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Associations between hypothalamic-pituitary-adrenal axis system gene variants and cortisol reactivity in preschoolers: Main effects and gene-environment interactions

Haroon I. Sheikh, *The University of Western Ontario*

Supervisor: Dr. Shiva M. Singh, *The University of Western Ontario*

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Associations between hypothalamic-pituitary-adrenal axis system gene variants and cortisol reactivity in preschoolers: Main effects and gene-environment interactions

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by

Haroon I. Sheikh

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Abstract

Exposure to stressful events during early development has consistently been shown to produce long lasting effects on the hypothalamic-pituitary-adrenal (HPA) axis, which may increase vulnerability to mood and anxiety disorders. Recently reported genetic association studies indicate that these disorders may be influenced, in part, by gene-environment interactions (GxE) involving polymorphisms within the corticotrophin-releasing hormone and monoaminergic system genes. However, little is known about how genetic variants and life stress work to shape children's neuroendocrine reactivity and emerging symptoms. Therefore, the aim of this thesis is to examine main effects of candidate genes and GxE on the neuroendocrine stress response and internalizing symptoms in a community sample of 409 preschoolers.

In Chapter 2 analyses show associations between variants of the *CRHR1* and *CRHBP* genes and children's cortisol responses to a standardized laboratory stress task. I also found evidence for GxE, where variants of the CRH system genes moderated the impact of childhood stress on early-emerging symptoms of depression and anxiety.

A functional polymorphism of the catechol-*O*-methyltransferase (*COMT*) gene, the *val*158*met*, has been implicated in the etiology of stress-related mood disorders. Therefore, in Chapter 3, I examined links between the *val*158*met* polymorphism, cortisol reactivity to stress, and internalizing symptoms. I found evidence for association between the *val*158*met* genotype and cortisol reactivity

to stress. Additionally, the *val*158*met* genotype moderated the link between childhood stress and emerging symptoms of anxiety.

Due to the proposed role of dopamine and serotonin gene polymorphisms in research on GxE in internalizing disorders, in Chapters 4 and 5, I examined whether associations between dopaminergic and serotonin candidate gene polymorphisms and childhood cortisol reactivity and internalizing symptoms were moderated by childhood life stress. Analyses showed evidence for GxE predicting children's symptoms. Specifically, polymorphisms of *DRD2* and *DAT1* genes moderated the effect of childhood stress on emerging symptoms of anxiety. With regard to serotonin pathway polymorphisms, I found associations between the serotonin transporter promoter polymorphism (5-HTTLPR) and children's anxious symptoms. Additionally, consistent with previously reported findings, the interaction between *MAOA* 30bp VNTR and childhood stress predicted child anxiety symptoms. Limitations of this work include a relatively small sample size for genetic analyses, as well as the examination of a limited number of markers at each gene. Additionally, I did not correct for multiple statistical tests in some analyses due to the hypothesis-driven nature of the work.

Taken together, the analyses show the complex underpinnings of individual differences in stress regulation, and highlight specific genetic vulnerabilities that influence early psychophysiological reactivity, that may in turn contribute to the development of stress-related disorders later in development.

Keywords: HPA axis, childhood stress, cortisol, haplotype, polymorphism, early development, serotonin, dopamine, gene-environment interactions, depression, anxiety.

Co-Authorship

Chapter 2 of this thesis contains material from the manuscript entitled, "Corticotrophin-Releasing Hormone System Polymorphisms are Associated with Children's Cortisol Reactivity" by Haroon Sheikh, Katie Kryski, Heather Smith, Elizabeth Hayden and Shiva Singh, which was published in *Neuroscience.* Chapter 3 contains materials from the manuscript entitled "Catechol-O-Methyltransferase gene *val*158*met* polymorphism and depressive symptoms during early childhood" by Haroon Sheikh, Dr. Lea Dougherty, Dr. Sarah Bufferd, Katie Kryski, Heather Smith, Dr. Elizabeth Hayden, Dr. Daniel Klein and Dr. Shiva Singh, which was published in *American Journal of Medical Genetics Part B*. In both studies Haroon Sheikh performed the experiments, analyzed the data and wrote the manuscripts. The stress task data and biological samples (buccal swabs and saliva) were collected by Katie Kryski and Heather Smith. The Child Behavior Checklist data were entered by numerous undergraduate and graduate students in the Hayden lab. The path analyses in Chapters 3 and 4 were conducted by Yuliya Kotelnikova and interpreted by Yuliya Kotelnikova and Haroon Sheikh. Dr. Hayden and Dr. Singh provided manuscript editing, feedback and supervision.

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Table of Contents

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of abbreviations

5-HTT	5-hydroxytryptophan
5'UTR	5'Untranslated region
3'UTR	3'Untranslated region
ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
AUC_i	Area under curve with respect to increase from baseline
AUC_g	Area under curve with respect to ground
BOLD	Blood oxygenation-level dependent
CBCL	Child Behavior Checklist
cDNA	complementary Deoxyribonucleic Acid
Chr	Chromosome
CNV	Copy number variation
COMT	Catechol-*O*-Methyltransferase
CRH	Corticotrophin-releasing hormone
CRHR1	Corticotrophin-releasing hormone receptor-1
CRHBP	Corticotrophin-releasing hormone binding protein
CS	Childhood stress
dbSNP	database single nucleotide polymorphisms
DA	Dopamine
DAT	Dopamine transporter
DRD2	Dopamine receptor Domain-2
DRD4	Dopamine receptor Domain-4

DNA	Deoxyribonucleic acid
DTI	Diffusion tensor imaging
DZ	Dizygotic twins
fMRI	functional magnetic resonance imaging
GWAS	Genome-wide association studies
GxE	Gene-environment interaction
h	Hour
HapMap	Haplotype Map
HPA	Hypothalamic-pituitary-adrenal
Kb	Kilobases
Leu	Leucine
LD	Linkage disequilibrium
LOD	Log-of-Odds
MAOA	Monoamine oxidase-A
Mb	Megabases
MDD	Major depressive disorder
Met	Methionine
µg	microgram
µL	MicroLitre
min	minutes
MZ	Monozygotic twins
ng	nanogram
OMIM	Online Mendelian Inheritance in Man

PCR	Polymerase chain reaction
PD	Panic disorder
PFC	Prefrontal cortex
pg	picogram
Phe	Phenylalanine
PLINK	Phase linkage
PPVT	Peabody Picture Vocabulary Test
PTSD	Post-traumatic stress disorder
RefSeq	Reference sequence
RNA	Ribonucleic acid
sec	seconds
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLC6A3	Solute carrier family 6, member A3
SLC6A4	Solute carrier family 6, member A4
tSNP	tagging-Single Nucleotide Polymorphism
UV	Ultraviolet
Val	Valine
vmPFC	ventro-medial prefrontal cortex

1 Introduction – Hypothalamus-pituitary-adrenal axis stress reactivity: The role of genetics, environment and implications for emerging risk for depression and anxiety

1.1 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is the primary physiological regulator of environmental stress in humans (Selye, 1973). In his seminal work, Selye defined stress as the result of an organism's failed attempt to respond appropriately to a physical challenge (Selye, 1936). Since then, this definition has been further elaborated to include psychological threats, including both anticipation and ideation of impending stressors, not just those actually present in the current environment (Schulkin et al., 1994). Expanding on this work, an alternative view of stress is encapsulated in the concept of allostasis and allostatic load (McEwen, 1998; Sterling & Eyer, 1998). Allostatic responses are those physiological changes that occur in response to environmental disturbances. These responses are not inherently negative, but instead play an important positive role in helping an individual adapt to a changing environment. Importantly, the concept of allostasis focuses on the mediators of adaptation, such that the HPA axis not only promotes adaptation to stressors, but also contributes to pathophysiology when it is overused or dysregulated (McEwen, 2006). For instance, inflammatory cytokines can stimulate the production of corticosteroids, which in turn can suppress inflammatory cytokine production. Similarly, the sympathetic and parasympathetic systems exert differential effects

on inflammatory cytokines, with the former stimulating their production and the latter inhibiting them (Sapolsky et al., 2000). However, under allostatic load when cortisol is too high, these systems become unbalanced leading to inhibition of an appropriate inflammatory response during immune challenge. Conversely, if corticosteroid levels are too low, a 'normal' immune response can become overactive and result in excessive inflammation (Karatsoreos & McEwen, 2011).

As described by McEwen and Wingfield (2003), when environmental stress is encountered, the HPA neuroendocrine cascade (Figure 1.1) initiates the release of corticotrophin-releasing hormone (CRH) in the hypothalamus into the hypothalamic-pituitary portal. CRH triggers the release of adrenocorticotrophic hormone (ACTH) in the anterior pituitary by binding to its receptor, the corticotrophin-releasing hormone receptor-1 (CRHR1). The cascade culminates at the adrenal cortex with the release of cortisol in primates and corticosterone in other mammals (e.g., rodents). Glucocorticoids in turn act on the hypothalamus and pituitary to suppress CRH and ACTH production in a negative feedback cycle, thus downregulating these hormones once the stressor has terminated. Cortisol is a potent glucocorticoid hormone and a key biomarker of HPA axis activity in mammals (McEwen, 2007) and its broad role in physiology and psychology are briefly reviewed in the following sections.

1.1.1 Cortisol and its importance in physiological metabolism

Cortisol is one of the few hormones essential for life; for example, adrenalectomy in humans is fatal unless glucocorticoids are administered

(Karatsoreos & McEwen, 2011). Cortisol exerts an anabolic effect, such as conversion of glucose to glycogen in the liver, and catabolic effects such as proteolysis and lipolysis in other tissues including skeletal muscle, adipose tissues and lymphoid tissues (Spiga et al., 2007). Further, cortisol also facilitates the function of other hormones. For example, cortisol must be present in order for glucagon to exert its calorigenic action, and for catecholamines such as dopamine, epinephrine and adrenaline to exert their lipolytic effects (Spiga et al., 2007). Cortisol also acts as an inhibitor of CRH and ACTH secretion by the hypothalamus and pituitary glands.

In addition to its effects on the organs and tissues directly involved in metabolic homeostasis, cortisol influences a number of other organs and systems. For example, cortisol maintains the responsiveness of vascular smooth muscle to catecholamines and therefore participates in blood pressure regulation (Danhof-Pont MB, van Veen & Zitman, 2011). When blood pressure is low, decreased muscle responsiveness, together with the associated hypovolemia caused by mineralocorticoid deficiency, can result in severe hypotension (Veldhuis et al., 1987).

Excessive cortisol production has been etiologically implicated in many medical conditions such as inflammatory diseases, musculoskeletal disorders, asthma, and heart disease (Negrao et al., 2000; Sharpley, 1998; Sternberg et al., 1990; Wright et al., 1998). Our understanding of the deleterious effects of excessive cortisol production (hypercortisolemia) comes from studies of people with Cushing's disease (for an in-depth review, see Prague, May & Whitelaw,

2013). Individuals with hypercortisolemia in Cushing's syndrome present with excessive abdominal fat, obesity and significantly higher risk factor for cardiovascular disease, Type-II diabetes, and stroke (Miller & O'Callaghan, 2002). Further, along with the effects of excessive cortisol exposure on physical health, maladaptive cortisol responses to stress have also been linked to psychiatric disorders such as depression, anxiety and panic disorders (McEwen, 2004, 2008).

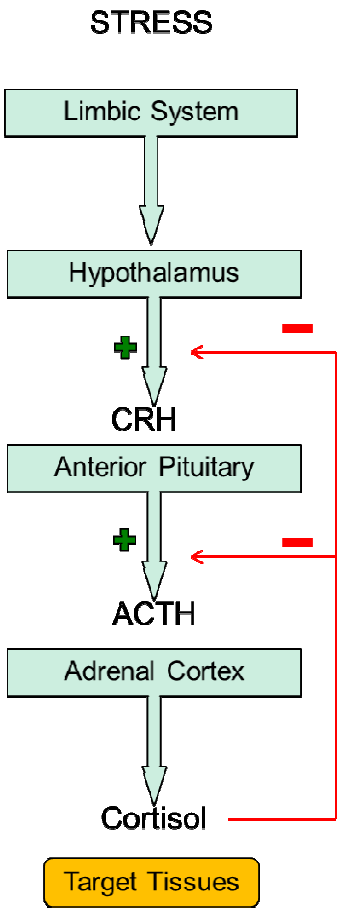

Figure 1.1. The hypothalamic-pituitary-adrenal axis. The red line indicates the negative feedback loop of the signalling cascade.

1.1.2 Cortisol function and depression

HPA axis dysregulation has been a well-documented neuroendocrine feature in individuals with depression and anxiety. Abnormal levels of cortisol in saliva, plasma, urine, and cerebrospinal fluid have long been known to be present during episodes of depression (Carroll, Curtis, & Mendels, 1976; Charney, 2004; Heim & Binder, 2012). Cortisol responses are also exaggerated after CRH and ACTH administration in patients with depression compared to healthy controls (Amsterdam et al., 1986; Nelson & Davis, 1997). Aberrant patterns of HPA axis activity is not only found in many adults with depression, but has also been reported in depressed children and adolescents. For example, compared to healthy controls, depressed children were found to exhibit a stronger ACTH response to CRH administration (Kaufman et al., 1997). Similarly, an increased cortisol response to psychosocial stress was observed in depressed children aged 3 through 6 compared to their healthy counterparts (Luby et al., 2003). A recent meta-analysis has confirmed these findings in patients with depression (Stetler & Miller, 2011). Literature also suggests that normalization of the HPA system may be necessary for stable remission of depression and successful antidepressant treatment has been shown to attenuate and normalize HPA axis abnormalities (De Bellis et al., 1993; Kling et al., 1994; Nemeroff et al., 1991; Ising et al., 2005).

1.1.3 Salivary cortisol as a biomarker for HPA axis reactivity

The primary biomarkers of the HPA axis include CRH, ACTH, and cortisol. In studies of human participants, CRH is measured in cerebrospinal fluid, which means that this biomarker must be measured in controlled clinical settings. In contrast, ACTH and cortisol can be assessed in blood, and unbound levels of cortisol can be measured in saliva. Unbound cortisol diffuses from the blood stream into salivary glands and is highly correlated with serum cortisol levels (McEwen, 2004). As cortisol is transferred from plasma to saliva within a few minutes, the short time lag makes it useful in research investigating HPA axis responses (Harmon et al., 2007). There are many advantages to using salivary biomarkers in stress research. First, sampling is non-invasive and eliminates the possibility of needle puncture injuries. Stress related to blood draws during collection, which may bias the results, is also minimized, making it useful in studies of children. Salivary biomarkers are also useful for research involving children as it avoids ethical concerns associated with invasive measures. Furthermore, saliva collection can be performed at participants' homes and does not require skilled healthcare professionals, making it highly useful in assessments in the subjects' natural environment, such as at a child's home. Cortisol is also relatively stable in saliva; thus, saliva samples can be stored at room temperatures for at least for a couple of weeks (Kirschbaum & Hellhammer, 2000). For these reasons, salivary cortisol has been widely used in HPA axis research and is considered a well-established biomarker of HPA axis function.

1.2 Correlates of the HPA axis response

As previously discussed, literature suggests that HPA axis dysregulation measured via cortisol reactivity is a prominent neuroendocrine feature in psychiatric disorders such as depression (Charney & Manji, 2004). However, the developmental correlates of the cortisol stress response are poorly understood (Krishnan & Nestler, 2008). Extant research points to the role of environment and individual diatheses in contributing to cortisol reactivity to stress. Therefore, the following sections will briefly review current knowledge of the environmental and genetic determinants of the HPA axis reactivity to stress.

1.2.1 Life stress during early childhood and the HPA axis

Early caregiving has been shown to contribute to the lifelong responsiveness of the HPA axis to stressors (Meaney et al., 1988, 1991). Perhaps the strongest evidence for the environmental regulation of the development of responses to stress comes from postnatal handling research with rodents. Handling involves a brief (i.e., 3–15 min), daily period of separation of the pup from the mother for the first few weeks of life, and results in hyper-reactivity to stress in adulthood (Levine et al., 1967; Zarrow et al., 1972; Meaney et al., 1989; Viau et al., 1993; Bhatnagar et al., 1995). As adults, rats exposed to extended positive maternal care show decreased fearfulness and more modest HPA responses to stress; such effects are apparent in animals tested as old as 26 months of age (Meaney et al 1988, 1992). The handling effects on the development of HPA responses to stress have important consequences for health. Glucocorticoid levels often rise with age in rats and are associated with hippocampal degeneration and the emergence of learning and memory deficits

(Landfield et al., 1981, Landfield & Pitler 1984, Sapolsky et al., 1984, Issa et al., 1990). Such age-related increases in basal and stress-induced pituitary-adrenal activity is significantly less apparent in the control or non-handled animals; thus, these animals show little evidence of hippocampal aging (Meaney et al., 1988, 1991).

