<td>0.224*</td>
<td>0.250*</td>
<td>-0.067</td>
<td>0.263*</td>
<td>0.198</td>
<td>0.295*</td>
<td>0.148</td>
<td>0.066</td>
<td>0.092</td>
<td>0.031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-0.060</td>
<td>0.034</td>
<td>-0.132</td>
<td>-0.015</td>
<td>0.196</td>
<td>0.077</td>
<td>0.134</td>
<td>0.263*</td>
<td>0.125</td>
<td>-0.123</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-0.174</td>
<td>-0.037</td>
<td>0.201</td>
<td>-0.071</td>
<td>0.285*</td>
<td>0.004</td>
<td>0.189</td>
<td>0.038</td>
<td>-0.084</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td>0.149</td>
<td>-0.085</td>
<td>0.197</td>
<td>0.148</td>
<td>0.129</td>
<td>0.172</td>
<td>0.186</td>
<td>-0.068</td>
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</tr>
<tr>
<td>6</td>
<td>0.209</td>
<td>0.308*</td>
<td>-0.043</td>
<td>0.164</td>
<td>0.111</td>
<td>0.185</td>
<td>0.166</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.596**</td>
<td>0.638**</td>
<td>0.812**</td>
<td>0.745**</td>
<td>0.585**</td>
<td>0.270*</td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>0.304**</td>
<td>0.603**</td>
<td>0.393**</td>
<td>0.470**</td>
<td>-0.040</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.676**</td>
<td>0.806**</td>
<td>0.649**</td>
<td>0.117</td>
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<tr>
<td>10</td>
<td>0.749**</td>
<td>0.702**</td>
<td>0.276*</td>
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</tr>
<tr>
<td>11</td>
<td>0.706**</td>
<td>0.212</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>0.262**</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: \( N = 80 \) infants. Pearson correlations were used to evaluate the association between CpG methylation. **p<0.01, *p<0.05.
Table 4.5 Mean, standard deviation, and range of methylation at individual CpG sites within the SLC6A4 promoter region at birth

<table>
<thead>
<tr>
<th>SLC6A4 CpG Site</th>
<th>Mean (Methylation %)</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.67</td>
<td>2.56</td>
<td>3.97 - 19.34</td>
</tr>
<tr>
<td>2</td>
<td>4.21</td>
<td>1.58</td>
<td>1.43 - 11.65</td>
</tr>
<tr>
<td>3</td>
<td>7.55</td>
<td>2.58</td>
<td>4.63 - 19.66</td>
</tr>
<tr>
<td>4</td>
<td>4.21</td>
<td>1.48</td>
<td>2.40 - 9.78</td>
</tr>
<tr>
<td>5</td>
<td>6.98</td>
<td>2.00</td>
<td>4.44 - 18.07</td>
</tr>
<tr>
<td>6</td>
<td>4.08</td>
<td>1.34</td>
<td>1.97 - 9.50</td>
</tr>
<tr>
<td>7</td>
<td>6.75</td>
<td>1.97</td>
<td>2.47 - 13.74</td>
</tr>
<tr>
<td>8</td>
<td>5.62</td>
<td>1.37</td>
<td>3.73 - 9.80</td>
</tr>
<tr>
<td>9</td>
<td>7.73</td>
<td>2.01</td>
<td>3.79 - 15.88</td>
</tr>
<tr>
<td>10</td>
<td>9.71</td>
<td>2.50</td>
<td>5.94 - 23.10</td>
</tr>
<tr>
<td>1 - 10 (mean)</td>
<td>6.55</td>
<td>1.52</td>
<td>4.15 - 12.74</td>
</tr>
</tbody>
</table>

Note: N = 80 infants.

Figure 4.12 Relationship between mean CpG SLC6A4 methylation at birth and salivary cortisol concentrations across the lab visit and evening in 5 - 7 year old children. Neonatal methylation of the CpG mean was associated with different cortisol stress response across the lab visit, whereby higher mean methylation was associated with a blunted cortisol stress response, compared to lower mean methylation, adjusting for age, sex, SRI exposure, time between Waking and Baseline, and maternal anxiety scores (HAM A) in the 3rd trimester and at 5 - 7 years (F= 4.177, p = 0.019, η² =0.092). CpG Methylation was evaluated as continuous variable and defined by a median split in Figure 4.12. Values shown as mean ± SEM. N = 64 children.
Table 4.6 The association between *SLC6A4* methylation at birth and cortisol reactivity at 5 - 7 years of age

<table>
<thead>
<tr>
<th>CpG Sites</th>
<th>Cortisol measures</th>
<th>Cortisol change scores and AUC&lt;sub&gt;G&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>Stress 1</td>
<td>Stress 2</td>
</tr>
<tr>
<td>1</td>
<td>p</td>
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</tr>
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<td></td>
<td>β</td>
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</tr>
<tr>
<td></td>
<td>η&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>2</td>
<td>p</td>
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<td>β</td>
<td>-0.457</td>
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<tr>
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<td>η&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.186</td>
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<td>β</td>
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<td>-0.372</td>
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<tr>
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<td>η&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.135</td>
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<td>8</td>
<td>p</td>
<td>0.000</td>
</tr>
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<td></td>
<td>β</td>
<td>-0.592</td>
</tr>
<tr>
<td></td>
<td>η&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>9</td>
<td>p</td>
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<td></td>
<td>β</td>
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<td>η&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>η&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.222</td>
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</table>

*Note:* General linear models adjusted for age, sex, time between Waking and Baseline, SRI exposure, and maternal anxiety scores in the 3<sup>rd</sup> trimester and at 5 - 7 years. CpG site 10 was not associated with cortisol stress measures. AUC<sub>G</sub>: Area under the curve with respect to ground. p: P-value; β: Standardized Beta; η<sup>2</sup>: Partial eta squared.
4.3 Aim 2 Discussion

Aim 2A evaluated the relationship between prenatal exposure to SRIs/3rd trimester maternal depressed/anxious mood on the methylation status of \( NR3C1 \, 1_F \), \( NR3C1 \, 1_D \), and \( SLC6A4 \) promoter regions at 5 - 7 years of age. Studies have shown that early environments have the potential to shape the structure and function of the developing brain, and may be associated with long term or permanent effects on subsequent child and adult physiology, behaviour and health.\(^{189,245}\) Furthermore, the methylation status of both \( NR3C1 \, 1_F \) and \( SLC6A4 \) in newborns was shown to be sensitive to prenatal maternal mood.\(^{52,202}\) Aim 2B and 2C evaluated the relationship between the methylation status of \( NR3C1 \, 1_F \) and \( SLC6A4 \) in cord blood DNA and \( NR3C1 \, 1_F \), \( NR3C1 \, 1_D \), and \( SLC6A4 \) in 5 - 7 year buccal epithelial DNA on cortisol patterns at 5 - 7 years of age.