Research with rodents has been complemented by work with nonhuman primates where maternal separation during early childhood leads to higher plasma cortisol levels (Bayart, et al., 1990; Levine, 1993; Sanchez et al., 2005). Primate infants separated from their mothers and raised with peers have greater HPA axis reactivity in adulthood (Heim & Nemeroff, 1999). In addition to maternal separation stress, poor early caregiving can be observed in non-human primates as well, where a small number of primate mothers are actively abusive to their offspring, subjecting them to behaviours that include dragging, crushing, hitting, and biting the infant (Maestripieri, 1998). The infants of abusive and nonabusive mothers were administered CRH and their basal cortisol and cortisol reactivity tested every 6 months for up to 3 years of age. The authors found no effects of abuse on basal cortisol levels but offspring of abusive mothers had greater cortisol reactivity to stress contexts (such as facing aggressive males of higher social rank) when compared to their nonabused counterparts (Sanchez et al., 2010).

In addition to research on links between poor early caregiving and abnormal cortisol response in rodents and non-human primates, similar findings have been reported in humans as well. For example, Heim and colleagues

(2002) and in a later study by Rao and colleagues (2008) showed that a history of childhood adversity was associated with increased neuroendocrine stress responses in adolescents and adult women. Elevated cortisol reactivity has also been documented in children in daycare centers with lower quality of care (Tout et al., 1998) and children with poor care in foster homes (Dettling et al., 2000). However, the literature also yields contradictory findings as well. For example Carpenter and colleagues (2007) and MacMillan and colleagues (2009) failed to replicate earlier findings of increased neuroendocrine reactivity to stress and found attenuated neuroendocrine stress responses in maltreated adolescents and adults, suggesting that the effect of exposure to adversity and caregiving on stress reactivity is not straightforward (Ellis & Boyce, 2008; Del Giudice, Ellis & Shirtcliff, 2011). However, it is of note that the literature has focused largely on extreme forms of negative early care such as physical and sexual abuse, although more normative aspects of early care are also likely relevant, and would have implications for a larger number of children. For example, a recent study by Hackman and colleagues (2013) explored the role of common parenting behaviours on adolescents' stress responses, demonstrating that positive early childhood parental care directly predicted adolescent cortisol reactivity to psychosocial stress.

1.2.2 Genetic bases of cortisol responses to stress

In addition to the influence of early care, genetic factors have been linked to cortisol response to stress. Literature from twin studies suggests that salivary cortisol responses to acute challenge are significantly influenced by genetic

factors. For example, in a seminal pilot study, the heritability of salivary cortisol responses was investigated in a sample of 124 monozygotic and 155 dizygotic twins who were exposed to laboratory stressors such public speaking, a complex arithmetic task, and exhausting physical exercise (Kirschbaum et al., 1992). The proportion of variance in cortisol reactivity attributable to heritable factors was estimated at 48%. Research over the past decade has supported these findings via replications in large independent samples (Bartels et al., 2003; Steptoe et al., 2009). Therefore, these heritability studies suggest a moderate genetic component of cortisol reactivity, and in addition to environmental influences on cortisol reactivity. However, only a small number of studies have examined associations between polymorphisms of the HPA axis genes and childhood cortisol reactivity.

Due to the genetic underpinnings of HPA axis function, and its links to complex traits such as internalizing symptoms, in psychiatric research, cortisol reactivity is considered an endophenotype of mood and anxiety disorders. Endophenotypes such as cortisol reactivity are quantifiable components in the genes-to-behaviors pathways, distinct from psychiatric symptoms, which make genetic and biological studies of etiologies for disease categories more manageable. Endophenotypes provided a means for identifying the "downstream" traits or facets of clinical phenotypes, as well as the "upstream" consequences of genes and, in principle, could assist in the identification of candidate genes in the hypothesized polygenic systems conferring vulnerabilities to disorders (Gottesman & Gould, 2003). Therefore, research is needed to

document specific genetic variants linked to individual differences in childhood cortisol reactivity to stress, the endophenotype of depression and anxiety.

1.2.3 Gene-environment interactions influence HPA axis response to stress: Implications for etiology of depression and anxiety

Although so far the extant literature suggests that genes and environmental factors influence early cortisol reactivity to stress, a large body of molecular genetics research has documented that the impact of genes on phenotypes is dependent on environmental exposure (e.g., Kendler et al., 2007). Therefore, it is the joint consideration of interactions between genetic diathesis and environmental exposure that can help unfold the complex biological pathways that contribute to psychological risk. However, limited research in adults has examined the role of gene-environment interactions as predictors of cortisol responses to stress. For example, Tyrka and colleagues (2009) have reported that an interaction between childhood trauma and *CRHR1* genotype predicted cortisol reactivity in a sample of adults. Expanding on this study, Heim and colleagues (2009) reported that interactions between the *CRHR1* genotype and history of childhood physical abuse predicted significantly higher increases in cortisol responses to stress stimuli in a standardized laboratory task in depressed patients when compared to healthy controls. Additionally, a study by Alexander and colleagues (2009) reported interactions between a functional polymorphism of the serotonin transporter gene (also known as 5-HTTLPR) and stressful life events predicted cortisol reactivity in a non-clinical sample. To my knowledge, the studies of adults briefly reviewed here are the only examples of GxE

predicting cortisol reactivity. However, no prior research has examined the role of GxE as a predictor of cortisol reactivity in young children; thus, this topic will be one focus of this dissertation.

Even though only a few studies have reported on the role of GxE in shaping cortisol responses to stress, a large literature has reported on GxE predicting stress-related psychopathologies such as depression and anxiety. Specifically, gene polymorphisms of the CRH system and the aminergic pathways (serotonin and dopamine) have been shown to interact with early childhood adversity to predict later risk for mood and anxiety disorders (for an in depth review see Mandelli & Serrtti, 2013). For example, the effect of early caregiving on risk for depression and anxiety was moderated by gene polymorphisms of serotonin (Eley et al., 2004; Huang et al., 2004; Karg et al., 2011), dopamine (Hayden et al., 2010), norandrenergic (Sun et al., 2008; Xu et al., 2009) and glutamate pathways (Sokolowski et al., 2013). In sum, based on these studies in addition to GxE role on intermediate phenotypes that may contribute risk for mood and anxiety problems, I also intend to examine whether GxE predict emerging symptoms.

1.2.4 Rationale for conducting study in preschoolers

As described above, studies of GxE (see reviews by Heim et al., 2009; Uher, 2007) indicate that variation in CRH and monoaminergic system genes may influence risk for depressive and anxious symptoms based on early childhood environment. One possible explanation for these findings is that a

critical period exists for the normative development of an emotional regulatory system. A few studies suggest that there might be timing-dependent effects of early stress on depression risk. For example, Agid and colleagues (1999) report that parental loss before 9 years of age was associated with higher depression risk compared to parental loss between 9 and 17 years of age. Although there are very few studies that have systematically evaluated neurobiological outcomes of childhood stress experienced at different developmental stages, studies of rodents and non-human primates suggest that the direction of the effects of early life stress on the CRH/HPA system depend on timing of the early life stress (Coplan et al., 2006). For example, early weaning of 21-day old rats causes faster myelination in the amygdala, decreased brain-derived neurotrophic factor protein levels in the hippocampus and prefrontal cortex, and reduced neurogenesis in the dentate gyrus (Kikusui & Mori, 2009). Although limited evidence from human studies for age-dependent effects of early caregiving have been reported, a study by Rogeness and McClure, (1996) showed abnormal dopamine metabolism in children who were maltreated in the first 3 years of life, but not in children maltreated later in childhood. Carpenter and colleagues (2004) reported that preschool life stress, but not later childhood stress, modulates adulthood cerebrospinal fluid CRH levels. The same group reported that cortisol response to stress in adults decreases with increasing onset age of emotional abuse (Carpenter et al., 2009). Taken together, the data described here suggest that early childhood is a critical period with consequences for later mental health outcomes.

In addition to the role of biological mechanisms underlying the phenotype in question (internalizing problems) and the environmental factors that may contribute to the expression of said phenotype, the study of emerging symptoms in preschoolers is an informative tool in the understanding of the etiology of depression and anxiety. For example, evidence exists that early symptoms, even subthreshold ones of anxiety and depression such as the ones found in preschoolers have heterotypic and homotypic continuity for later disorders. Specifically literature from our group and others has shown that early emerging symptoms of depression and anxiety predict internalizing symptoms later in life (Costello et al., 2003; Klein et al., 2008; Luby et al., 2009). Therefore findings in this study would help inform early prevention and intervention.

1.3 Neurotransmitters as mediators of links between early life stress and HPA axis response

The search for candidate genes that shape cortisol responses to stress must be informed by an understanding of the neurobiological systems that regulate the function of the HPA axis. The hypothalamus and pituitary glands are heavily innervated by aminergic neuronal inputs from the limbic system region including the prefrontal cortex, hippocampus, and amygdala (Herman et al., 1996). The two major aminergic neurotransmitter systems linked to HPA axis regulation are the dopaminergic and serotonergic systems: these are briefly reviewed here.

1.3.1 The dopaminergic system

Dopamine (DA) is released predominantly from the ventral tegmental area of the brain and is involved in the control of locomotion, cognition, affect, and neuroendocrine secretion (Bunney et al., 1980; Jaber et al., 1996; Smythe, 1977). At the synaptic level, DA exerts its effects through its interaction with the DA receptors. Molecular biology and pharmacology studies have identified two classes of receptors consisting of at least five DA receptor subtypes: D1, D2, D3, D4, and D5, with the most widely expressed subtypes in the prefrontal regions of the brain being the D2 and D4 receptors (LaHoste et al., 2000). On binding an agonist, DA receptors activate the adenylate cyclase second messenger system, elevating intracellular cyclic adenosine monophosphate concentrations. Cyclic adenosine monophosphate increases protein kinase-A activity with resulting changes in expression levels of a wide range of proteins within the cell (Dunlop & Nemeroff, 2007). DA signalling is terminated via either its reuptake by the dopamine transporter (DAT) into the presynaptic neurons or DA inactivation by two catabolic enzymes, namely, the catechol-*O*-methyltransferase (COMT) and the monoamine oxidase-A (MAOA).

DA innervation of the medial prefrontal cortex (mPFC) appears to be particularly sensitive to mild and brief stress (Deutch et al., 1985). In addition to genetic influences, animal studies of rodents and non-human primates show that both acute and chronic stress influence the functioning of the DA system. For example, rodents exposed to brief stressors such as low intensity foot shock and short periods of restraints show DA release and metabolism in the prefrontal cortex (Deutch & Roth, 1990; Thierry et al., 1998; Kalivas & Duffy, 1989). In

contrast, chronic stress over time may enhances DA degradation in areas that receive DA innervations, such as the hypothalamus and hippocampus, leading to overall DA depletion and DA mediated signalling in these important stress regulating brain regions (Roth et al., 1988). Thus, mPFC DA metabolism and release is related to the occurrence of stress, and suggests the importance of DA in stress regulation (Abercrombie et al., 1989; Deutch et al., 1985; Deutch & Roth, 1990; Mantz et al., 1989).

1.3.2 The serotonergic system

Serotonin (5-HT) is involved in a wide variety of processes including anxiety, arousal, vigilance, aggression, mood, impulsivity, and regulation of appetite (Charney & Manji, 2004). There is both anatomical and functional evidence for a regulatory role of 5-HT on stress-induced HPA activity (Dinan, 1996; Phelix et al., 1992). Animals exposed to a variety of stressors, including restraint and maternal separation, show an increase in 5-HT turnover in limbic regions, such as the medial prefrontal cortex (Adell et al., 1988; Inoue et al., 1994; Pei et al., 1990; Petty & Sherman, 1983), amygdala, and hypothalamus (Kaehler et al., 2000). Animals exposed to social stress such as maternal separation during early life, also had an increase in activation of 5-HT receptors in the hippocampus and dentate gyrus, two regions widely implicated in regulation of stress stimuli (McKittrick et al., 1995). Studies of non-human primates have provided evidence that increased 5-HT metabolism during exposure to inescapable stress prevents anhedonic behaviours, evinced by lack of caloric intake (loss of appetite), decrease in sexual activity and withdrawal

from social exploration (Ronan et al., 2000), behaviours usually associated with depression in humans. 5-HT antagonists also produce behavioural deficits while drugs that enhance 5-HT neurotransmission (SSRI) are effective in reversing anhedonia-like symptoms (Martin et al., 1990; Sherman & Petty, 1980). Additionally, injection of 5-HT into the frontal cortex after stress exposure in adult rats reverses stress-related behavioural deficits, such as anhedonia-like symptoms described above (Sherman & Petty, 1982).

There are two key regulators of serotonin levels in the limbic system. First, is the 5-HT transporter (5-HTT), which regulates serotonin neurotransmission through precise control of extracellular 5-HT levels (Lesch et al., 1996). This process is driven by the 5-HTT, which belongs to the sodium ion dependent transporter family (Nelson, 1998; Rudnick & Clark, 1993). 5-HTT assumes the uptake of extracellular 5-HT across the cell membrane of neuronal and non-neuronal cells, such as serotonergic neurons of raphe nuclei, basal nuclei and platelets. Dysregulation of the tightly controlled external concentration of 5-HT is linked to a host of metabolic and psychiatric disorders, including depressive, anxious, and obsessive-compulsive disorders (Charney & Manji, 2004). The second regulator of synaptic 5-HT levels is the MAOA enzyme, which catabolizes serotonin in the neuronal synapse (Martin et al., 1990). In brains of MAOA knockout mice, 5-HT concentrations were increased up to nine-fold compared with wild-type mice. Additionally, adrenergic concentrations in the prefrontal cortex and basal nuclei were increased up to two-fold; however, relative to 5-HT, only a small increase in DA levels was observed in infant mice brains (Cases et

al., 1995), suggesting that MAOA has a greater affinity for degrading serotonin when compared to DA. MAOA deficient mice show a significant increase in brain serotonin levels and attenuated reactivity to stress, suggesting a link between serotonergic metabolism and HPA axis function (Shih et al., 1999). Due to its ability to regulate serotonin availability in the brain, MAOA inhibitors have been widely used in psychiatric populations for the treatment of depression, anxiety and posttraumatic stress disorder (Liebowitz et al., 1990). Doyle and colleagues (1996) reported that an increase in salivary MAOA activity was also correlated with levels of life stress. Additionally, MAOA inhibitor therapy in adult patients diagnosed with posttraumatic stress disorder leads to normalization of diurnal cortisol rhythm (Liebowitz et al., 1990). Taken together, these studies suggest a role of serotonin and DA pathways in HPA responses to stress and stress-related psychopathologies such as mood and anxiety disorders.

1.4 Candidate genes examined in the current study

Based on current literature implicating the dopamine and serotonin systems in HPA axis responses to stress (Figure 1.2), a logical starting point for examining the underpinnings of psychological risk is to focus on gene variants implicated in aminergic signalling. This method (also known as the gene-pathway approach; Mormede et al., 2002) of candidate gene identification utilizes *a priori* knowledge of the metabolic pathway involved taking advantage of data from previous cellular, biochemical, molecular, or pathological experiments. Candidate genes and their variants investigated in this study are listed in Table 1.1. DA signalling is widely documented in regulating HPA axis response to stress and

stress-related disorders such as depression and anxiety. Therefore, the current study will explore common variants of the DA pathway, specifically the D2 and D4 receptors (Chapter 4). Additionally, as both COMT and DAT directly affect the availability of synaptic DA, thereby controlling the intensity of DA neurotransmission in response to stress, genetic variants of the *COMT* and *DAT* genes will be investigated (Chapters 3 & 4). Similarly, as the serotonin transporter gene (commonly known as 5-HTT) and MAOA also regulate the availability of the limbic systems extracellular serotonin, the current thesis will also examine the role of the variants of the two genes in stress response and emerging risk for psychopathology in Chapter 5.

As discussed in previous sections, the intensity of the cortisol response at the adrenal level is based on the release of CRH in the hypothalamus (McEwen, 2007). The availability of CRH in the brain is directly regulated by the CRHBP protein, which sequesters and inactivates CRH and its receptor in the pituitary gland (CRHR1) that initiates the release of ACTH. However, the common genetic variants that may be associated with cortisol stress reactivity in young children are unknown. Therefore, the current study aims to examine the genetic variation of the entire coding and regulatory regions of the CRH system genes. These associations will be based on constructing haplotypes based on linkage disequilibrium. Haplotypes refer to combinations of marker alleles which are located closely together on the same chromosome and which tend to be inherited together (van West et al., 2004). Methods based on haplotypes can be more powerful than those based on single markers in association studies of mapping

complex disease genes. The power of single marker-based methods depends on the linkage disequilibrium (LD) between the tested marker locus and the disease-susceptibility locus (Akey et al., 2001). LD information contained in flanking markers is not incorporated into such methods, which can result in potential reduction of power. In addition, even if the tested marker locus is in strong LD with the disease locus, power can be quite low if the frequencies of the marker and disease alleles are different (e.g., Kaplan & Morris, 2001). Therefore, haplotype-based association methods are generally regarded as being more powerful than methods based on single markers (Akey et al., 2001; Morris and Kaplan, 2002) as these analyses can detect the combined effects of multiple sequence variants on promoter activity or protein structure and/or function (Devlin & Roeder 1999; Drysdale et al. 2000; Joosten et al. 2001). The work of the International HapMap Project (International HapMap Consortium 2003; International HapMap Consortium 2005), dedicated to describing the patterns of human genetic variation by developing a map of the linkage maps in the human genome, provides a valuable resource for efficient SNP selection in haplotype-based association studies and forms the basis of Chapter 2 of this thesis.

Table 1.1. List of genes investigated for associations with childhood cortisol reactivity to stress and emerging internalizing symptoms.

Gene[1]	Chromosome	Functional mutation	Behavioural associations
CRH	8q13	None reported	↑behavioural inhibition in children at risk for panic disorder (Smoller et al., 2005)
CRHR1	17q12	None reported	Increased risk for internalizing problems (Bradley et al, 2008)
CRHBP	5q11	18 amino acid exon 7 deletion leads to truncated messenger RNA	Suicidal ideation (Roy, 2012)
COMT	22q11	Exon 4 Val→Met substitution at position 158 leads to changes in enzyme activity	Linked to multiple internalizing disorders
MAOA	X	30bp VNTR polymorphism in gene promoter region leads to decreased function	Polymorphism linked to externalizing behaviours such as aggression
SLC6A4 (5-HTTLPR)	17q11	44-48bp VNTR polymorphism in gene promoter region leads to decreased transporter protein	↑ depression and anxiety incidence among short allele carriers (Wust et al., 2009)
SLC6A3 (DAT-1)	5p13	30bp VNTR in exon 4 leads to differential dopamine binding	Associated with major depression and bipolar disorder (Szczepankiewicz et al., 2011)
DRD2	11q23	Single nucleotide polymorphism in the promoter region	Associated with cortisol reactivity in mice and non-primate humans
DRD4	11p15	48bp VNTR polymorphism in exon 3 leads to altered receptor function	Interaction between this variant and early life stress predict anxiety problems

Note: *BDNF*: Brain derived neurotrophic factor; *SLC6A3*: Solute carrier family member-3 (dopamine transporter); *SLC6A4*: Solute carrier family member-4 (serotonin transporter); *CRH*: Corticotrophin releasing hormone; *CRHR1*: Corticotrophin releasing hormone

receptor-1; *CRHBP*: Corticotrophin releasing hormone binding protein; *COMT*: Catechol-O-Methyltransferase; *MAOA*: Monoamine oxidase-A; *DRD2*: Dopamine receptor D2; *DRD4*: Dopamine receptor D4; bp: base pairs; VNTR: Variable number tandem repeat. Val: valine; Met: methionine; Leu: leucine; Phe: phenylalanine; Gly: glycine; Ile: isoleucine.

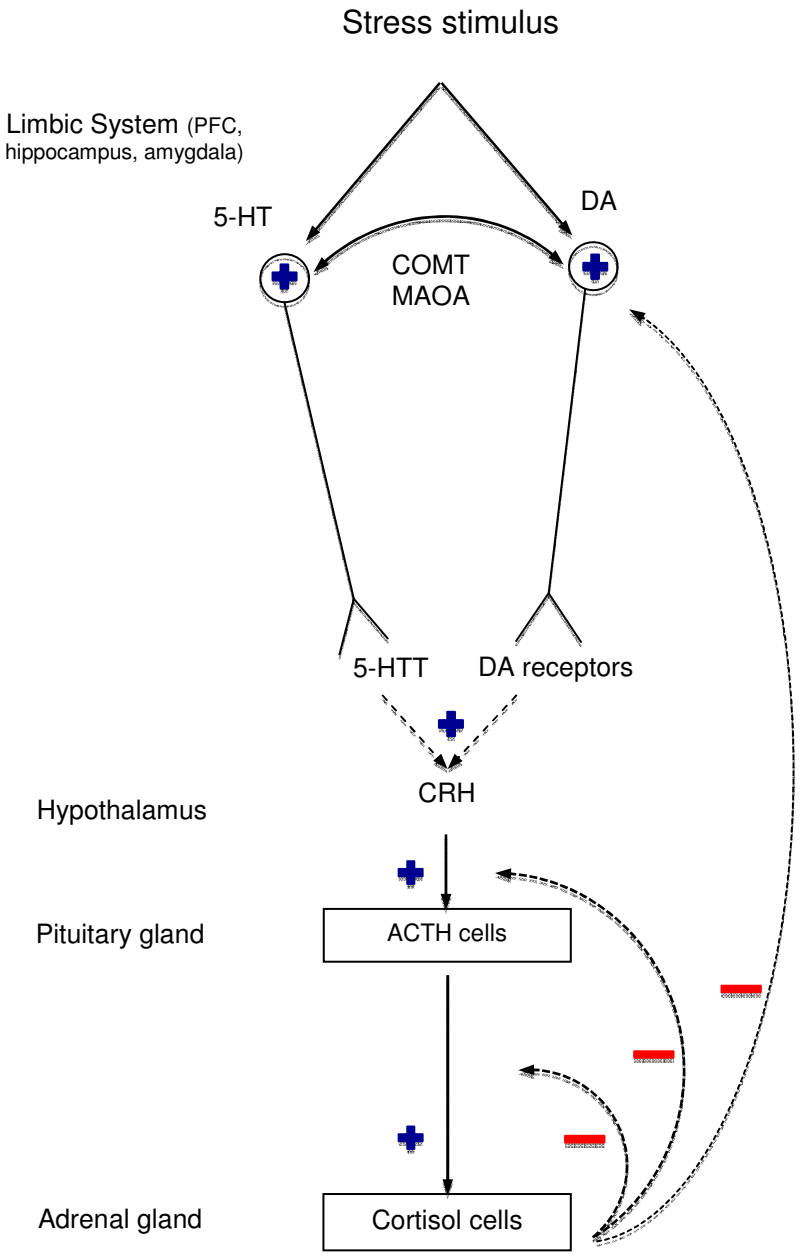

Figure 1.2. Overview of the limbic regulation of the HPA axis via central nervous system neurotransmitters. Activation and inhibition of the signalling cascade is denoted by the "+" and "-" signs. Note: CRH: Corticotrophin releasing hormone; ACTH:

Adrenocorticotrophic hormone; COMT: Catechol-O-Methyltransferase; MAOA: Monoamine oxidase; DA: Dopamine.

1.5 Hypotheses and thesis objectives

The main focus of this thesis is to investigate the link between genetic variants of the dopamine, serotonin and CRH pathway genes and cortisol responses to stress in a community sample of preschoolers. **I hypothesize that the genetic variants of the monoaminergic (DA and 5-HT) and CRH pathway genes will be associated with individual differences in children's cortisol reactivity to stress. Further, GxE between candidate gene polymorphisms and life stress will predict risk for emerging symptoms of depression and anxiety in preschool-aged children.**

Given the role of the HPA axis response to stress in the etiology of internalizing problems, I will also examine associations between monoaminergic and CRH pathway gene variants and emerging risk for psychopathology. Specifically, the following chapters of this dissertation focus on three objectives (see conceptual model in Figure 1.3):

1. Examine the main-effects of CRH system, serotonin and dopaminergic system gene variants on early age cortisol reactivity to stress.
2. Examine the main-effects of CRH system, serotonin and dopaminergic system gene variants on early age emerging symptoms of depression and anxiety.
3. Investigate whether gene-environment interactions between candidate gene variants and childhood stress predict cortisol

response to stress and/or emerging symptoms in a community sample of preschoolers.

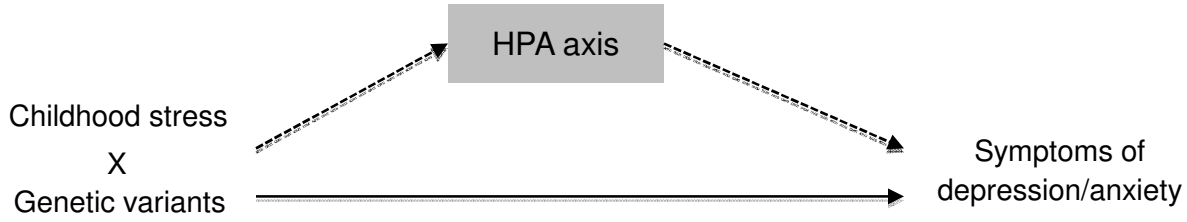

Figure 1.3. A conceptual model of life stress and genetics interacting through HPA axis physiological response to influence emerging symptoms.

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2 – Genetic variation of the CRH pathway genes and childhood cortisol response to stress: The role of early life stress and implications for emerging risk

2.1 Introduction

As discussed in Chapter 1, dysregulation of the HPA axis in patients with major depressive disorder (MDD), demonstrated by abnormal plasma ACTH and cortisol concentrations, is one of the most consistent biological markers identified in the literature (Gillespie & Nemeroff, 2005; Holsboer et al., 1984). HPA axis dysfunction is also often found among patients with anxiety, including panic disorder (Abelson et al., 2007), social anxiety disorder (Condren et al., 2002) and generalized anxiety disorder (reviewed in Martin et al., 2009). However, a significant gap in knowledge remains in research on human participants regarding the genetic mechanisms underlying cortisol reactivity to stress, which may be an important mediator of genetic risk for depression and anxiety. Research using murine and primate models has explored this question in greater detail (McEwen, 1998), with extant literature implicating genes expressing the cascade initiation molecule, the corticotrophin-releasing hormone (CRH), and its components, the CRH receptors and CRH binding protein (CRHBP) in regulating glucocorticoid reactivity in response to stress (Behan et al., 1995; Roy et al., 2012). In this cascade, CRHBP is a passive ligand trap that neutralizes active CRH by binding to it, thereby regulating bioavailability of free CRH and influencing downstream release of glucocorticoids (Jahn et al., 2005). Null mutants of the murine *Crh* gene (*Crh* -/-) have markedly impaired production of

both ACTH and corticosterone under conditions of stress compared to wild-type mice (Muglia, 1995). Other pituitary neuropeptides, such as oxytocin and vasopressin, do not compensate for the loss of corticosterone reactivity under stress (Schmidt et al., 2003; Venihaki & Majzoub, 2002), highlighting the importance of *CRH* gene expression in normal HPA axis function. Consistent with this, murine *Crhr1* (*Crhr1* -/-) knockouts lack a typical corticosterone response to stress. Furthermore, conditional knockouts of *Crhr1* in the hypothalamus and pituitary lack a stress-induced corticosterone response (Schmidt et al., 2003, 2006). Translational research on the effects of stress in *CRHBP* knockout mice is limited, but these knockouts do exhibit higher free CRH in their serum and increased anxious behaviours compared to wild-type animals, supporting the role of *CRHBP* gene in the HPA axis function (Gammie et al., 2008). These findings implicate the importance of CRH system genes in maintaining HPA axis function and regulating glucocorticoid reactivity to stress.

Based on findings from animal studies, and the role CRH system genes play in cortisol response in animals, a parallel line of research in humans has increasingly focused on the GxE between CRH system genes and the early environment in patients with internalizing disorders. For example, variants of both *CRHR1* and *CRHBP* genes have been shown to interact with early adversity, such as child abuse or maltreatment, to predict risk of depression and even suicidality later in life (Bradley et al., 2008; Heim et al., 2009; Grabbe et al., 2010; Roy et al., 2012). Additionally, genetic variation in the *CRHR1* gene has also been linked to consolidation of aversive memories in adults and is thought to

mediate the pathway from childhood maltreatment to adulthood depression (Polanczyk et al., 2009). Similarly, variation in the DNA region containing the *CRH* gene has also been linked to behavioural inhibition, a personality trait associated with increased risk for internalizing disorders (Smoller et al., 2003, 2006). Taken together, these studies suggest that CRH system genes may play a role in moderating the effect of childhood adversity on behaviour outcomes.

Little is known about whether links between CRH system gene variation and cortisol reactivity or emerging risk for psychopathology exist in early childhood. Therefore, the aim of this chapter is to examine the links between common variation of the *CRH* system genes and early life cortisol reactivity and emerging symptoms. The second aim of this chapter is to extend the adult literature on CRH pathway gene variants as moderators of the effect of childhood adversity on later psychiatric problems (Bradley, 2008; Ciccetti, 2011). Specifically, we examined whether interactions between the CRH system gene variants and childhood adversity predicted either cortisol response to stress or emerging symptoms of anxiety or depression in young children.

2.2 METHODS

2.2.1 Participants

Participants were an unselected community sample of 409 children (201 boys; 49.1%) between 36 and 47 months of age (M = 40.72, SD = 3.51) from southwestern Ontario, recruited for a study of child emotional development (see Table 2.1 for further demographic details). Informed consent was obtained from

children's parents before data were collected. Children with significant medical or psychological problems were excluded from participation via a screening procedure administered by trained study personnel at the recruitment stage. The sample was mostly Caucasian (90.5%) and of average cognitive ability (M = 111.94, SD = 14.32) as assessed by the Peabody Picture Vocabulary Test–Fourth Edition (Dunn & Dunn, 1997; PPVT-4). The study protocols were reviewed and approved by the University of Western Ontario Human Research Ethics Review panel. Finally, as allele frequencies and correlation structures between SNPs in a gene differ across populations, we limited all analyses to the subsample of 371 Caucasian children in the sample.

Table 2.1. Study sample demographics

Characteristic	Participants No. (%)
Sex	
Male	201 (49.1)
Female	208 (50.9)
Caregiver-identified Race/Ethnicity	
Caucasian	371 (90.5)
Asian American	6(2.3)
Other	28 (68)
Parent Education	
Some high school	7(1.8)
High school graduate or some college	212 (52.8)
College graduate or beyond	181 (45.4)
Household Income	
<$20K	22 (5.5)
$20-40K	45 (11.0)
$40-100K	219 (53.5)
>$101K	120 (29.5)

2.2.2 DNA preparation

DNA samples were collected from all participants using buccal swabs (Epicentre, Madison, WI, USA) and stored according to the manufacturer's instructions. Qiagen DNA MicroKit® (Mississauga, ON, Canada) was used to extract DNA from epithelial cells and stored according to the manufacturer's instructions. DNA was successfully extracted from the buccal swab samples of all participants.

2.2.3 SNP selection and genotyping

A SNP marker panel (Table 2.1) was used to tag the full-length *CRH* (RefSeq NM_006180, 1.27 kilobases [kb]), *CRHR1* (RefSeq NM_001145146, 27.3 kb) and *CRHBP* (RefSeq NM_001882, 16.6 kb) according to Human HapMap Project phase II data for the central European population. The Tagger program (http://www.broad.mit.edu/mpg/tagger/; de Bakker et al., 2005) was used to determine the minimum set of SNPs necessary to capture or ‘‘tag’’ all HapMap SNPs (through linkage disequilibrium) with minor allele frequencies >5% among Caucasians. Tag-SNPs (tSNP) validated in Caucasian populations were selected from public databases (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/; HapMap, http://www.hapmap.org) flanking ±10 kb of the respective gene coding regions. This strategy allowed us to genotype variants in the gene promoter regions, which could be functionally important in gene transcription. Specifically, this tagging approach allowed us to select a limited number of SNPs that account for the entire genetic variation across a sequence of DNA by taking into account SNPs that are highly correlated with each other. SNPs tagged had a minimum r^2 of 0.80 with the tagging SNP (mean r^2 was 0.98). The suggested number of tSNPs was four for *CRH*, seven for *CRHR1* and seven for *CRHBP*.