4.3.1 \( NR3C1 \) Methylation and Cortisol Patterns at 5 - 7 Years

Children exposed to higher 3rd trimester and concurrent maternal anxious mood displayed lower \( NR3C1 \, 1_F \) CpG 2 methylation in buccal cells at 5 - 7 years of age, adjusting for age, sex, and prenatal SRI exposure. In contrast, children exposed to higher 3rd trimester maternal anxious mood alone exhibited higher CpG 2 methylation. Interestingly, although the range of children’s age in this study was small (5.11 – 7.72 years), age was associated with the methylation status at CpG 5 and 6 - 13 mean of \( NR3C1 \, 1_F \), whereby increasing age was associated with lower methylation levels.

There is increasing evidence for involvement altered DNA methylation patterns as a mechanism for short and long-term status of GR expression and glucocorticoid-feedback sensitivity.\(^{144,246}\) GR levels are transcriptionally controlled by multiple untranslated alternative
first exons, and each exon has its own promoter providing a mechanism for tissue-specific 
NR3C1 expression. In rats, Meaney and colleagues have shown that maternal behaviour can lead to 
epigenetic variations in Nr3c1 expression and GR function. Ngfi-a regulates the expression of 
the Nr3c1 17 promoter in rats, and patch methylated NR3C1 1F promoter constructs that 
mimicked the methylation status in human hippocampal tissue from abused suicide victims, was 
shown to alter NGFI-A binding and induced transcription. Although I observed differential 
methylation along neighboring CpG sites of the NGFI-A binding site, CpG 3 and 4 methylation 
status at 5-7 years appeared to be unaffected by prenatal maternal mood and SRI exposure.

An association between early environmental exposures and methylation at the NGFI-A 
binding site has been observed in other tissues, apart from buccal DNA. For example, 3rd 
trimester maternal mood was associated with higher DNA methylation in cord blood at CpG 3 
(NGFI-A consensus-binding site) of the exon 1F promoter. Higher DNA methylation at the 
NGFI-A binding site in hippocampal brain tissue was also found in suicide victims exposed to 
child abuse. Additionally, maternal cortisol concentrations in the 2nd trimester were positively 
associated with the methylation state of CpG 4 in the NR3C1 exon 1F promoter (equivalent to 
CpG F38.39 in Hompes et al) in cord blood mononuclear cells. A study in adults found 
greater methylation in leukocytes at CpG sites 1 and 3 were associated with childhood adversity 
and maltreatment, while a trend for an effect of childhood adversity measure was found with 
CpG 5 methylation, and no correlation was found with methylation of CpG 2, 4 and 6. In 
contrast, Moser et al found that the NGFI-A binding site (CpG 3 and 4 in this current study) 
was unmethylated in post-mortem hippocampal specimens from the 32 subjects with various 
psychiatric disorders. Interestingly, CpG 9 (CpG 6 in this study), located in NR3C1 exon 1F, was
highly methylated in all subjects. However, given no information on early environment was provided and subjects were older and suffered from disorders including Parkinson’s Disease and dementia, these findings may not reflect the epigenetic landscape of the NR3CI exon 1_F promoter for all individuals. Similarly, Alt et al showed the 1_F promoter to be uniformly unmethylated in brain regions from adults with major depressive disorder and controls.

An interaction between pre- and postnatal maternal mood showed children exposed to higher 3rd trimester and concurrent maternal anxious mood displayed lower NR3CI 1_F CpG 2 methylation, compared to children exposed to higher 3rd trimester and lower concurrent maternal anxious mood. Given NR3CI 1_F methylation has been negatively associated with GR expression, chronic exposure to maternal anxiety (i.e. pre- and postnatal) may influence GR expression and cortisol regulation. However, adding to the variability of previous findings, these outcomes may highlight the tissue, disease, and species dependent specificity of 1_F promoter methylation. Thus, experimental confirmation is required to determine a role for DNA methylation in transcriptional regulation of the human NR3CI gene.

Exon 1_D shows a high degree of similarity with the rat homologue exon 1_10, and was found to represent at least 50% of total Nr3c1 transcripts in rats. In humans, higher scores on a questionnaire regarding antenatal anxiety was positively associated with the methylation state of the NR3CI 1_D promoter region in cord blood mononuclear cells, while 1st trimester maternal cortisol concentrations were negatively associated with a CpG site in the 1_D promoter. Multiple transcription factors bind various regions of the GR promoter, and studies have suggested that Ying Yang 1 (YY1) and GR itself may regulate this region. Specifically, three transcription factor binding sites for YY1 are located 114, 158, and 330 base pairs upstream of the 1_D promoter. However it is not established if these elements control the
transcription starting at exon 1D. Although I expanded the analysis to regions outside of the NR3C1 1F promoter, I did not see an effect of prenatal maternal mood or SRI exposure on methylation status of the 1D promoter in early childhood.

The Diurnal Rhythm

At 5 - 7 years of age, higher mean methylation of NR3C1 1F CpGs 6 - 13 was associated with higher Waking and Wake + 20 minute cortisol concentrations, and a greater AUCG, indicating total cortisol output over the day. These findings remained significant when controlling for age, sex, and maternal mood scores in the 3rd trimester and at 5 - 7 years.

Consistent findings were found with the NR3C1 1D promoter methylation status. Specifically, higher CpG 4 methylation was associated with a lower Waking cortisol and a greater increase in cortisol between Waking and Wake + 20 minutes, and higher CpG 2 methylation was associated with a smaller decline in cortisol from Waking to Evening. These findings suggest that after waking up in the morning, children with higher levels of methylation exhibited a greater increase in cortisol concentrations, which did not fall over the day to the same extent as children with lower methylation levels. Together these findings suggest that higher methylation in two NR3C1 promoter regions is associated with a higher and flattened diurnal cortisol rhythm at 5 - 7 years of age.

A flatter diurnal slope in 15 year olds and a high, flattened daytime cortisol profile in 14-15 year olds was associated with prenatal anxiety and depression. Although no relationship was found between prenatal maternal mood and SRI exposure with measures of the diurnal rhythm at 5 - 7 years, an association between variable methylation patterns in the NR3C1 exon 1F
and 1D promoter region and basal cortisol measures suggests that epigenetic mechanisms may be associated with the capacity to regulate the diurnal cortisol rhythm.