All SNPs were genotyped using a TaqMan allelic discrimination assay (Livak, 1999), developed for use on the StepOne® instrument (Applied Biosystems, Foster City, California). Assay identification numbers and primer sequences for all of the TaqMan probes are provided in Table 2.2. Polymerase chain reactions were performed in 10-µL reaction volumes in 48-well plates and

contained 10 ng of DNA. Thermal cycler conditions were 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The SDS 2.2 software (Applied Biosystems) was used for allelic discrimination. For quality control, 10.0 % of the samples were randomly chosen and genotyped as duplicates across and within a 48-well plate. The genotyping success rate for the *CRHBP* and *CRHR1* genes was 99.1 % or 369 participants, and 98.1 % or 364 participants for the CRH gene. The final number of participants genotyped is provided in Table 2.1 for each gene region.

Table 2.2. The CRH system genes: description of tSNPs.

SNPs	Chromosome location		Nucleotide variant	MAF in study sample	MAF in HapMap, CEU populations[a]	HWE *p*-values
CRH	Chr 8	Gene				
rs9694082	67066780	3' region	C > G	0.18	0.19	0.51
rs6999100	67082732	Intronic	T > C	0.32	0.37	0.70
rs1870394	67102818	5' region	C > T	0.34	0.37	0.72
rs11996294	67114738	5' region	G > T	0.26	0.28	0.49
N =364						
CRHBP	Chr 5					
rs1715751	76239704	Intergenic	C > T	0.36	0.34	0.15
rs1715747	76250378	5' region	T > C	0.45	0.48	0.44
rs32897[b]	76250972	Intronic	T > C	0.22	0.20	0.26
rs7728378	76259350	Intronic	T > C	0.41	0.47	0.91
rs10062367	76264354	Intronic	G > A	0.24	0.24	0.42
rs10473984[b]	76264982	3' UTR	G > T	0.12	0.10	0.49
rs10514082	76266447	Intergenic	A > G	0.17	0.14	0.33
N = 369						
CRHR1	Chr 17					
rs12944712	43871147	3' region	G > A	0.28	0.31	0.25
rs16969853	43880159	Intronic	A > C	0.13	0.13	0.83
rs242924	43885367	Intronic	G > T	0.42	0.46	0.13
rs171441	43893345	Intronic	A > G	0.15	0.13	0.26
rs1396862	43902997	Intronic	G > A	0.12	0.12	0.24
rs17763104[b]	43905795	Intronic	G > A	0.13	0.15	0.65
rs17689966	43910455	5' region	G > A	0.40	0.39	0.54
N = 369						

Note: CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein; Chr, Chromosome; MAF, Minor allele frequency; SNP, Single Nucleotide Polymorphism; HWE, Hardy-Weinberg equilibrium.

[a] Frequency in HapMap European (CEU) populations of the minor allele.

[b] SNPs associated with cortisol reactivity (See Figure 2.1).

Table 2.3: List of tag-SNP-specific primers and their annealing temperatures.

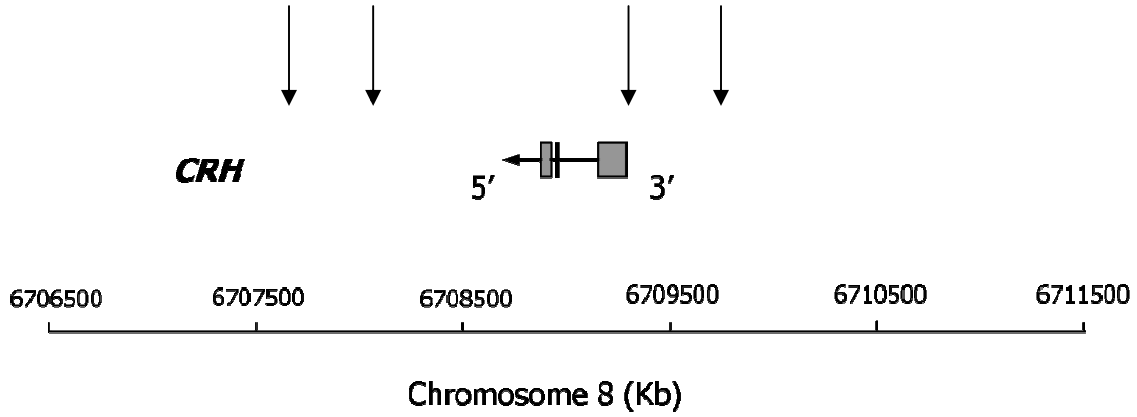
Gene		Allele-specific primer [5' to 3']	Annealing temp. (℃)
CRH			
	rs9694082	AACAAAACAAATTTGAGTTCACTCT**[C/G]**CTGGTGCATGGTGACCTCACACCTG	60
	rs6999100	ATGTATGCATGACATTTTGTTTATC[**C/T**]GCTTCTCCAAGGATGGACTCTTGGG	60
	rs1870394	TAAGGTCAGGGTAATAGAAAAGAAT**[C/T]**ATTCATCATAAGTTTTAAAACTCCC	62
	rs11996294	AGAGCCAATCCTTCAGTAAGATGTC**[G/T]**ATATAAATGCACTGCCCTGAGGTTA	59
CRHR1			
	rs12944712	TAAAGCTGACAGGGCAGGAGACCTG**[A/G**]GGTTGGAGCTGACTCAGCCACTTCT	62
	rs16969853	AAGGGAAGACTAGCCCTTTGCCTGG**[A/C]**ATTTGGCTTCATTTTCTGACGAATC	60
	rs242924	GCATGGCTGCTGCTGGGCAAAAATG**[G/T]**AGAGGGTCCCTGCACCTGAGTGTCT	60
	rs171441	AAGGAGGGCCAACTTCATTTAGCTG**[A/G]**TTCTTCCCTGCAGGGCCAGGGTAGA	62
	rs1396862	GTTGGACCAGGGCTTCTGAACTGCA**[A/G]**AGGTGCTTTTTCCTAAAACCAAGCT	63
	rs17763104	CGGGGTTGCCCTGATGGTTTAAGAC**[A/G]**ATAACAGATATGAAAATCCTCTGTA	62
	rs17689966	AAGCACTGTCCCTCCCCATGCCATC**[A/G]**AGGTGGACGCAGATGACCCTTCCTC	60
CRHBP			
	rs1715751	CACATGCAGAAACCAGAATGGGGCC**[C/T]**GAGGCAAAAGGAAAAGTCACTGACA	62
	rs1715747	ACTTTAAGGGATGAGGAGTTACTTC**[C/T]**TTTGAATGAGCAAAGAAAGCGGTTT	60
	rs32897	GTTCTTGACATTTTAAAGTAATATG**[C/T]**GATGATATTTTTAAAAAATGAGAAA	60
	rs7728378	GATTAAAAAAAAATAAGCACTCCCC**[C/T]**AATATTTTCTACATTGGAAGGTGAG	61
	rs10062367	ATGAGGAGAAAGACTGAATTCAATT**[A/G]**CACTATTCTATAACTAATTATAAGT	60
	rs10473984	AATTTACAGTACCTTTACAGAAGGA**[G/T]**AAAGGTGCCTTCTTCAAAAGGTTTT	60
	rs10514082	CTTCATTTCATAGAAACTAAATTCT**[A/G]**TGAAGCAGGAGGTTGAACCCTTTTC	60

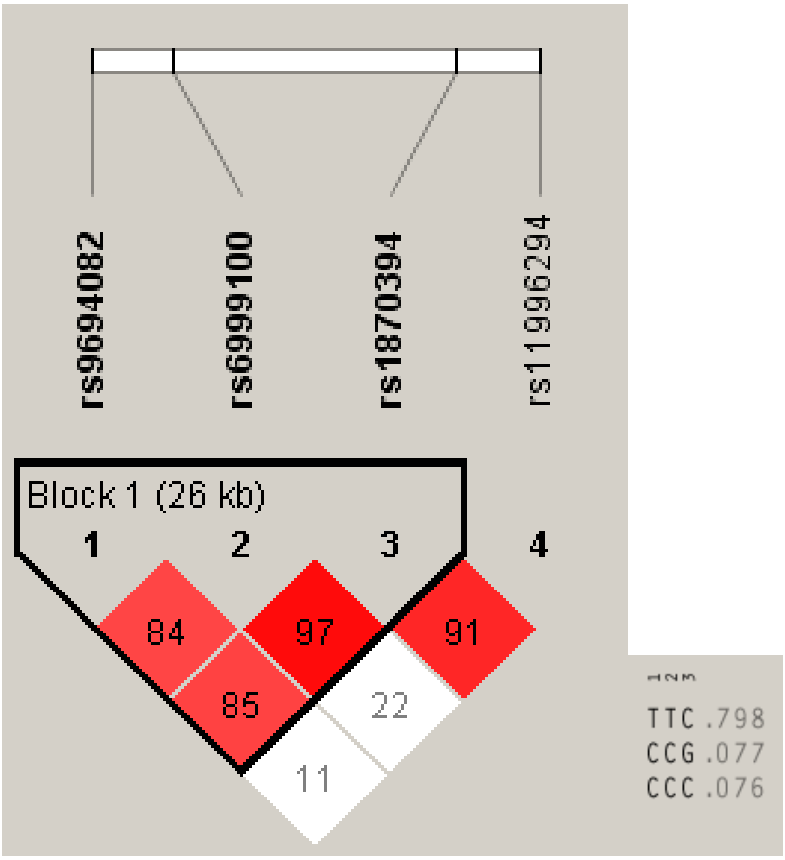
Note: CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein.

2.2.4 Linkage Disequilibrium

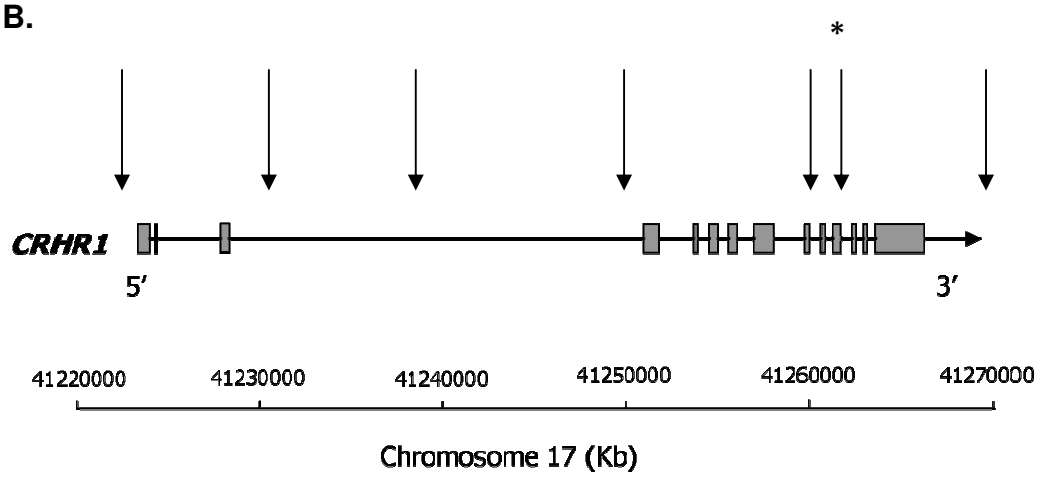
The linkage disequilibrium (LD) pattern and haplotype block delineation were determined using Haploview software version 4.0.51. Blocks were defined using the confidence interval method described by Gabriel et al. (2002) as implemented in Haploview software. Our sample showed a modest LD in the *CRH*, *CRHBP* and *CRHR1* gene coding regions represented by one LD block in of 26 kb, 8 kb and 22 kb respectively (Figure 2.1).

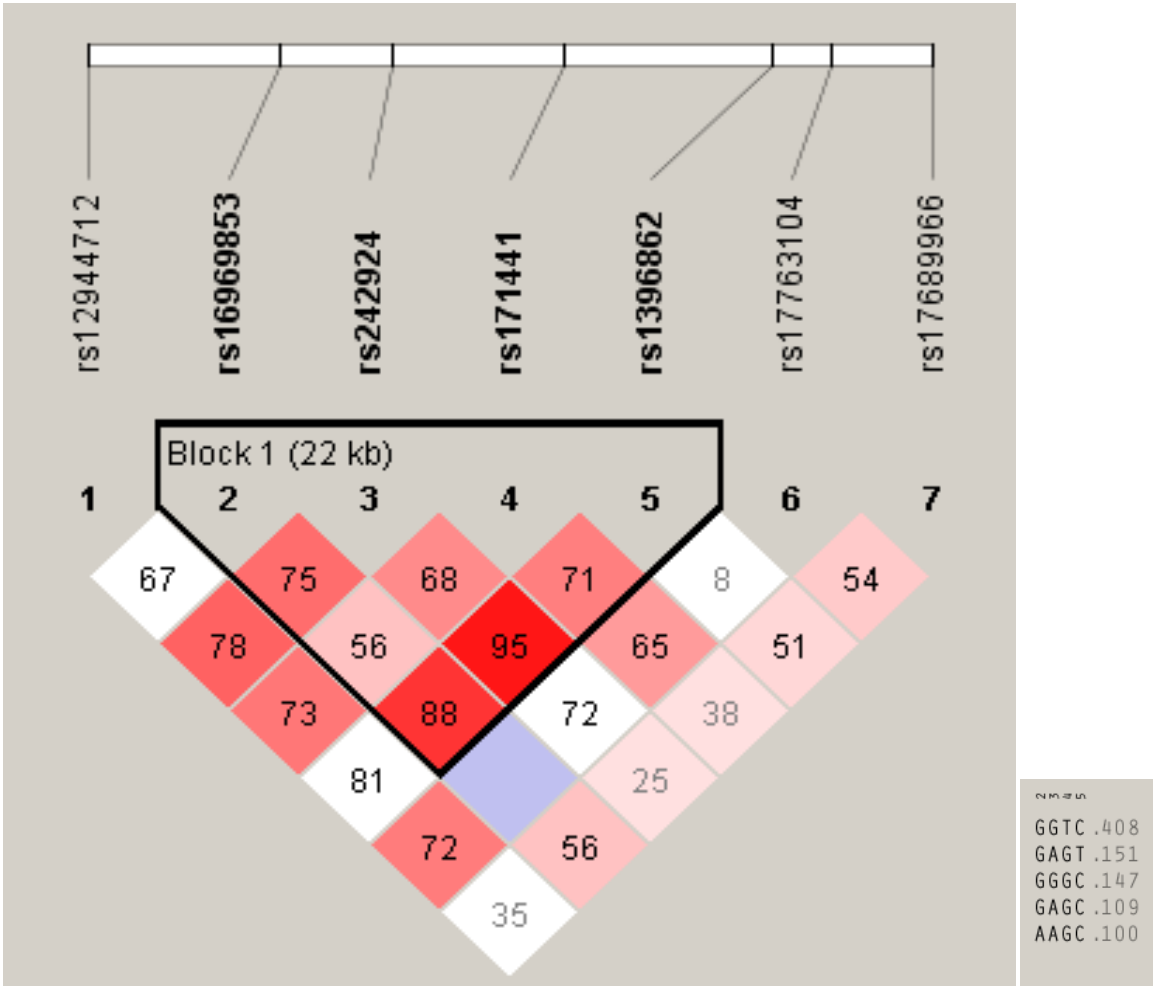
A.