The Cortisol Stress Response

Higher NR3C1 1F CpG 3 methylation was associated with lower Baseline salivary cortisol concentrations and an increased cortisol change between Baseline and Stress 2. Additionally, lower cortisol concentrations at Stress 1, and greater change in cortisol between Stress 1 and Stress 2 were associated with higher NR3C1 1F CpG 1 methylation levels at 5 - 7 years. In general, given higher methylation of CpGs 1 and 3 of the NR3C1 1F promoter was associated lower cortisol concentrations at Stress 1, but a larger increase between Baseline and Stress 2, and Stress 1 and Stress 2, these findings indicate children with higher methylation levels displayed lower cortisol concentrations and a delayed stress response suggesting a reduced ability to regulate cortisol following a stressor. Given that increased methylation of the NR3C1 1F promoter has been associated with decreased GR mRNA levels, a reduced ability to respond to a stressor may reflect lower GR expression and an altered HPA negative feedback mechanism. However, GR expression was not quantified in these subjects so I cannot determine the influence of methylation at this point.

In a study evaluating NR3C1 1F promoter methylation status in adults exposed to childhood maltreatment or adversity, no association was found between methylation of CpG sites 1, 3, and 4 and cortisol responses to a dexamethasone/CRH test, while an attenuated cortisol response was associated with higher methylation of CpG 5 and the mean of CpGs 7 - 13 leukocytes. Tyrka et al found only CpG 5 methylation to be associated with both childhood adversity and HPA reactivity. Building on previous findings showing an association between
higher CpG 3 methylation at birth and both higher 3rd trimester maternal depressed mood and stress reactivity at 3 months,202 I have shown higher CpG 3 methylation at 5 - 7 years is associated with a diminished, delayed stress response, suggesting a sustained programming effect at the NGFI-A binding site (CpG 3).

In contrast with exon 1F, a lower Baseline cortisol concentration and AUCG was associated with lower methylation levels of exon 1D CpGs 5, 6, 7, 8 and the mean. Furthermore, lower methylation at CpGs 5, 6, and 7 was associated with lower cortisol at Stress 1, and lower cortisol concentrations at Stress 2 were associated with higher CpG 6 and 7 methylation. Given the same physiological cortisol response was displayed, yet opposing methylation patterns were shown in exon 1F and 1D, this finding highlights the tissue specific GR expression of alternative first exons.192

In addition, McEwen et al250 suggests that a chronic stress exposure may initially involve hyperactivation of HPA activity, but in response to an excessive and prolonged stress exposure a hyporeactive response may result from a counter-regulatory adaptation state. I found a blunted stress response was associated with exposure to higher 3rd trimester and 5 - 7 year maternal anxious mood, suggesting a chronic exposure to high levels of maternal anxious mood; it is possible a potential shift in reactivity may explain these opposing findings. Furthermore, no associations were found between NR3C1 1F methylation at birth and cortisol patterns at 5 - 7 years of age.

4.3.2 SLC6A4 Methylation and Cortisol Patterns at 5 - 7 Years

A significant interaction between 3rd trimester and 5 - 7 year maternal anxious mood was associated with CpG 6, 8, and 9 methylation levels. Specifically, children of mothers with
higher 3\textsuperscript{rd} trimester and 5 - 7 maternal anxious mood exhibited lower \textit{SLC6A4} methylation levels, compared to children only exposed to higher 3\textsuperscript{rd} trimester maternal anxious mood, displaying higher methylation levels. As mentioned previously, although this interaction was between maternal mood scores collected 5 - 7 years apart, increased maternal anxious mood in the 3\textsuperscript{rd} trimester and at 5 - 7 years may indicate chronic exposure to maternal mood disturbances between birth and early childhood. Interestingly, a study evaluating \textit{SLC6A4} methylation in buccal DNA at 5 and 10 years of age, found children who were victims of bullying between these time points showed increased methylation at 10 years of age, compared to non-bullied children, suggesting that epigenetic processes are responsive to social environments in early childhood.\textsuperscript{251} This finding supports the capability of 5 - 7 year maternal mood to moderate an association between prenatal maternal mood and \textit{SLC6A4} methylation in early childhood.

Higher maternal depressed mood during the 2\textsuperscript{nd} trimester of pregnancy and reduced methylation at CpG 6 and 9 in the \textit{SLC6A4} promoter was observed in newborn cord blood leukocytes.\textsuperscript{52} In addition, higher methylation of the \textit{SLC6A4} promoter was associated with a greater vulnerability to major depression and mood disorders.\textsuperscript{183}

Interestingly, a much larger region of the \textit{SLC6A4} containing 81 CpG sites was quantified in a lymphoblast cell line, and methylation of 4 CpG residues was associated with SLC6A4 mRNA levels\textsuperscript{252}, with CpG 8 from this current study being one of them (bp 872 in Philibert \textit{et al}.\textsuperscript{252}). \textit{In vitro} methylation of the promoter in luciferase reporter constructs was found to suppress \textit{SLC6A4} transcriptional activity, demonstrating a functional role of methylation on \textit{SLC6A4} gene expression.\textsuperscript{253} Using a program to determine potential transcription factors that may interact with certain CpG sites, Wang \textit{et al}.\textsuperscript{253} found the \textit{SLC6A4} promoter region to be enriched with Sp1 binding sites and other transcription factors, such as ER, GR, Krox-20, RXR-
beta, and RAP1. Additionally, several other potential binding sites in the promoter include a TATA-like motif and cAMP response element (CRE). Given these findings, increased SLC6A4 expression as a function of reduced SLC6A4 methylation may result in increased 5-HT re-uptake and lower intrasynaptic 5-HT. Together these findings suggest that methylation of the SLC6A4 promoter may be an important regulator of serotonergic function and may provide an underlying mechanism for early life exposures on long-term developmental outcomes.

Significantly higher SLC6A4 methylation was observed in T cells and monocytes from individuals with a history of aggression during childhood. Using positron emission technology, Wang et al. found higher mean methylation of SLC6A4 at specific CpG sites was associated with lower in vivo brain 5-HT synthesis, demonstrating an association between peripheral measures of DNA methylation and central 5-HT synthesis. Furthermore, Riese et al. evaluated the methylation status of 69 CpGs in the SLC6A4 promoter in peripheral blood leukocytes and amygdala tissue from an adult female donors, and found 9 CpGs were largely correlated between tissues, with CpG 10 in this study being one of these sites. Together these studies provide evidence for peripheral SLC6A4 methylation status as a potential biomarker of central function.

The Diurnal Rhythm

Our understanding of the role of SLC6A4 methylation linking early life with changes in cortisol regulation is only just emerging. To my knowledge, this is the first study to date investigating the links between SLCA64 methylation and cortisol regulation in children as a function of prenatal exposure to SRIs and maternal depressed/anxious mood, controlling for concurrent maternal mood.
At 5 - 7 years of age, methylation status of CpG 2 and 10 in *SLC6A4* promoter methylation were associated with diurnal cortisol across the day. Interestingly, lower methylation at 5 - 7 years in the *SLC6A4* promoter was associated with a typical diurnal pattern defined by an higher CAR and steeper slope across the day. These findings contrast with the association observed between higher *SLC6A4* methylation and dysregulated cortisol responses to stress.

In rats, Vazquez *et al.* observed administration of glucocorticoids during the early postnatal period altered adrenocortical responses to stress, however, basal corticosterone and mineralcorticoid mRNA expression did not differ from the control group. Since glucocorticoids act through high-affinity MRs and lower affinity GRs, MRs are thought to play a significant role in HPA function when cortisol concentrations are low. Furthermore, in an animal model, hippocampal MR mRNA exhibited a significant diurnal rhythm, while a lack of diurnal rhythm of GR mRNA was found in the paraventricular nucleus (PVN) of the hypothalamus. This finding led Herman *et al.* to propose diurnal fluctuations may be more central to MR regulation, while PVN GRs may be more related to stress regulation. Thus, it is possible early adverse environments may only display unfavorable outcomes when cortisol demands rise above basal functioning levels. These findings may explain the discordance between *SLC6A4* methylation at 5 – 7 years with diurnal patterns and stress responses, and may be why we observed an association with alterations in the stress response and not the diurnal rhythm.

*The Cortisol Stress Response*

Higher methylation of CpGs 4 and 10 of the *SLC6A4* promoter at 5 - 7 years of age was associated with smaller change scores between Baseline and Stress 1, and higher methylation levels at CpG sites 4, 7, and 10 was associated with reduced change in cortisol between Baseline
and Stress 2. Together these findings suggest that higher methylation levels of the $SLC6A4$ promoter at 5 - 7 years is associated with dysregulated cortisol stress responses. Similar findings were shown in 12-year-old children displaying higher levels of $SLC6A4$ methylation and lower cortisol responses to a laboratory stressor.\textsuperscript{251}

Importantly, a highly significant association was observed between $SLC6A4$ methylation at birth and cortisol stress responses in early childhood. A main effect of methylation levels at CpGs 1, 4, 6, 7, 8, and the mean at birth revealed higher $SLC6A4$ methylation was associated with a flatter cortisol slope throughout the stress challenge and into the evening. In particular, higher methylation levels of all CpG sites analyzed at birth, except CpG 10, were associated with lower cortisol concentrations at Stress 1 and 2, a flatter slope between Stress 1 and 2 with Evening cortisol, and a reduced AUC\textsubscript{G}. This relationship remained significant after controlling for age, sex, time between Waking and Baseline, and maternal mood scores in the 3\textsuperscript{rd} trimester and at 5 - 7 years. In addition, prenatal SRI exposure displayed a main effect in all these analyses. These findings suggest that the fetal environment may have rendered some children able to prompt an appropriate stress response, and others not. Given 5-HT transmission may be regulated by both $SLC6A4$ methylation status and SRI exposure, altered 5-HT levels may be the underlying factor driving this observed programming effect. Furthermore, $SLC6A4$ methylation at birth may be a possible marker of early exposure to a stressful event that could alter long-term cortisol stress responses.

Importantly, serotonin has been shown to modulate the activity of the HPA axis.\textsuperscript{258} Prenatal SRI exposure elevates early 5-HT levels and may influence neurodevelopmental processes and decrease signaling later in life.\textsuperscript{122} Evidence from animal studies show that an altered or complete loss of 5-HTT function results in neurobehavioural alterations and
behavioural changes in response to stress. For instance, Slc6a4 -/- mice exhibit increased intrasynaptic 5-HT and depression and anxiety related behaviours. In a rodent model, the effects of maternal care on hippocampal GR expression are mediated by increased serotonergic neurotransmission and expression of the NGFI-A transcription factor. Two CpG sites contained in the NGFI-A consensus sequence of Nr3c1 17 are potential targets of DNA methylation, rendering them susceptible to early adverse environments. Liu et al. found a strong correlation between the magnitude of the HPA stress response in adult offspring and maternal care, suggesting that the effects of early life environment on the developing HPA regulatory system reflects a time of plasticity when factors such as maternal care are able to alter the trajectory of stress responses later in life. Additionally, administration of a serotonin receptor agonist was shown to block the effects of postnatal handling on GR expression further confirming 5-HTs critical role in mediating environmental effects on the development of GR binding in the hippocampus.

Given higher SLC6A4 methylation was associated with lower SLC6A4 expression and a subsequent rise in 5-HT neurotransmission, the blunted stress response at 5 - 7 years observed in association with higher methylation may appear as a contrasting observation. However, increased 5-HT levels have also showed altered fetal neurobehavioural development. Modifications of SLC6A4 promoter methylation status has been associated with early-life stress, childhood adversities, an increased risk for depression and stress-related disorders later in life. Although adverse early environments are associated with both higher and lower methylation levels, these alterations are thought to affect HPA reactivity through disruption of 5-HT transmission. Serotonin not only acts as a modulatory neurotransmitter in the mature brain, but also acts as a trophic factor during early developmental periods.
Furthermore, prenatal SRI exposure elevates early 5-HT levels that may influence neurodevelopmental processes and decrease 5-HT signaling later in life.\textsuperscript{122} In this way, elevated 5-HT levels in response to higher SLC6A4 methylation at birth and SRI exposure during a critical window of neurodevelopment may have the potential to shape a pathway of health or disease across the lifespan.

Overall, healthy brain development is dependent on finely tuned 5-HT levels,\textsuperscript{94} and the formation of pathways towards health or disease begins long before birth.\textsuperscript{87} The association found between SLC6A4 methylation at birth and cortisol regulation at 5 - 7 years suggests that epigenetic regulation of the serotonergic pathway may exert its main effects on biological function during early development. Although these findings are a first step towards understanding how SLC6A4 promoter methylation may play a role linking early environment with measures of HPA regulation in childhood, further studies are required to characterize precise mechanisms mediating the association between the HPA and serotonergic regulatory systems.
Chapter 5: AIM 3: 5-HTTLPR GENOTYPE AND CORTISOL PATTERNS AT 5 - 7 YEARS OF AGE

The 5-HTTLPR variant located in the promoter region of SLC6A4 moderates the effects of early life stress and vulnerability to mood disorders, and variable cortisol responses have been associated with 5-HTTLPR alleles. In addition, SLC6A4 promoter methylation plays a role in governing mRNA expression; but this is dependent on the 5-HTTLPR genotype.