CRH
5′
3′
6706500
6707500
6708500
6709500
6710500
6711500
Chromosome 8 (Kb)

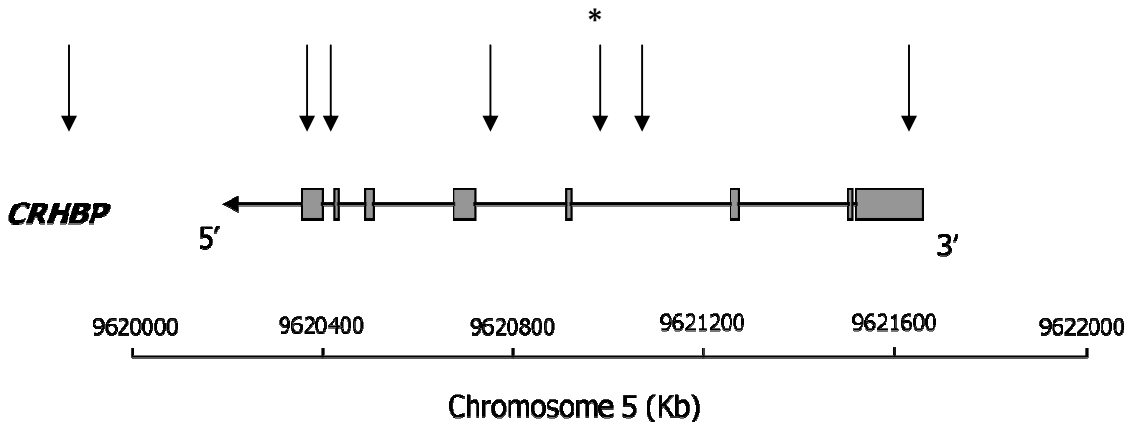

rs9694082
rs6999100
rs1870394
rs11996294
Block 1 (26 kb)
1
2
3
4
84
97
91
85
22
11
TTC .798
CCG .077
CCC .076

B.


*
CRHR1
5′
3′
41220000
41230000
41240000
41250000
41260000
41270000
Chromosome 17 (Kb)


rs12944712
rs16969853
rs242924
rs171441
rs1396862
rs17763104
rs17689966
Block 1 (22 kb)
1
2
3
4
5
6
7
67
75
68
71
8
54
78
56
95
65
51
73
88
72
38
81
25
72
56
35
GGTC .408
GAGT .151
GGGC .147
GAGC .109
AAGC .100

C.


*
CRHBP
5′
3′
9620000
9620400
9620800
9621200
9621600
9622000
Chromosome 5 (Kb)

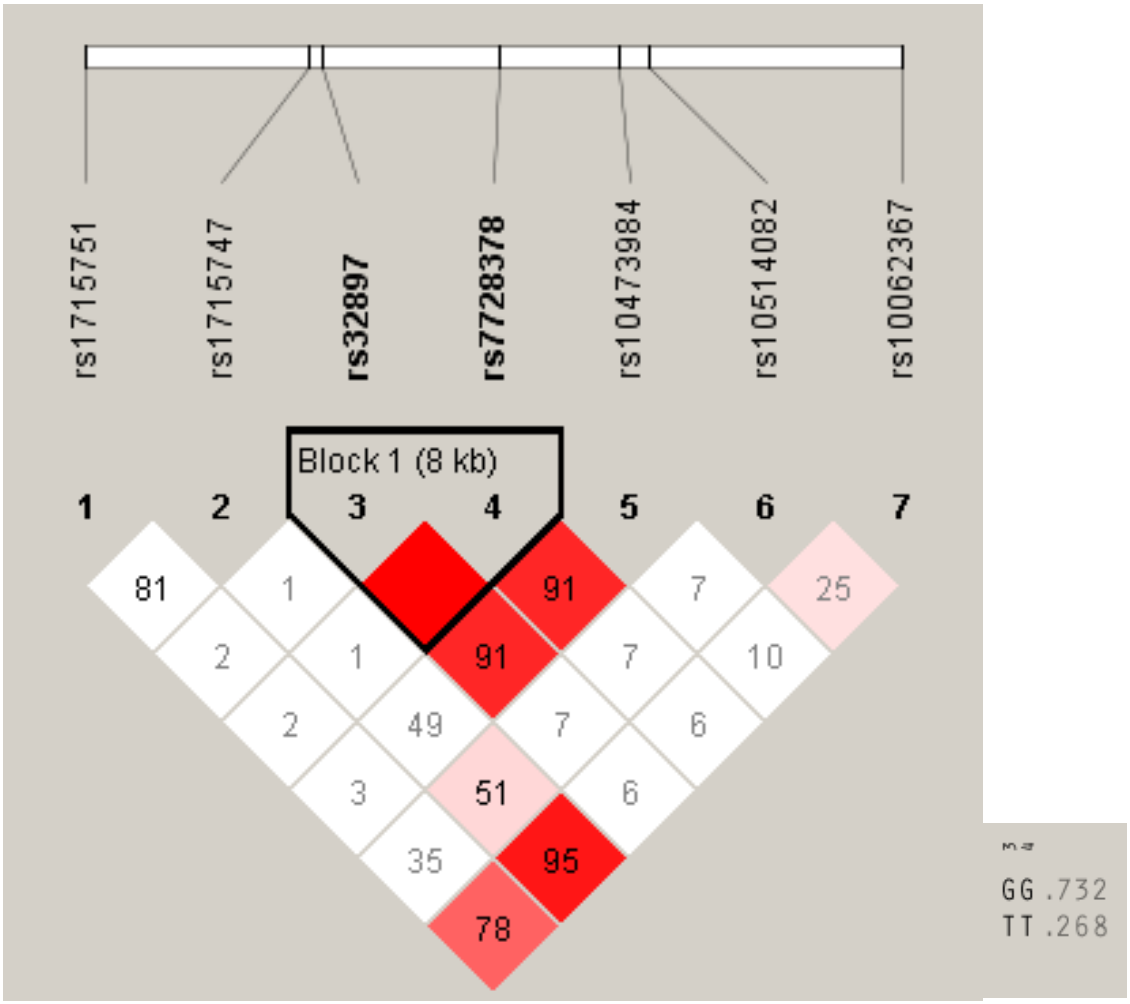

rs1715751
rs1715747
rs32897
rs7728378
rs10473984
rs10514082
rs10062367
Block 1 (8 kb)
1
2
3
4
5
6
7
81
1
91
7
25
2
1
91
7
10
2
49
7
6
3
51
6
35
95
78
GG .732
TT .268

Figure 2.1. The 5' to 3' gene structures, respective chromosome tracks and r^2-based linkage disequilibrium (LD) structures of CRH system genes included in this study. **A**, *CRH*; **B**, *CRHR1* and **C**, *CRHBP* LD structures, spanning across 31 kb, 25 kb and 47 kb respectively. Boxes and lines represent exons and introns. Arrows represent location of tSNPs in relation to the gene coding region. Average distance between SNPs was 7.75 kb for *CRH*, 3.57 kb for *CRHBP* and 4.10 kb for *CRHR1*. Inset shows expected haplotype frequencies for each block based on HapMap CEU populations. dbSNP information for each SNP is presented in Table 2.2. SNPs associated with cortisol reactivity are marked by an asterisk.

2.2.5 Stress task and cortisol sampling procedure

The stress task and cortisol sampling procedures for this study are described in greater detail previously (Kryski et al., 2011) and are outlined in brief here. Several steps were taken to increase the accuracy with which children's baseline cortisol levels were indexed. First, to eliminate the influence of a novel laboratory setting, which has been shown to increase children's cortisol (Donzella et al., 2008), cortisol data were collected during a visit to the family's home by a female experimenter whom the child had met previously during a laboratory visit unrelated to the present study. All visits began between 12:00 pm and 3:30 pm to minimize the effects of diurnal variation on cortisol samples (de Weerth, Zijl & Buitelaar, 2003; Donzella et al., 2008). Caregivers were asked to prevent children from eating or drinking for a half hour prior to the visit to minimize the influence of food/drink on cortisol assays (Magnano, Diamond & Gardner, 1989; Schwartz et al., 1998). None of the children were taking corticosteroids. Finally, the visit began with the child and a familiar experimenter playing quietly with a set of unexciting toys to allow any effect of the experimenter's arrival on children's cortisol to dissipate. After 30 minutes of quiet play, a baseline salivary cortisol sample was collected, followed by the stress task described below.

The stress task was designed to emphasize social evaluation under motivated and uncontrollable circumstances, which has been shown to elicit large cortisol responses in both adults (Magnano, Diamond & Gardner, 1989) and preschool-aged children (Dickerson & Kemeny, 2004; Gunnar, Talge & Harrera, 2009). Briefly, each child attempted to complete a matching task by

matching coloured game pieces to animal icons based on an answer key. A large toy replica of a traffic light was placed adjacent to the board, and the child was instructed that the traffic light would show how much time they had to complete the task and win a prize, with “green” indicating that they had time to work, and “red” indicating that they were out of time. The experimenter surreptitiously controlled the traffic light via remote control to that no child could complete the task on time during any of the three trials conducted. The mean duration of the task for children who completed all three trials was 15.01 min (SD = 1.5), including the instruction period. Supporting the validity of this task as a means of inducing stress, our group has previously shown that it successfully elicited a mean increase in children’s cortisol, and that child negative affect increased as a result of participating in this task (Kryski et al., 2011). Following the stress task, the child and experimenter resumed quiet play while the remaining cortisol samples were collected every 10, 20, 30, 40, and 50 minutes.

To obtain cortisol, children chewed on a 2-inch absorbent cotton dental roll until it was wet with saliva, which was expunged into a micro tube and frozen at -20℃. Studies consistently report high correlations in saliva to serum cortisol concentrations (Daniel et al., 2006; Dorn et al., 2009; Eatough et al., 2009). Saliva samples were assayed in duplicate using salivary cortisol enzyme immunoassay kit (Salimetrics, PA, USA). Optical density was read on a standard plate reader at 450 nm and corrected at 650 nm (Molecular Devices, Sunnyvale, CA, USA). All samples from the same child were assayed in the same batch with no duplicates varying more than 5%. The average intra- and interassay

coefficients were 3.5 and 5.1%. Standard curve and concentration of unknown samples were generated according to manufacturer's instructions using a 4-parameter sigmoid minus curve fit. Cortisol data were skewed and were therefore $\log_{10}$ transformed prior to all analyses, a standard procedure with human cortisol data (Schwartz et al., 1998).

Three aspects of cortisol stress reactivity were examined. I calculated individual cortisol response for each participant by calculating post-stress-task cortisol change scores [baseline cortisol – peak cortisol post stress task], hereafter referred to as "baseline to peak change." I also calculated the area under the curve (AUC) as a measure of cortisol response. The AUC is comprised two types of information; first, the intensity of the response or AUC_i, and second, the total response as a function of time or AUC_g (Figure 2.2). With endocrinological data, AUC_g is assumed to be a measure more related to total hormonal output, whereas AUC_I is a parameter that emphasizes the changes over time and is more related to sensitivity of the system (Fekedulegn et al., 2007; Pruessner et al., 2010).

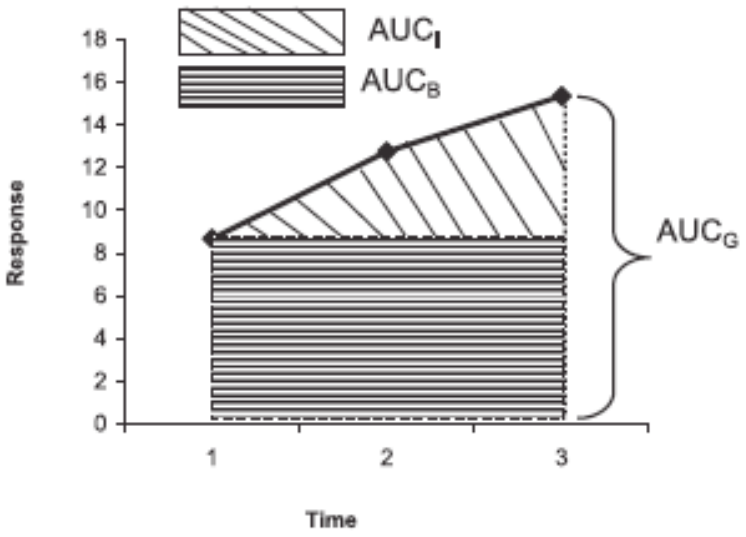


Figure 2.2. Cortisol response over 3 time points in an arbitrary dataset. AUC_I is the area under the curve above the referent baseline measurement (shaded region). (Adapted from Fekedulegn et al., 2007).

2.2.6 Behaviour measures

The child's primary caregiver completed the Child Behavior Checklist (CBCL; Achenbach, 2001), a standardized parent report measure of the frequency and intensity of child behavioural and emotional problems exhibited in the last 6 months. Since I was primarily interested in the presence and severity of children's specific internalizing disorder symptoms, alternative scale scores derived to be consistent with DSM-IV diagnostic criteria for disorders were used (Lengua et al., 2001) including empirically-derived scales indexing depression (α = 0.81) and anxiety (α = 0.77).

2.2.7 Assessment of life stress

Several factors known to contribute to children's early development were examined: familial chronic stress (Burge & Hammen, 1991); marital discord (Hammen, Brennan, & Shih, 2004); and socioeconomic status (SES; Lupien et al., 2000). The UCLA Life Stress Interview was used to assess chronic stress as reported by the primary caregiver. Trained Ph.D. students in clinical psychology gathered information from each child's primary caregiver related to their intimate relationships, close friendships, social life, family relationships, childcare hassles, work, finances, health of the primary caregivers, and health of close family members (Adrian & Hammen, 1993; Hammen, 1991). The interviewer then assigned Likert style ratings to the level of stress present in each domain from low (1) to high (5). Reliability was assessed by having a second coder rerate the chronic stress descriptions for 12 of the interviews (average ICC = 0.78). Life stress ratings for each domain were averaged to create an average life stress variable.

Primary caregiver reports of relationship adjustment were collected using the Dyadic Adjustment Scale (DAS; Spanier, 1976). The DAS is a 32-item questionnaire of marital adjustment designed for use with either married or unmarried cohabiting couples (Spanier, 1976). The instrument provides a global score of dyadic adjustment which can range from 0 to 151, with higher scores reflecting higher level of dyadic adjustment. For the purposes of this study, the global score were reverse-coded so that higher scores reflect greater marital discord. Previous research has demonstrated good psychometric properties

(Baillargeon, Dubois, & Marineau, 1986). The internal consistency for global dyadic adjustment was good (α = 0.83).

Family income was reported by one of the child's caregivers as an index of family socio-economic status. This data was collected on a generic demographic form along with other basic demographic characteristics. Family income scores were generated that ranged from < $20,000 coded as 1 to income > $100,000 coded as 5. For the purposes of this study family income was reverse coded so that higher scores reflect lower family income and greater socioeconomic stress. Following Evans and colleagues (2013), an aggregate index of children's cumulative stress exposure was created by first standardizing and then averaging these variables. This overall life stress aggregate, which isI referred to as "childhood stress" or CS, was used in all subsequent analyses.

2.2.8 Data Analyses

Descriptive statistics for children's genotypes, gender, age, cortisol reactivity, CS and symptoms are presented in Table 2.1. Next, bivariate correlations between the main outcome measures were tested using Pearson correlation analysis (Table 2.1). I used linear regression to assess whether SNPs in the *CRH*, *CRHR1* and *CRHBP* genes predicted child cortisol reactivity scores. I first considered single-SNP analyses that regressed cortisol reactivity on tSNP genotype (coded under additive, recessive & dominant models). I also investigated whether epistatic interactions between CRH system polymorphisms predicted cortisol reactivity using regression models. I established the significance of genotype–mean cortisol reactivity using permutation-based

procedures (Schmidt et al., 2002, 2003a) that randomly assigned the sample cortisol reactivity scores to subjects (sampled without replacement) while holding each subject's genotype fixed. This permutation method is preferred as it accounts for LD among SNPs in a haplotype block therefore conserving power compared to commonly used correction techniques such as the Bonferroni method (Schmidt et al., 2003b). For each analysis, the empirical P value was based on 10,000 permutations. I conducted these analyses using appropriate components of PLINK (Purcell et al., 2007).

To examine whether CRH gene pathway tSNPs moderated associations between CS and child internalizing symptoms, multiple regression was used as implemented in the macro developed by Hayes (2012). This macro uses a regression-based path analytical framework for estimating direct and indirect effects in simple and multiple moderation models, two- and three-way interactions in moderation models along with simple slopes and regions of significance for probing interactions. Bootstrap methods are implemented for inference about indirect effects in both unmoderated as well as mediated moderation models. All predictor values were centered as needed.