Therefore, for Aim 3 I investigated whether 5-HTTLPR genotype and SLC6A4 methylation at 5 – 7 years converge to influence variable cortisol patterns in children with prenatal SRI exposure and higher 3rd trimester maternal mood scores. The following sub-aims were addressed in Aim 3 (See Figure 5.1 for schematic representation):

Aim 3A: To determine the relationship between children’s 5-HTTLPR genotype and both the diurnal rhythm and cortisol stress response in early childhood.

Aim 3B: To determine the relationship between children’s 5-HTTLPR genotype and SLC6A4 methylation status, and whether an association between genotype and methylation is related to both the diurnal rhythm and cortisol stress response in early childhood.
Figure 5.1 Sub-aims addressed in Aim 3.
5.1 **Aim 3 Methods**

5.1.1 **Aim 3A: 5-HTTLPR Genotype and Cortisol Patterns at 5 - 7 Years**

Analyses of diurnal rhythm included 93 children with genotypes: l/l (N = 27), l/s (N = 41), or s/s (N = 15). Analyses of the cortisol stress response included 60 children with genotypes: l/l (N = 22), l/s (N = 29), or s/s (N = 9). **Table 5.1** displays descriptive child and maternal information, and saliva sample collection times defined by 5-HTTLPR genotype.

Repeated measures analysis and general linear models assessed the relationship between 5-HTTLPR genotype and diurnal and stress response cortisol measures, with genotype as a fixed factor, and age, sex, prenatal SRI exposure, and 3rd trimester/5 - 7 year maternal mood scores (anxiety and depression) as covariates.

5.1.2 **Aim 3B: 5-HTTLPR Genotype, SLC6A4 methylation, and Cortisol Patterns at 5 - 7 Years**

Analyses of 5-HTTLPR genotype and SLC6A4 methylation included 103 children with genotypes: l/l (N = 38), l/s (N = 47), or s/s (N = 18). A multivariate regression model was used to evaluate the association between 5-HTTLPR genotype and children’s SLC6A4 methylation status. These analyses included prenatal SRI exposure as a fixed factor, and age, sex, and maternal mood scores in 3rd trimester and at 5 - 7 years as covariates.

Corresponding to general linear models used to assess diurnal patterns and cortisol stress responses in Aim 2A and 2B, an interaction term between SLC6A4 methylation at specific CpG sites and 5-HTTLPR genotype was evaluated in association with cortisol measures.
Table 5.1 Children’s 5-HTTLPR genotype and descriptive information of children and their mothers and cortisol collection times

<table>
<thead>
<tr>
<th>Child 5-HTTLPR Genotype</th>
<th>( l/l ) (n = 37)</th>
<th>( l/s ) (n = 41)</th>
<th>( s/s ) (n = 15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diurnal Rhythm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal anxiety (HAM-A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>7.50 ± 5.55</td>
<td>7.35 ± 5.89</td>
<td>10.93 ± 6.03</td>
<td>0.106</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>948 ± 7.02</td>
<td>7.30 ± 6.33</td>
<td>8.00 ± 4.62</td>
<td>0.237</td>
</tr>
<tr>
<td>Maternal depression (HAM-D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>6.12 ± 5.05</td>
<td>5.73 ± 5.76</td>
<td>8.53 ± 5.83</td>
<td>0.361</td>
</tr>
<tr>
<td>5 - 7 years</td>
<td>10.09 ± 7.51</td>
<td>7.35 ± 6.39</td>
<td>9.23 ± 6.35</td>
<td>0.245</td>
</tr>
<tr>
<td>Childs age, years (SD)</td>
<td>5.97 ± 0.58</td>
<td>6.00 ± 0.51</td>
<td>6.23 ± 0.81</td>
<td>0.352</td>
</tr>
<tr>
<td>Prenatal SRI exposure, n (%)</td>
<td>17 (45.9)</td>
<td>14 (34.1)</td>
<td>6 (40.4)</td>
<td>0.568</td>
</tr>
<tr>
<td>Sex, % M/F</td>
<td>37.8/62.2</td>
<td>36.6/63.4</td>
<td>53.3/46.7</td>
<td>0.501</td>
</tr>
<tr>
<td><strong>Cortisol collection time (hh:mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>7:33 ± 0:59</td>
<td>7:39 ± 0:51</td>
<td>7:55 ± 1:13</td>
<td>0.443</td>
</tr>
<tr>
<td>Wake + 20 min</td>
<td>7:57 ± 1:01</td>
<td>8:04 ± 1:01</td>
<td>8:07 ± 0:58</td>
<td>0.814</td>
</tr>
<tr>
<td>Evening</td>
<td>18:36 ± 0:57</td>
<td>18:49 ± 0:55</td>
<td>18:57 ± 1:09</td>
<td>0.407</td>
</tr>
<tr>
<td>Stress Reactivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cortisol collection time (hh:mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>7:41 ± 1:04</td>
<td>7:33 ± 0:42</td>
<td>7:54 ± 0:46</td>
<td>0.447</td>
</tr>
<tr>
<td>Baseline</td>
<td>10:42 ± 0:58</td>
<td>10:45 ± 1:07</td>
<td>11:23 ± 1:22</td>
<td>0.410</td>
</tr>
<tr>
<td>Stress 1</td>
<td>12:07 ± 1:06</td>
<td>12:18 ± 1:15</td>
<td>12:43 ± 1:53</td>
<td>0.358</td>
</tr>
<tr>
<td>Stress 2</td>
<td>12:30 ± 1:08</td>
<td>12:40 ± 1:26</td>
<td>1:26 ± 12:59</td>
<td>0.577</td>
</tr>
<tr>
<td>Evening</td>
<td>18:38 ± 1:05</td>
<td>18:40 ± 1:18</td>
<td>18:28 ± 1:23</td>
<td>0.893</td>
</tr>
</tbody>
</table>

*Note:* Continuous values are displayed as mean ± standard deviation (SD). Groups were compared using one-way ANOVAS for continuous variables, and Chi-Square tests for categorical variables. Collection times were analyzed using decimal time and converted to hh:mm in table. N’s vary across maternal mood scores because of missing data.
5.2 Aim 3 Results

5.2.1 Aim 3A: 5-HTTLPR Genotype and Cortisol Patterns at 5 - 7 Years

Genotype frequencies for the 5-HTTLPR variant were 39.78% l/l, 44.1% l/s, 16.1% s/s, similar to previous reports. Table 5.2 displays children’s cortisol concentrations for the diurnal rhythm and cortisol stress response.

Children’s 5-HTTLPR genotype was not associated with the diurnal cortisol rhythm across the day or with diurnal cortisol patterns at individual time points or cortisol change scores (p > 0.05), adjusting for age, sex, prenatal SRI exposure, and 3rd trimester/5 - 7 year maternal mood scores (anxiety and depression).

Furthermore, Children’s 5-HTTLPR genotype was not associated with cortisol stress responses across the lab visit and evening (p > 0.05), and no associations were observed between 5-HTTLPR genotype and cortisol concentrations at specific time points or cortisol change scores (p > 0.05).

5.2.2 Aim 3B: 5-HTTLPR Genotype, SLC6A4 Methylation Status, and Cortisol Patterns at 5 - 7 Years

The methylation status of the SLC6A4 promoter was not associated with 5-HTTLPR genotype (p > 0.05). No relationships were observed between SLC6A4 methylation and 5-HTTLPR genotype on diurnal cortisol patterns and cortisol stress response at 5 - 7 years of age (p > 0.05).
<table>
<thead>
<tr>
<th>Table 5.2</th>
<th>Children’s 5-HTTLPR genotype and salivary cortisol concentrations at 5 - 7 years of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child 5-HTTLPR Genotype</td>
<td></td>
</tr>
<tr>
<td><strong>Diurnal Rhythm</strong></td>
<td><strong>I/I (n= 37)</strong></td>
</tr>
<tr>
<td><strong>Cortisol concentration (nmol/L)</strong></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>14.29 ± 5.13</td>
</tr>
<tr>
<td>Wake + 20 min</td>
<td>14.31 ± 6.60</td>
</tr>
<tr>
<td>Evening</td>
<td>3.72 ± 3.65</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>102.45 ± 39.67</td>
</tr>
<tr>
<td><strong>Stress Reactivity</strong></td>
<td><strong>I/I (n= 22)</strong></td>
</tr>
<tr>
<td><strong>Cortisol Concentration (nmol/L)</strong></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.03 ± 2.12</td>
</tr>
<tr>
<td>Stress 1</td>
<td>6.09 ± 3.52</td>
</tr>
<tr>
<td>Stress 2</td>
<td>5.95 ± 1.48</td>
</tr>
<tr>
<td>Evening</td>
<td>3.01 ± 5.10</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>37.63± 19.28</td>
</tr>
</tbody>
</table>

*Note: Values are displayed as mean ± standard deviation (SD). Groups were compared using one-way ANOVA t-tests with log transformed cortisol concentrations.*
5.3 Aim 3 Discussion

The 5-HTTLPR genotype moderates the relationship between stress and depression. A 44 bp insertion/deletion polymorphism has been found to influence SLC6A4 transcription, with the short (s) allele less transcriptionally efficient than the long (l) allele. 

However, recent discordant meta-analyses found either no relationship or a relationship for the moderating role of 5-HTTLPR genotype between stress and depression. Additionally, imaging studies in adult human brains have found no association between 5-HTTLPR and 5-HTT expression, suggesting that other mechanisms are involved in the association between serotonergic and HPA systems, or the 5-HTTLPR variant may exert its main effects during early development. In the latter suggestion, SLC6A4 genotype may therefore function as a key moderator of early life environment and prenatal SRI exposure by influencing additional long-term risk or resilience through altered 5-HT availability.

The association between 5-HTTLPR genotype and HPA reactivity has been evaluated by several studies. In response to a laboratory stressor, higher and prolonged cortisol responses were seen in girls and young adults with an s/s genotype, compared to individuals with an l/s or l/l genotype. In contrast, no association between 5-HTTLPR genotype and cortisol stress response was found in a group of adolescents and young adults. Similarly, I found no association between 5-HTTLPR genotype with either diurnal cortisol or stress reactivity in 5 - 7 year old children. A study evaluating children and both younger and older adults, found no association between genotype and cortisol responses in the 8-11 year old children, suggesting that age may be a critical variable in 5-HTTLPR mediated gene-environment interactions. Given that age was significantly associated with diurnal cortisol in my analysis, it would be interesting to re-evaluate the children as they reach adolescence. Wankerl et al found a
significant interaction between sex and 5-HTTLPR on mean diurnal cortisol concentrations in males, but not females. However, with restraints of a smaller sample size, I did not explore an interaction of this type.

SLC6A4 methylation may be a source of these conflicting findings, as methylation status was shown to affect mRNA transcription and may be altered in result of early adverse environments. A study using 49 lymphoblast cell lines found the methylation status of 81 CpG sites located within a CpG island in the SLC6A4 promoter was associated with mRNA transcription, and the extent of methylation was dependent on 5-HTTLPR genotype. A follow-up study with 192 lymphoblast cell lines confirmed these findings, which lead Philibert et al to suggest that counteracting effects of methylation with 5-HTTLPR genotype may be an adaptive mechanism to maintain a desired level of gene transcription. At 5 - 7 years of age, I did not find an association between methylation status of the SLC6A4 promoter and 5-HTTLPR genotype. However, I examined 10 CpG sites representing a portion of the region evaluated by Philibert et al, and SLC6A4 mRNA levels were not quantified. Similarly, in 24 year old adults, Olsson et al found no association between depressive symptoms during adolescence and SLC6A4 methylation in buccal DNA or 5-HTTLPR genotype.

In this current aim, neither diurnal cortisol measures nor stress responses in 5 - 7 year-old children were associated with 5-HTTLPR genotypes, and 5-HTTLPR genotype was not associated with SLC6A4 methylation. Given SLC6A4 methylation status was associated with children’s cortisol, these findings provide evidence that methylation is regulated by complex factors which may not be associated with 5-HTTLPR genotype.

The association with stressful life events, 5-HTTLPR genotype, and depression is a well-documented finding. However, it was proposed that an interaction between 5-HTTLPR and
environment may not directly be associated with depression, but rather reflect the involvement of 5-HT with the human stress response. Further studies are required to identify specific mechanisms by which 5-HTT levels regulate HPA function.
Chapter 6: GENERAL DISCUSSION

6.1 Discussion of Results and Concluding Remarks

Our capacity to regulate neuroendocrine responses to stressful events via the HPA axis is of particular importance to setting pathways to mental health and illness across the life span. Because altered stress reactivity is associated with an increased risk of mood disorders, including anxiety and depression, it is important to understanding how early life experiences influence the development of HPA stress reactivity.