2.3 RESULTS

2.3.1 Associations between study variables and CRH system gene polymorphisms

We first looked at whether links existed between study demographics and cortisol reactivity measures. Table 2.4 presents correlations between all major

study variables. Cortisol response measures (i.e., AUC_i, AUC_g and baseline to peak cortisol change scores) were highly positively correlated with each other. As baseline to peak cortisol change and AUC_i are highly correlated and capture the intensity of cortisol response, we chose to present only the AUC_i as a dependent variable in subsequent sections. As is frequently found in the literature on cortisol reactivity, higher family income was associated with lower cortisol reactivity (Chen et al., 2010; Lupien et al., 2001) and with fewer depressive and anxious symptoms. Child sex was associated with symptoms such that girls had more anxious and depressive symptoms compared to boys in the sample. No significant associations between child sex and the three measures of cortisol response were found, as expected based on the literature (Mackinaw-Koons & Vasey, 2000; Stroud et al., 2004). Anxious symptoms were positively correlated with all measures of cortisol reactivity (AUC_i, AUC_g and baseline to peak cortisol change). All symptom measures were positively correlated. Finally, the CS measure was positively associated with all measures of cortisol response and internalizing symptoms, but negatively correlated with family income.

Table 2.4. Correlations among study variables

	1	2	3	4	5	6	7	8	9
1. baseline to peak change [peak – baseline]	--								
2. AUC_i	.881***	--							
3. AUC_g	.686**	.724***	--						
4. PPVT	-.026	.006	.003	--					
5. Family Income	-.074	-.090	-.123*	.092	--				
6. Child Sex	.002	.030	-.008	.064	.009	--			
7. Anxious Symptoms	.116*	.114*	.117*	.041	-.125*	.133*	--		
8. Depressive Symptoms	-.018	-.002	.006	-.032	-.120	.144**	.313**	--	
9. Childhood stress	.146**	.141*	.176**	-.118*	-.624**	.017	.161**	.264**	--
Mean	0.065	.050	.180	111.98	3.76	1.498	1.258	1.273	0.096
(SD)	(0.053)	(.090)	(.123)	(14.11)	(1.10)	(.500)	(1.442)	(1.533)	(.645)

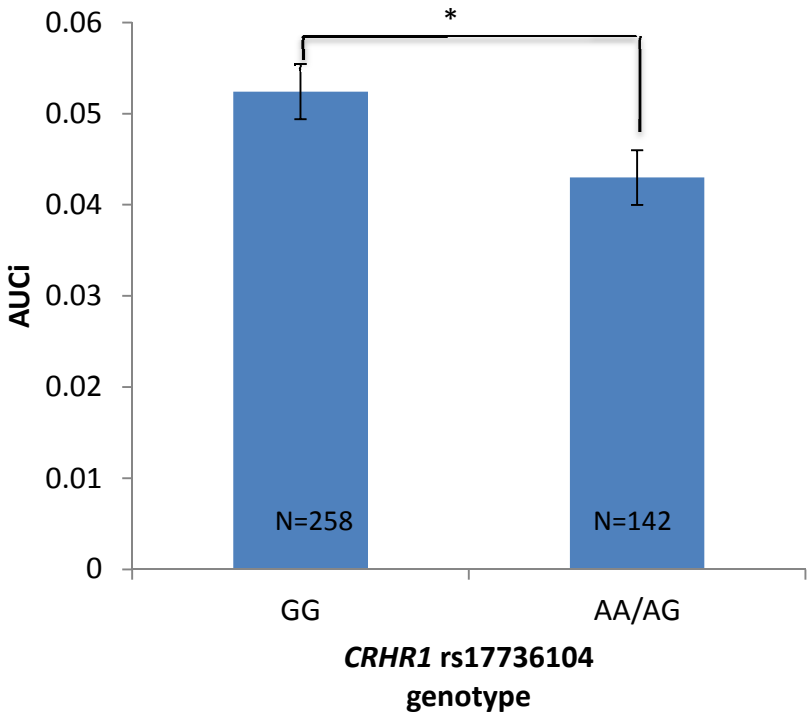
Note: Child gender was coded as males = 0 and females = 1; Family income was coded as 1 = < $20,000; 2= $20,000-$40,000; 3= $40,001-$70,000; 4 = $70,001-$100,000; 5 = > $100,001. AUC_i = area under curve with respect to baseline; AUC_g = area under curve with respect to ground; PPVT = Picture Vocabulary Test–Fourth Edition (Dunn & Dunn, 1997).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

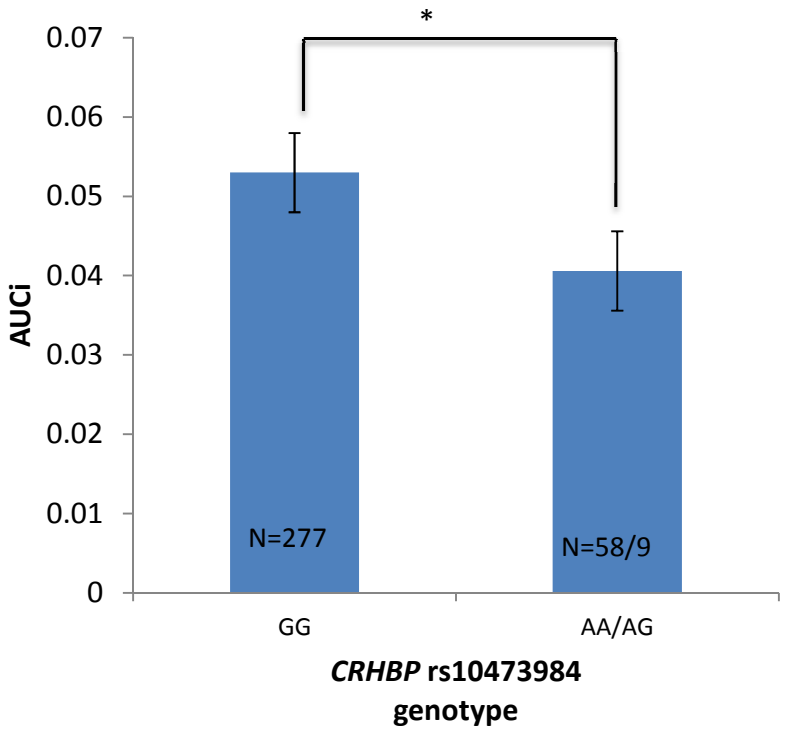
Regression analyses showed no significant associations between demographic variables and the 18 tSNPs genotypes in this study (*ps* > 0.11). Of the 18 tSNPs genotyped, only two tSNPs were significantly associated with cortisol reactivity or AUC_i after correcting for multiple tests, *CRHR1* tSNP rs17763104 and *CRHBP* tSNP rs10473984. The *CRHR1* tSNP rs17763104 genotypes predicted child AUC_i under an ancestral recessive model, with the *CRHR1* tSNP rs17763104 GG homozygote children having significantly lower cortisol reactivity (AUC_i) relative to heterozygotes and AA homozygotes (β = 0.13, *se* = 0.05, *t* = 1.98, *p* = 0.01) (Figure 2.3A). Similarly, an association also existed between children's AUC_i and *CRHBP* tSNP rs10473984 when coded under a recessive model (β = 0.05, *se* = 0.03, *t* =1.14, *p* = 0.02), such that the GG homozygote children had significantly higher AUC_i than their counterparts who have at least one copy of the T allele (Figure 2.3B).

An association also existed between children's total cortisol response or AUC_g and *CRHBP* tSNP rs10062367 and cortisol reactivity when coded under a recessive model (β = -0.04, *se* = 0.01, t = -2.74, *p* < 0.01), such that the GG homozygous children had significantly higher AUC_g than children with at least one copy of the A allele (Figure 2.3C).

A.


*
0.06
0.05
0.04
0.03
0.02
0.01
0
AUCi
N=258
N=142
GG
AA/AG
CRHR1 rs17736104
genotype

B.


*
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
AUCi
N=277
N=58/9
GG
AA/AG
CRHBP rs10473984
genotype

C.

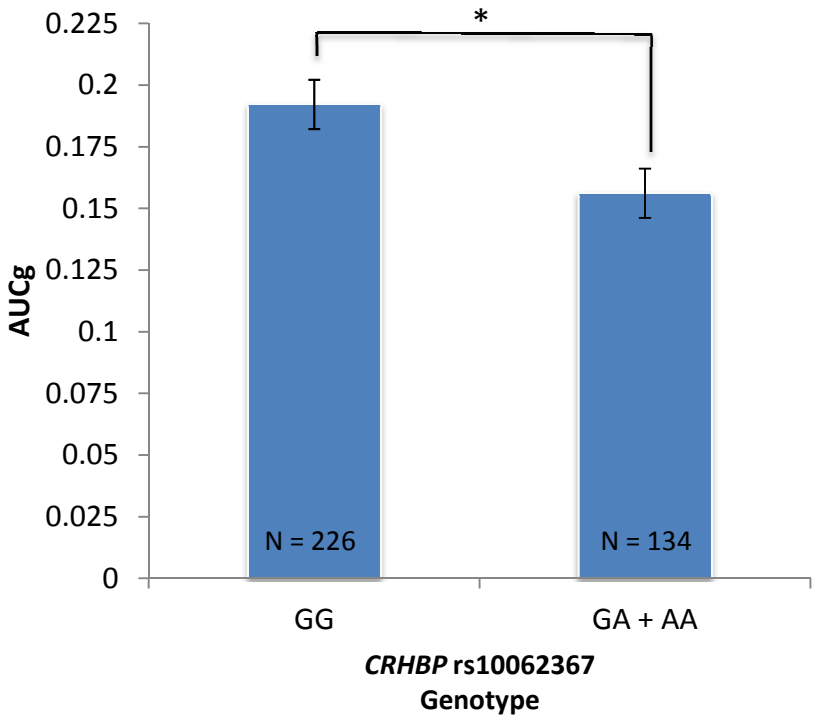


Figure 2.3. Distribution of AUC_i (±SEM) in children with the (**A)** *CRHR1* rs17763104 and (**B)** *CRHBP* rs10473984. AUC_g as a function of *CRHBP* rs10062367 genotypes (**C)**.

Note: CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein; tSNP, tagging Single Nucleotide Polymorphism. Inset, N, represents number of children in each genotypic group. * $p < 0.05$.

2.3.2 CRHR1 haplotypes are associated with cortisol reactivity to stress

One haplotype block extended distally across the gene and included four tSNPs from rs16969853 to rs1396862 (Figure 2.1B). There were five haplotypes with a frequency > 0.05 accounting for 94% of haplotype diversity. Figure 2.4 shows the relationship between *CRHR1* haplotype carriers inferred from the 22kb LD block and children's cortisol reactivity measured via AUC_i. Children with the GGTC and AAGC haplotypes had significantly lower cortisol reactivity than the GAGT, GGGC and GAGC haplotype groups (all *ps* < 0.05) but these two haplotypes were not significantly different in cortisol reactivity (all *ps* > 0.05). All four tSNPs were in strong LD for the 26kb *CRH* gene region. There were four haplotypes with a frequency of > 0.05 that accounted for 99% of haplotype diversity. Similarly two tSNPs were in strong LD in a 8kb *CRHBP* gene region (all *ps* > 0.23). There were two haplotypes with a frequency of > 0.05. The *CRHBP* haplotypes were also not associated with measures of cortisol reactivity (all *ps* > 0.14).

No links were found between the *CRHR1* haplotypes and cortisol response measured via AUC_g (all *ps* > 0.05).

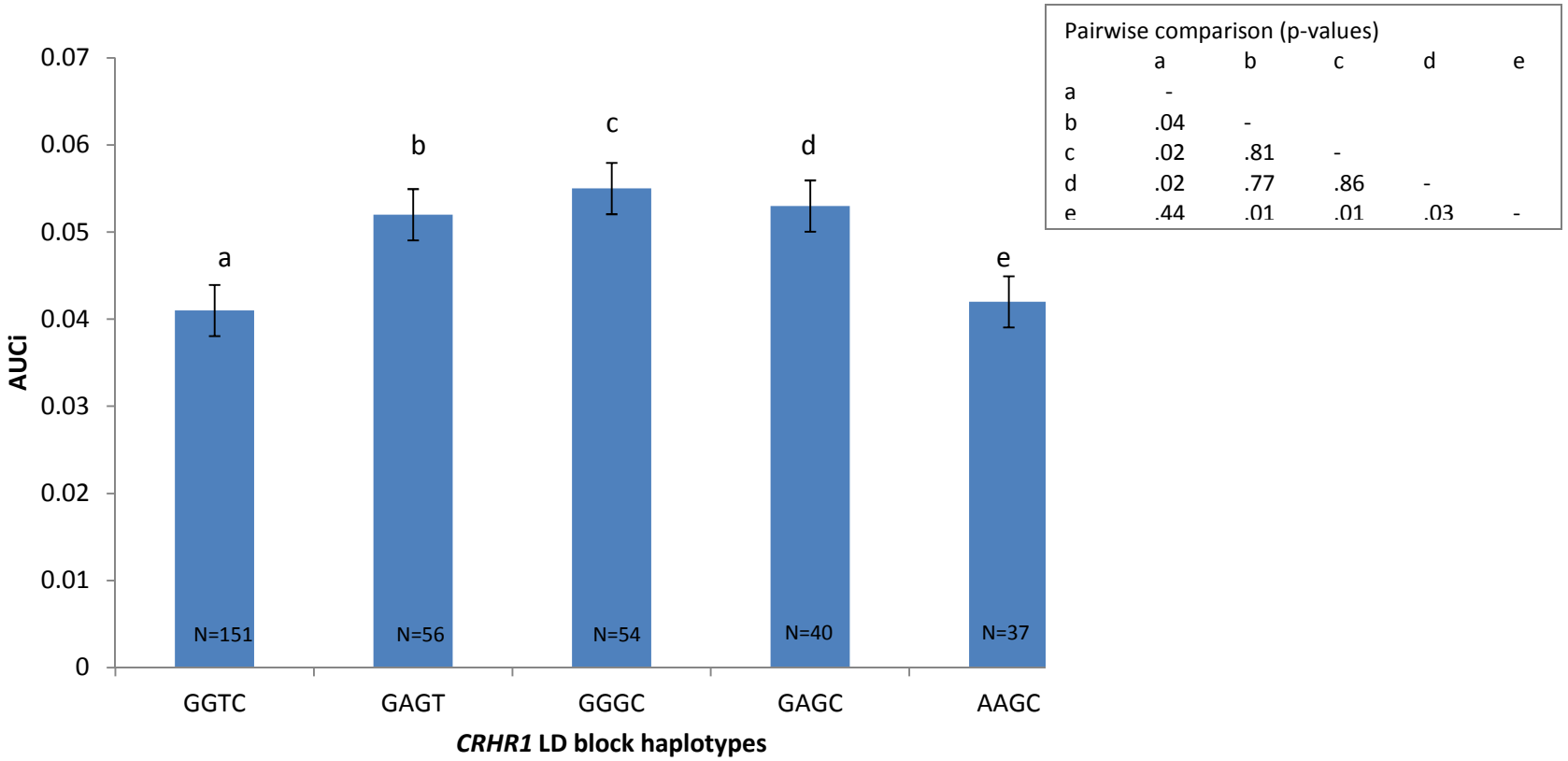


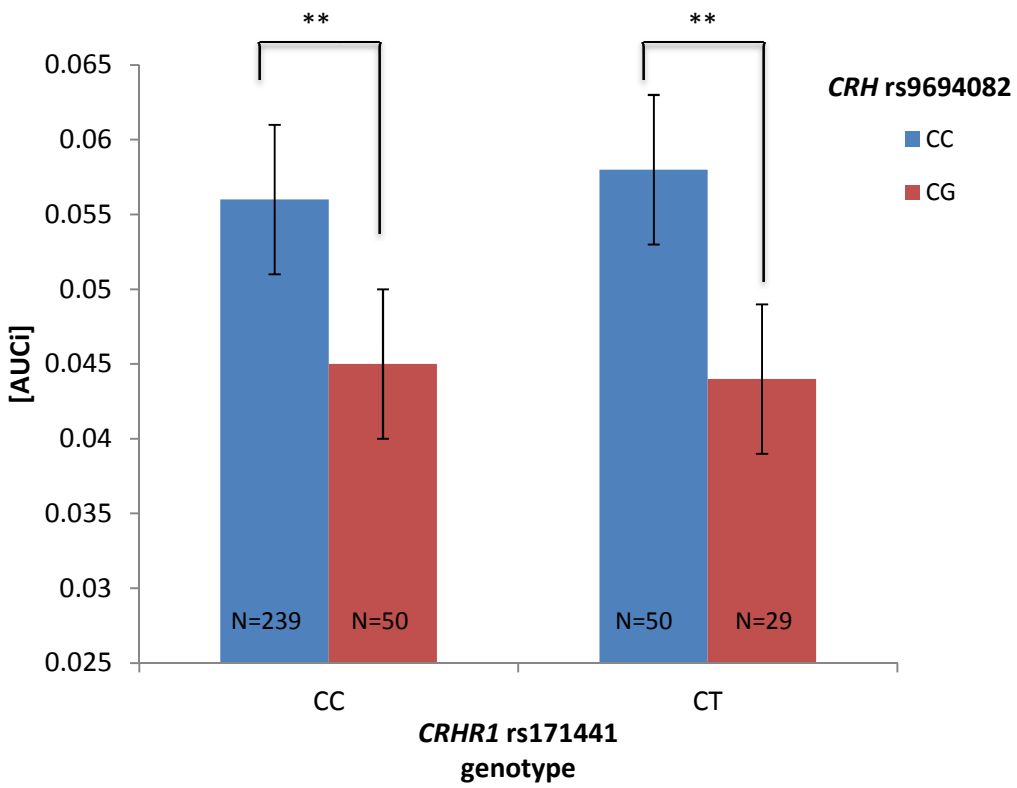
Figure 2.4. Mean salivary AUC_i as a function of *CRHR1* haplotypes in linkage block 1 (Figure 2.1). Two haplotype groups showed significantly lower cortisol reactivity compared to the rest of the haplotype groups.