Prenatal exposure to maternal depression and anxiety may influence the developing HPA system and increase vulnerability to psychopathology later in life. In this study, I found blunted cortisol stress responses in 5 - 7 year old children was associated with prenatal exposure to SRIs and higher 3rd trimester maternal anxious/depressed mood, compared to children with no exposure to SRIs and mothers with lower levels of 3rd trimester depressed and anxious mood. This result corroborates earlier findings showing a flattened daytime cortisol profile and diminished cortisol-awakening response in 15 year olds exposed to antenatal maternal anxiety. Previous studies support the association between in utero exposure to maternal mood and altered cortisol reactivity in childhood and adolescence, however, it remains unclear how childhood environment and social relationships modify the consequences of early adverse environments. Thus, inconsistencies between the association of early life exposures and the degree and direction HPA reactivity in childhood and adolescence may be attributed to whether studies accounted for concurrent maternal mood.

In this study, children with prenatal exposure to SRIs and higher maternal depressed/anxious mood (3rd trimester and at 5 - 7 years) displayed a blunted cortisol stress response, compared to children exposed only to higher 3rd trimester or concurrent maternal
mood. Previously, higher afternoon salivary cortisol concentrations were shown in 4.5-year-old children exposed to maternal stress during infancy and concurrently compared to children exposed only to higher maternal stress in childhood. Together these findings suggest that early life adversity associated with maternal mood disturbances during and following pregnancy may elicit an altered sensitization to subsequent life stressors, while current environmental conditions may further provoke or repress an altered stress response.

Contrasting results between early adverse environments and both hypo- and hyperreactive stress responses highlights the complexity of alterations in HPA function. These findings suggest that it is dysregulation in stress responses, rather than simply elevated reactivity, that may increase susceptibility to stress-related mood disorders. Blunted stress reactivity may reflect an adaptive stress response as a consequence of down-regulation of HPA reactivity following chronic stress exposure. However, decreased sensitivity for initiating a stress response may induce long-term effects on neural circuits regulating stress, emotion, reactivity and behaviour.

The association between early life stress and the subsequent development of mood disorders may be mediated by changes in 5-HT neurotransmission and the HPA systems coordination of behavioral, autonomic, immune, and endocrine components implicated in the regulation of the stress response. In response to early-life adversity, rodent and non-human primates show increased neurophysiological and HPA axis vulnerability, and alterations in cellular processes involving neural circuit formation. In humans, prenatal maternal mood and SRIs affect central fetal 5-HT concentrations involved in regulating the development of serotonergic neurons and target tissues that may influence the developing HPA axis. Maternal depression during pregnancy has been associated with higher maternal cortisol concentrations in
urine, and increased fetal exposure to cortisol may be a key mechanism linking an adverse *in utero* environment with an altered HPA activity in offspring.

Epigenetic modifications to DNA may provide a mechanism linking the dynamic interaction between early life experiences, genetic variations and activity, and risk for psychopathology. During the prenatal and early postnatal period, environmental signals may ‘get under the skin’ and result in epigenetic changes. If these are accompanied by altered gene expression they could change the trajectory of fetal brain development with long-term phenotypic consequences.

There is considerable experimental evidence illustrating an association between early life stress and epigenetic changes in animal models. In rodents, the effects of early maternal care on hippocampal GR expression and the HPA stress response in adult offspring was associated with epigenetic modifications where NGFI-A binds the the *Nr3c1* promoter. Hypermethylation of the NGFI-A binding site in response to low maternal care decreased NGFI-A binding and reduced GR expression and efficiency of glucocorticoid-mediated negative feedback on HPA axis activity.

Although less conclusive, growing evidence from human studies are supportive of findings from animal models. Higher 3rd trimester maternal depressed mood was associated with higher cord blood methylation of the potential NGFI-A binding site (CpG 3) in the *NR3C1* 1F promoter, and infants with higher CpG 3 methylation displayed heightened HPA stress reactivity at 3 months. In 5 - 7 year old children, higher methylation of CpG 1 and 3 in the *NR3C1* 1F promoter in buccal DNA was associated with reduced and delayed cortisol stress responses. Higher methylation at the NGFI-A binding site in hippocampal tissue that has been shown to reduce GR expression in rodents, is also evident in humans. Thus, the observed association
between CpG 3 methylation in the NR3C1 \(1_F\) promoter and cortisol stress responses in early childhood may have resulted through altered GR expression and HPA feedback regulation.

Children with higher SLC6A4 promoter methylation at birth displayed blunted cortisol concentrations with stress. This finding offers evidence for the effect of early life environment on the human epigenome and long term HPA cortisol reactivity. SLC6A4 is a key regulator of 5-HT levels which play a critical role during early developmental periods, and is central to the development and function of the HPA axis.\(^{84,268}\) In this way, altered 5-HT neurotransmission may influence fetal brain development via epigenetic regulation of SLC6A4, further illustrating findings in rodent models showing that long term stress responses are shaped via 5-HT moderation of the \(Nr3c1\) NGFI-A binding site in rodents pups.\(^{15}\) Further research is required to explore the biological pathways of the complex influences taking place between the serotonergic system and HPA axis during development.

Examination of methylation status prior to, concurrently with, and following adverse early social experience, may shed light on whether maternal mood disturbances changes are long lasting. The methylation status of CpG sites located in NR3C1 \(1_F\) and SLC6A4 promoter regions at 5 - 7 years of age were associated with 3\(^{rd}\) trimester and concurrent maternal depressed/anxious mood. Between 5 and 10 years of age, higher SLC6A4 methylation in buccal DNA from bullied children, compared to non-bullied children, was suggested as an environmentally mediated effect of victimization on HPA axis reactivity.\(^{251}\) Building on this finding and the interactive relationship I observed between 3\(^{rd}\) trimester and concurrent maternal mood on children’s cortisol stress responses, these results suggest that epigenetic modifications of NR3C1 \(1_F\) and SLC6A4 at 5 - 7 years may be a function of prenatal maternal mood that also extends to postnatal maternal mood in early childhood. Thus, the processes involved in the
establishment and structural organization of stress regulatory systems may be dependent on the characteristics, predictability, and structure of information from both an early and ongoing social environmental context.  

Recent studies have also shown early life adversity to be associated with epigenetic modifications of other genes, apart from SLC6A4 and NR3C1. For example, corticotropin-releasing hormone (CRH) initiates the HPA stress response, and maternal stress during pregnancy leads to lower Crh methylation in the hypothalamus and amygdala of rat offspring. Brain derived neurotrophic factor (BDNF) actions are vital for both brain development and plasticity, and adult rats with adverse maternal care during the first postnatal week showed higher Bdnf methylation and changes in Bdnf expression in the prefrontal cortex. Adult females with childhood adversities and a variant in the monoamine oxidase (MAOA) gene (enzyme that metabolizes 5-HT), had a higher risk of developing depression, and lower methylation of MAOA was found in saliva samples of depressed females compared to controls. Thus, evidence continues to show early life experiences on neurobiological and behavioral outcomes may involve methylation at specific gene loci.

A number of studies with both animal models and human subjects have provided substantial evidence that early environment experiences can shape the structure and function of the brain and peripheral organ systems with long term physiological vulnerability to stress, behavior and health across the lifespan. Although less is known regarding how altered HPA dysfunction may confer susceptibility of adverse mental health outcomes, epigenetic mechanisms are being increasingly recognized as critical factors linking early life experiences and risk for psychopathology. The World Health Organization recently highlighted the importance of maternal mental health during pregnancy and effects on development processes.
and risk of disease in offspring.\textsuperscript{286} Thus, determining the long-term consequences of early life stress during sensitive periods of development associated with gestational maternal mood disturbances should be an important public health concern.

Overall, I found an association between prenatal exposure to SRIs and 3\textsuperscript{rd} trimester maternal depressed/anxious mood and altered cortisol stress reactivity in early childhood. Furthermore, the relationship between these prenatal exposures and subsequent stress responses may be moderated by epigenetic mechanisms involving the serotonergic and HPA regulatory systems. An interactive relationship between pre- and postnatal maternal mood scores with cortisol stress responses and methylation levels at 5 - 7 years suggests that an early adverse environment may shape or ‘program’ a subsequent HPA stress response. However, the ultimate impact of this programming may not become evident without appreciating a sensitivity to a postnatal maternal environment that shifts stress responses towards either vulnerability or resilience to stress-related mood disorders across the lifespan.

Broader implications also need to be considered. First, the association between prenatal mood and altered cortisol reactivity implies that an intervention to reduce prenatal mood symptoms could have significant clinical implications for improving childhood outcomes. Second, exposure to concurrent maternal mood appeared to moderate the relationship between prenatal mood and stress reactivity in early childhood, suggesting that intervention with at-risk children whose early environment rendered them vulnerable to altered cortisol reactivity, may redirect their trajectory towards stress-related mood disorders.
6.2 Limitations, Strengths, And Future Directions

6.2.1 Limitations

A number of key limitations need to be noted. In this study I assessed DNA methylation status in buccal epithelial cells. Thus, caution is needed when interpreting results, as methylation in the brain may differ from peripheral measures. Buccal cells and brain tissue share an ectodermal origin and may therefore be more similar to neuronal lineages than other peripheral tissues. However, the question still remains to what extent is methylation in buccal epithelial cells a marker for central functioning.

Neonatal gene-specific methylation was analyzed from genomic DNA extracted from whole cord blood, which consists of different cells types that may carry different epigenetic marks. However, similarity of findings with studies using leukocytes, whole cord blood, and hippocampal tissues is encouraging, but data regarding differential control of GR expression in various tissues are very limited. Furthermore, the difference in methylation status between subjects was quite small, and the NR3C1 and SLC6A4 promoter regions have very low levels of methylation. Currently, the biological impact of small changes in methylation status is not clear. As NR3C1 and SLC6A4 mRNA expression was not quantified, I can only speculate regarding the effect of the differential methylation on gene expression.

The number of children included in the thesis was relatively small and sample size varied between aims. This was due to missing data for early life measures (such as maternal mood scores in the 3rd trimester), or data at 5 - 7 years (such as missing cortisol samples on study day). For instance, 33 children had complete data from all eight time points on the study day (Aim 1B), while 111 children had complete methylation data at 5 - 7 years (Aim 2A). Although a
larger sample size would allow for more robust statistical result, this becomes a challenge with longitudinal prospective cohorts.

Lastly, multiple comparisons, such as Bonferroni corrections, were not applied in this thesis. As a consequence, some significant findings observed may be a result of type I errors. Although this thesis focused on exploring trends between methylation and cortisol patterns at 5-7 years, correction for multiple comparisons will be applied in the next steps of data analyses for these findings, and will be included in manuscripts submitted for publication.

6.2.2 Strengths

This study had a number of key strengths. First, the data acquired for this study comes from a longitudinal prospective cohort with human subjects. Thus, in-depth collection of clinical measures from multiple time points enables control for many potential confounders, and does not have to account for recall-biases associated with retrospective collection. A further strength was the comprehensive protocol used for salivary cortisol collection and analysis. Taking the mean from samples collected from three time points over the course of four days increased the reliability of characterizing the diurnal pattern in individual subjects. Furthermore, very few studies have examined children’s cortisol stress reactivity, in addition to baseline diurnal patterns, and the association with prenatal exposure to SRIs and maternal depressed/anxious mood. Lastly, DNA methylation status was determined using bisulphite pyrosequencing technology that enables precise quantification of site-specific DNA methylation status.
6.2.3 Future Directions

To extend these findings, it would be of interest to evaluate the influence of antenatal maternal mood on the cognitive, behavioural, and emotional outcomes at 5 - 7 years of age. In particular, analyzing the relationship between cortisol concentrations and test scores from study day may reveal behavioural correlates of an altered stress response.

While methylation of the CpGs in the NR3C1 1F, NR3C1 1D, and SLC6A4 promoters were associated with cortisol stress responses in 5 - 7 year old children, more direct evidence, such as the relationship between methylation and mRNA levels, is required to determine the functional consequence of altered methylation status.

Principle component analysis (PCA) can be used to understand correlations of defined parameters and reduce the dimension of summary measures to a few meaningful components. Since a variety of diurnal and stress-related cortisol measures were analyzed in this study, PCA may reveal relationships between cortisol concentrations and changes in cortisol over time, which may not be identified using linear regression analyses.

Furthermore, it would be interesting to examine methylation patterns and genetic variations within NR3C1 and other genes associated with the HPA stress response. For instance, sensitivity of the GR to cortisol was shown to be influenced by the FK506-binding protein 5 (FKBP5), which may also moderate the effect of prenatal psychological symptoms with emotional and behavioural problems in children.

Lastly, it would be of great interest to evaluate these children at a later age and determine whether early life experiences leave persistent epigenetic, physiological, and behavioural marks beyond early childhood.
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