Note: Inset represents pairwise comparisons between haplotype groups.

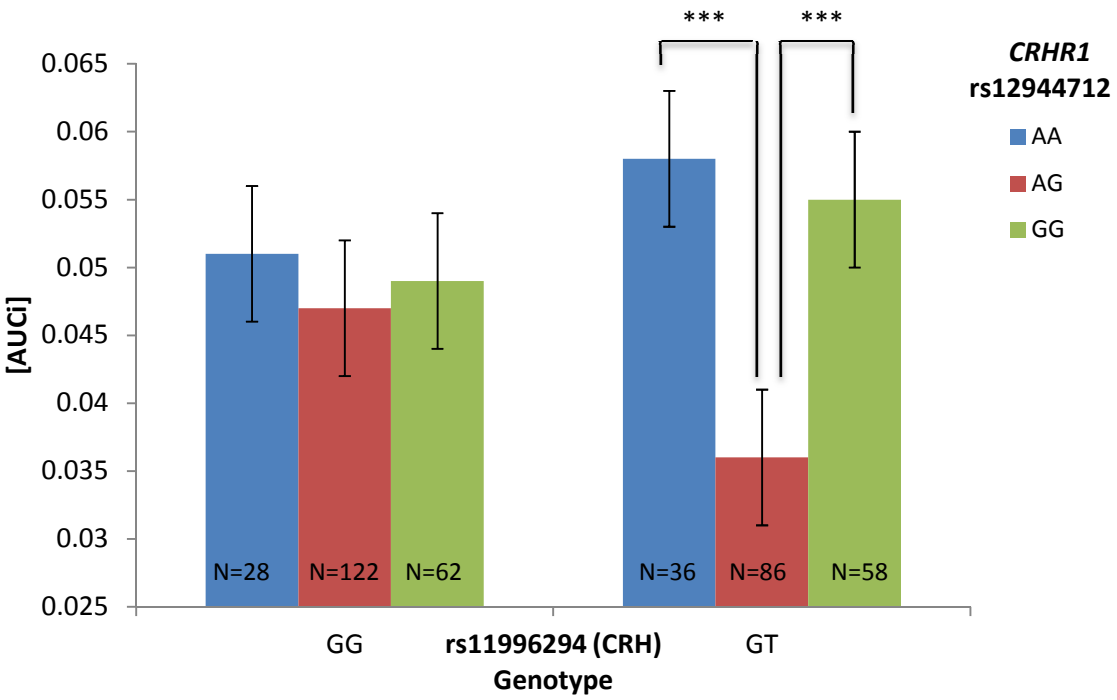
2.3.3 Gene–gene interactions and cortisol reactivity

I also investigated pairwise interactions between tSNPs genotypes in predicting cortisol reactivity, finding effects of three tSNP pairs, illustrated in Figure 2.5. Regression analyses showed a significant interaction between two tSNP pairs on chromosome 8 and 17. The first significant interaction was between *CRH* rs9694082 and *CRHR1* rs171441 (β = -1.10, *se* = 0.52, corrected $p < 0.01$; Figure 2.5A), such that *CRHR1* rs171441 heterozygotes had significantly lower cortisol reactivity than their homozygous counterparts (all *ps* < 0.01). Figure 2.5B illustrates the second significant interaction that was found between *CRH* rs11996294 and *CRHR1* tSNP rs12944712 (β = -2.97, *se* = 0.50, corrected $p < 0.01$) such that carriers who were heterozygous for the CRH rs11996294 tSNP but homozygous for the rs12944712 G or A alleles had significantly higher cortisol reactivity than their rs12944712 GA heterozygous counterparts (all *ps* < 0.001). Figure 2.5C illustrates the interaction between the *CRHR1* rs171441 and *CRHBP* rs32897 predicting child cortisol reactivity (β =1.41, *se* = 0.25, corrected $p = 0.01$). Carriers homozygous for the rs32897 G allele substitution had significantly higher cortisol reactivity compared to carriers with at least one copy of the rs32897 A allele (all *ps* < 0.01).

A.


**
**
0.065
0.06
0.055
0.05
0.045
0.04
0.035
0.03
0.025
[AUCi]
N=239
N=50
N=50
N=29
CC
CT
CRHR1 rs171441 genotype
CRH rs9694082
CC
CG

B.


***
***
0.065
0.06
0.055
0.05
0.045
0.04
0.035
0.03
0.025
[AUCi]
N=28
N=122
N=62
N=36
N=86
N=58
GG
rs11996294 (CRH)
GT
Genotype
CRHR1 rs12944712
AA
AG
GG

C.

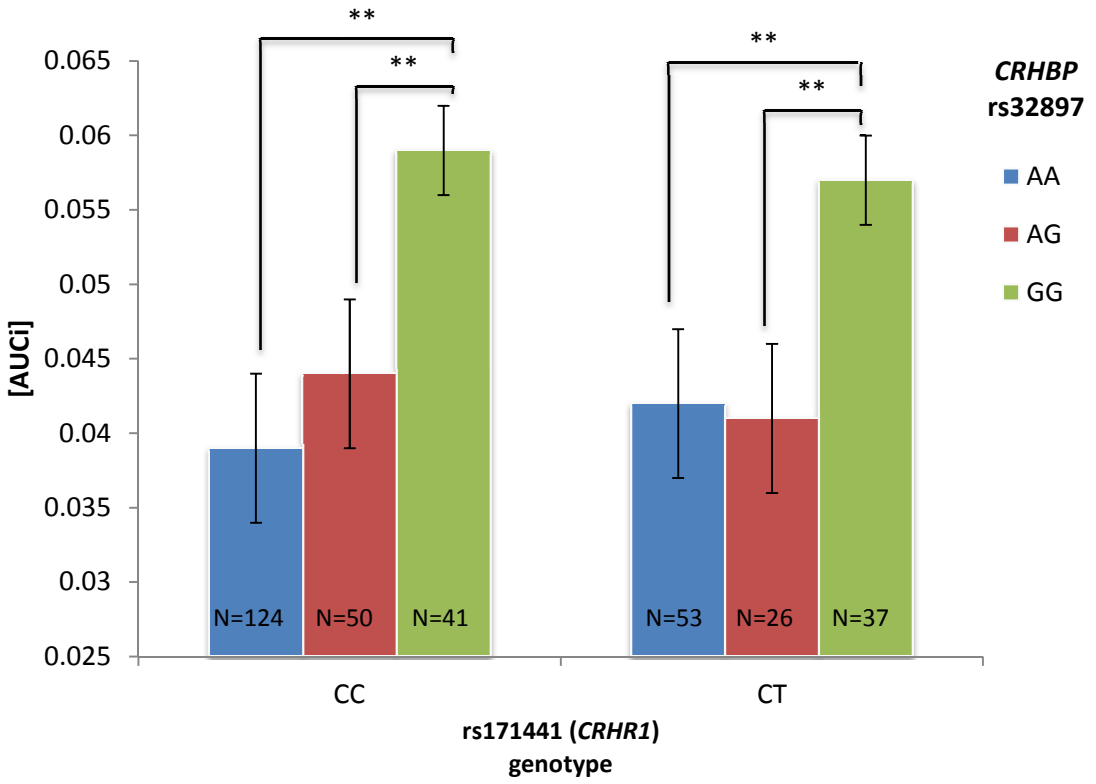


Figure 2.5. Gene-gene interactions predict cortisol reactivity measured via AUC_i. A, Interaction effect between *CRHR1* tSNP rs171441 and *CRH* tSNP rs9694082 genotypes with respect to AUC_i. B, Interaction effect between *CRHR1* tSNP rs12944712 and *CRH* tSNP rs11996294 genotypes with respect to AUC_i. C, Interaction effect between *CRHR1* tSNP rs171441 and *CRHBP* tSNP rs32897 genotypes with respect to AUC_i. Data are presented as mean AUC_i (±SEM).

Note: CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein; Chr, Chromosome; tSNP, tagging-Single Nucleotide Polymorphism. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Gene-gene interactions also predicted overall cortisol response or AUC_g. Figure 2.6A illustrates the interaction effect between *CRHR1* tSNP rs242924 and *CRHR1* tSNP rs12944712 genotypes predicting AUC_g (β =2.81, *se* = 0.25, corrected $p = 0.001$) such that children heterozygous for the two tSNPs had lower cortisol response than children who were not heterozygous for these tSNPs. Figure 2.6B illustrates the interaction between *CRHR1* tSNP rs16969853 and *CRH* tSNP rs17689966 genotypes predicting child AUC_g (β =2.07, *se* = 0.18, corrected $p < 0.01$) such that children homozygous for the G allele had lower cortisol response when compared to children who were either heterozygous or homozygous for the A allele.

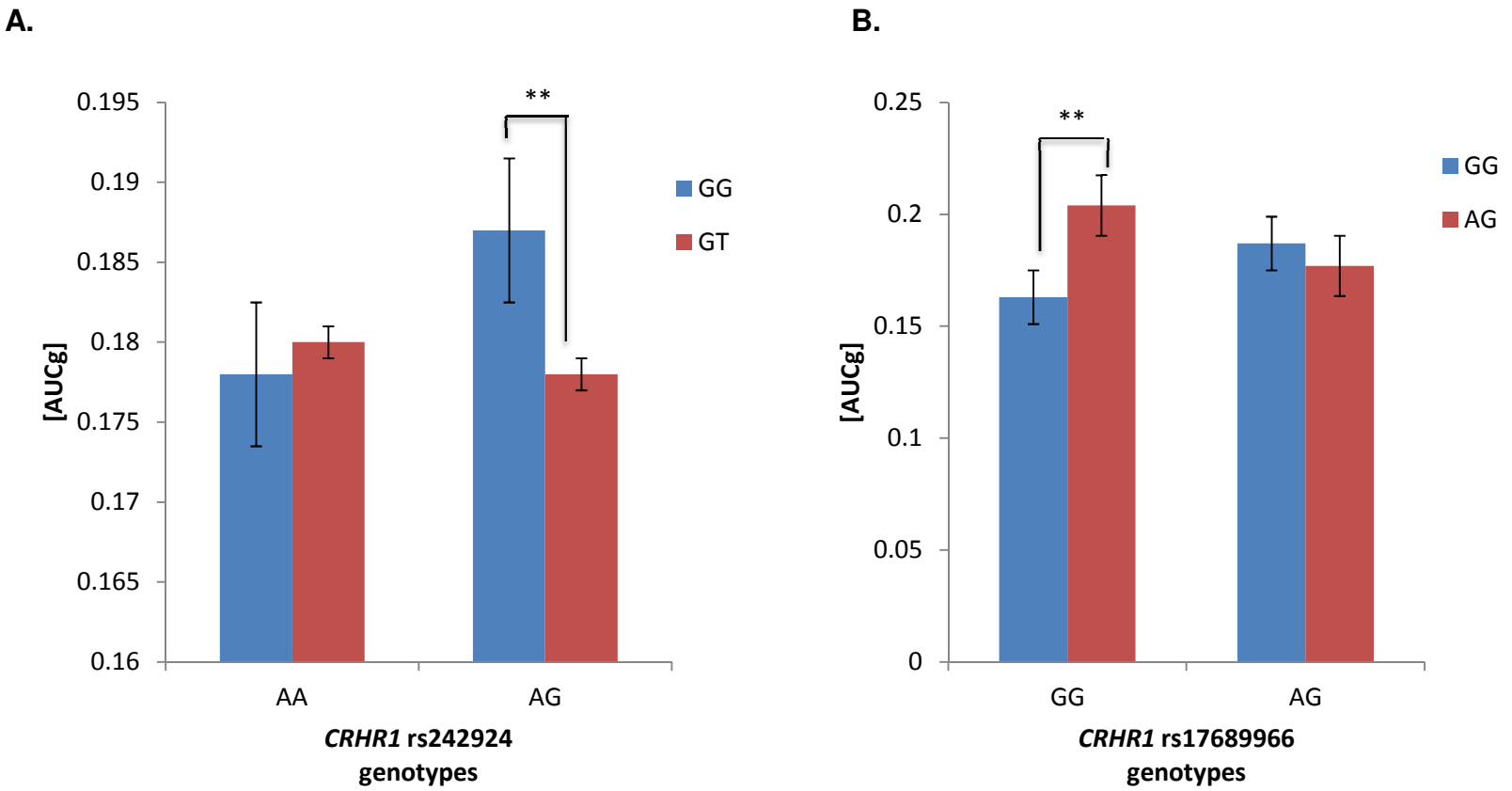


Figure 2.6. Gene-gene interactions predict total cortisol response measured via AUC_g. A, Interaction effect between *CRHR1* tSNP rs242924 and *CRHR1* tSNP rs12944712 genotypes **(A)** and *CRHR1* tSNP rs16969853 and *CRH* tSNP rs17689966 genotypes **(B)** as a function of AUC_g.

Note: AUC_g, area under curve with respect to ground; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein; Chr, Chromosome; tSNP, tagging-Single Nucleotide Polymorphism. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

2.3.4 CRH pathway tSNPs are associated with emerging symptoms of depression and anxiety

Based on the hypotheses that the CRH system gene variants may contribute to depressive and anxious symptoms, I examined such links in this sample of preschoolers. Results of these analyses are presented in Table 2.5. I found significant main effects of the tSNPs and genotype on children's symptoms of depression and anxiety. First, the *CRH* gene tSNP, rs9694082, was associated with child anxious symptoms (Figure 2.7A), such that the carriers for at least one copy of the G allele had significantly higher anxious symptoms than their counterparts homozygous for the C allele ($t = 2.30$, $df = 368$, $p = 0.02$).

Similarly, the *CRHR1* tSNP, rs242924, was also associated with symptoms of depression and anxiety in preschoolers (results presented in Figures 2.7B and 2.7C). Children who carried at least one copy of the G allele had significantly higher depressive ($t = -2.48$, $df = 368$, $p = 0.01$) and anxious symptoms ($t = -2.27$, $df = 368$, $p = 0.02$) compared to children homozygous for the T allele.

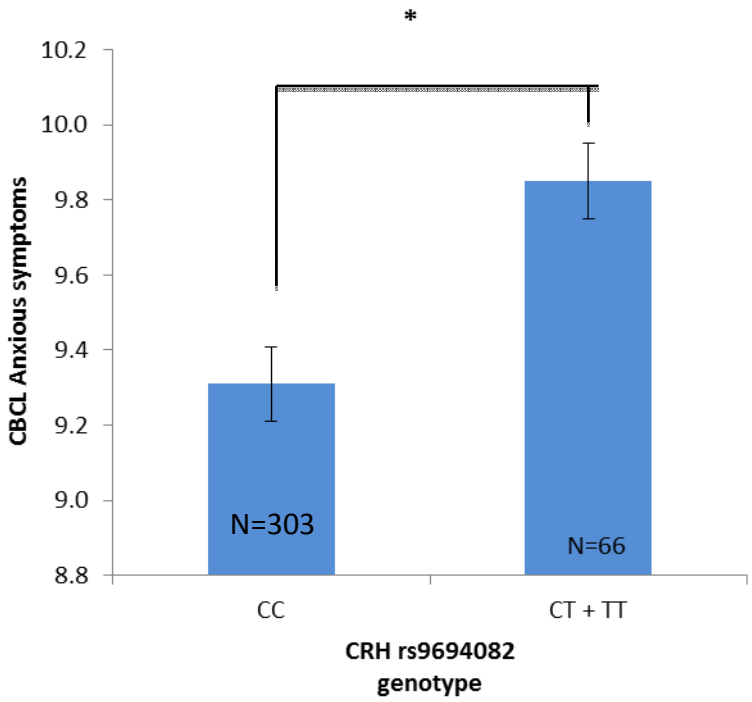
One tSNP in the *CRHBP* gene region was also associated with child anxious symptoms (Figure 2.7D), such that children who were carriers of the major allele or A allele had significantly higher anxious symptoms than children carrying the minor allele or G allele ($t = 3.67$, $df = 360$, $p < 0.01$).

Table 2.5. Main effects of CRH system gene variants on emerging depressive and anxious symptoms.

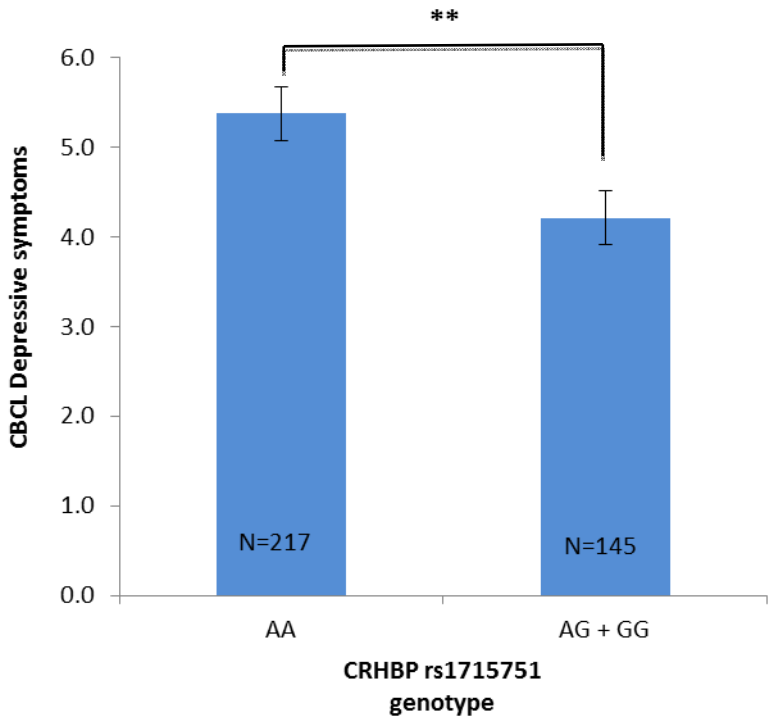
		Depressive symptoms		Anxious symptoms	
Gene *(N=369)*	tSNP	Additive model	Allelic model	Additive model	Allelic model
CRH		*p-value*		*p-value*	
	rs9694082*	0.58	0.41	0.06	**0.02**
	rs6999100	0.92	0.85	0.65	0.71
	rs1870394	0.60	0.51	0.73	0.66
	rs11996294	0.16	0.06	0.75	0.25
CRHR1					
	rs12944712	0.19	0.20	0.80	0.55
	rs16969853	0.55	-	0.81	-
	rs242924**	0.26	**0.01**	**0.05**	**0.05**
	rs171441	0.81	0.14	0.97	0.31
	rs1396862	0.53	0.94	0.13	0.66
	rs17763104	0.22	0.27	0.47	0.68
	rs17689966	0.18	0.15	0.60	0.49
CRHBP					
	rs1715751**	0.32	0.08	0.15	**0.01**
	rs1715747	0.41	0.26	0.86	0.73
	rs32897	0.98	0.88	0.99	0.94
	rs7728378	0.48	0.31	0.29	0.31
	rs10062367	0.71	0.56	0.94	0.82
	rs10473984	0.08	0.11	0.37	0.22
	rs10514082	0.55	0.31	0.36	0.58

Note: CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein.

A.


*
10.2
10.0
9.8
9.6
9.4
9.2
9.0
8.8
CBCL Anxious symptoms
N=303
N=66
CC
CT + TT
CRH rs9694082
genotype

B.


**
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CBCL Depressive symptoms
N=217
N=145
AA
AG + GG
CRHBP rs1715751
genotype

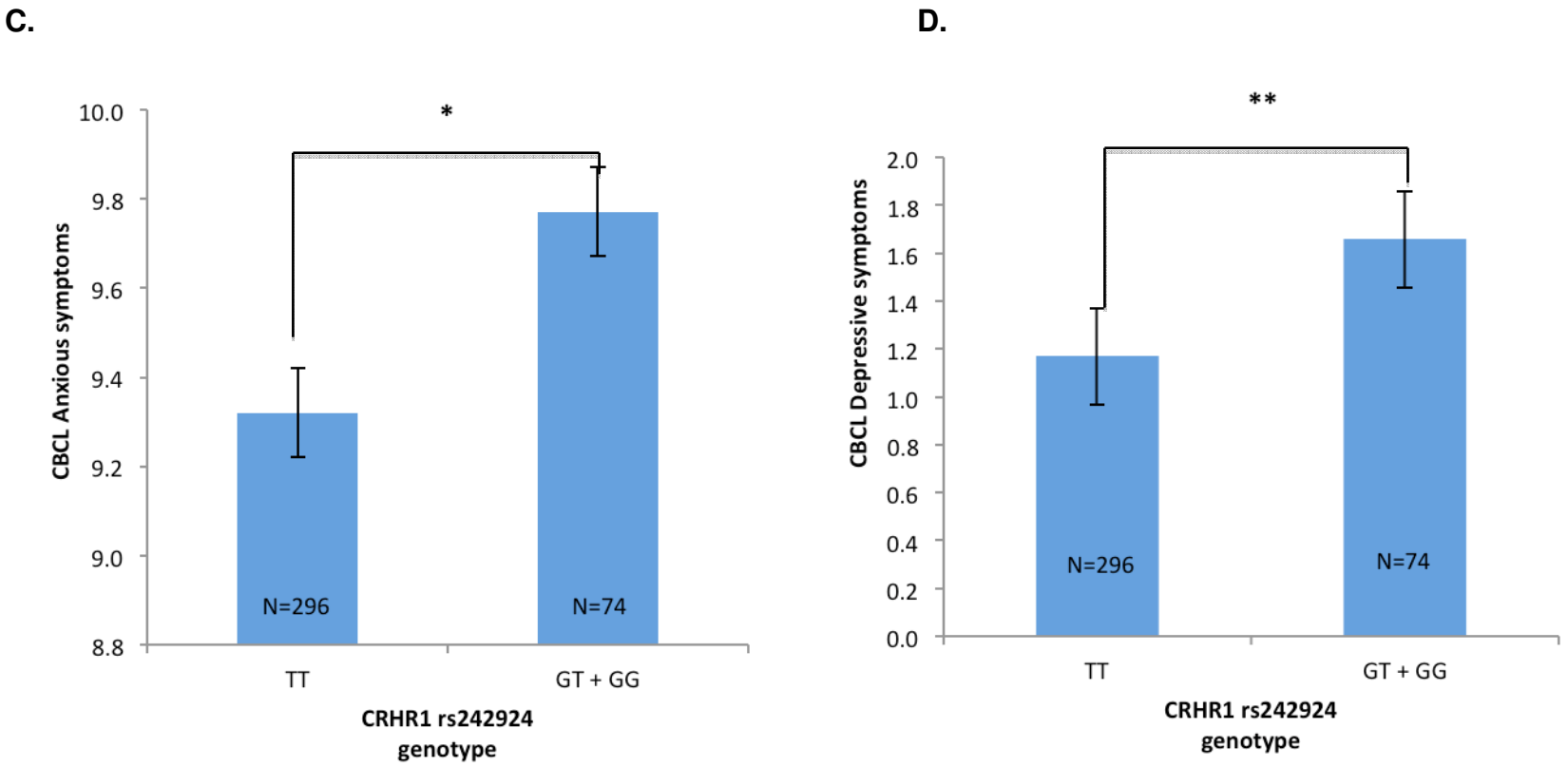


Figure 2.7. CRH pathway gene tSNPs are associated with emerging symptoms of depression and anxiety. Results derived from the best genetic model are reported. Symptoms are plotted as a function of genotype for **A)** *CRH* tSNP, rs9694082, and anxiety symptoms; **B, C)** *CRHR1* tSNP, rs242924, and depressive and anxious symptoms; **D)** *CRHBP* tSNP rs1715751 and depressive symptoms in preschoolers. *$p < 0.05$; **$p < 0.01$.

2.3.5 Interactions between CRH system gene variants and childhood stress predict cortisol response to stress in preschoolers

In the next set of analyses, I examined whether the interactions between CRH system tSNPs and CS were associated with children's cortisol AUC_i (Table 2.6). I found two tSNPs in the *CRHBP* coding region which acted as moderators of the association between CS and children's AUC_i. The first significant interaction term included *CRHBP* rs1715747 tSNP and CS. To understand the moderation effect, I plotted the association between cortisol and CS for the two allelic groups (Figure 2.8A). My analysis showed that children with at least one copy of the G allele showed higher AUC_i, as CS levels increased (β = 0.09, se = 0.02, $p < 0.001$, 95% CI: 0.04 – 0.13, $N = 71$); in contrast, the association between AUC_i and CS in children homozygous for the T allele was not fully significant (β = 0.02, *se* = 0.01, $p = 0.07$, 95% CI: -0.00 – 0.03, $N = 300$).[1]

I also found a similar evidence for moderation in the *CRHBP* gene variant as well. The interaction term for *CRHBP* rs32897 tSNP and CS was also significant (Table 2.6). I plotted the levels of CS for the two allelic groups (Figure 2.8B). My analysis showed that children with at least one copy of the G allele showed higher AUC_i as CS levels increased (β = 0.21, se = 0.04, $p < 0.001$, 95% CI: 0.13 – 0.29, $N = 219$); in contrast, the association between AUC_i and CS in

[1] The gene-environment interaction term did not predict AUC_g, $p > 0.66$.

children homozygous for the T allele was not significant (β = 0.01, *se* = 0.01, *p* = 0.31, 95% CI: -0.01 – 0.03, *N* = 148).[2]

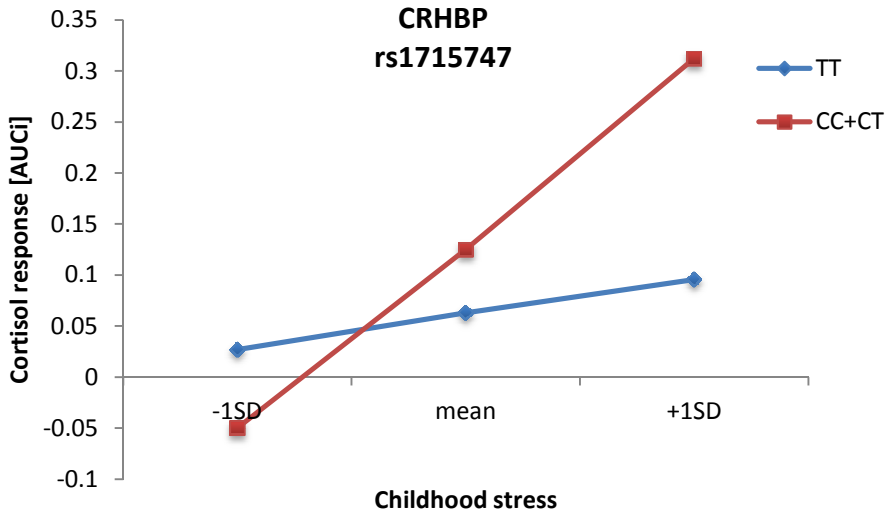
[2] Similar to rs1715747 tSNP, the interaction between the rs32897 tSNP and CS did not predict AUCg, $p > 0.23$.

Table 2.6. Interaction between CRH system tSNPs and childhood stress (CS) effect on stress response measured via cortisol AUC_i.

Gene	SNP x CS	model	*β*	*se*	*p-value*
CRH					
	rs9694082	REC	-0.01	0.02	0.59
	rs6999100	REC	0.00	0.02	0.99
	rs1870394	ADD	-0.02	0.02	0.25
	rs11996294	REC	-0.02	0.02	0.17
CRHR1					
	rs12944712	ADD	0.00	0.01	0.96
	rs16969853	ADD	0.01	0.02	0.73
	rs242924	ADD	0.01	0.01	0.95
	rs171441	REC	-0.02	0.03	0.37
	rs1396862	ADD	0.01	0.02	0.69
	rs17763104	ADD	0.03	0.02	0.18
	rs17689966	REC	0.02	0.02	0.33
CRHBP					
	rs1715751	ADD	-0.02	0.02	0.26
	rs1715747*	DOM	0.24	0.01	**0.04**
	rs32897***	REC	0.19	0.04	**0.00**
	rs7728378	ADD	0.01	0.01	0.70
	rs10062367	REC	-0.13	0.02	0.07
	rs10473984	ADD	0.00	0.02	0.91
	rs10514082	ADD	0.02	0.02	0.41

Note: SNP, single nucleotide polymorphism; AUC, area under curve; ADD, additive model; REC, recessive model; DOM, dominant model; CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein.

A.



B.

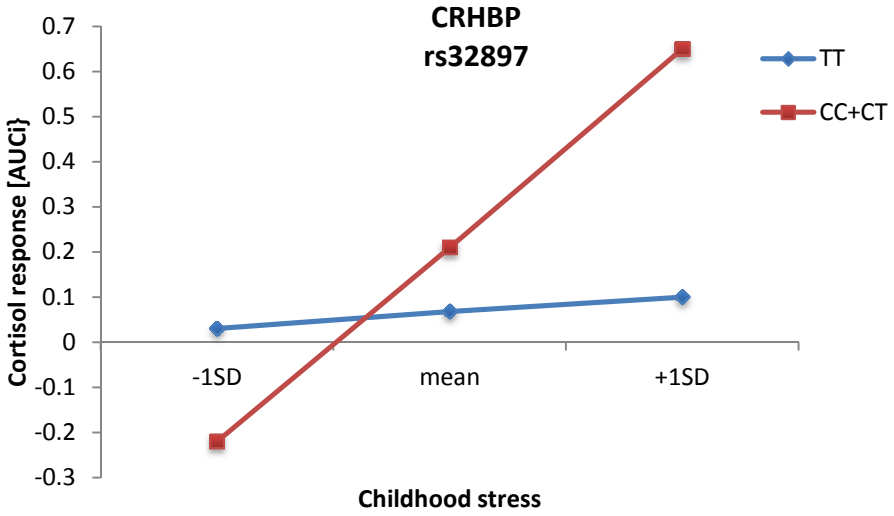


Figure 2.8. Relationship between childhood stress and AUC_i by *CRHBP* tSNP rs1715747 **(A),** and **(B)** *CRHBP* tSNP rs32897 genotypes.

2.3.6 GxE between CRH system genetic variation and childhood stress predict emerging symptoms of depression and anxiety

Based on recent findings (Heim et al., 2009), I investigated whether CRH pathway gene variation would moderate the link between CS and internalizing symptoms in preschoolers. I found three tSNPs which acted as moderators of the association between CS and children's depressive or anxious symptoms. The interaction terms for *CRHR1* rs242924 tSNP and CS were significant for both depressive and anxious symptoms (Table 2.7). I plotted levels of child depressive and anxious symptoms as a function of CS and *CRHR1* rs242924 tSNP genotype (Figure 2.9A). My analysis showed that children with at least one copy of the G allele showed an increasing levels of depressive symptoms as the levels of CS increased ($\beta = 0.20$, $se = 0.07$, $p < 0.001$, 95% CI: 0.06 – 0.34, $N = 275$); in contrast, the link between depressive symptoms and CS in children homozygous for the T allele was not significant ($\beta = 0.06$, se = 0.13, $p = 0.38$, 95% CI: -0.07 – 0.14, $N = 96$). Similarly, my analysis showed that children with at least one copy of the G allele showed increasing levels of anxious symptoms as CS increased ($\beta = 0.58$, se = 0.16, $p < 0.001$, 95% CI: 0.26 – 0.90, $N = 275$); in contrast, the link between anxious symptoms and CS in children homozygous for the T allele was not significant ($\beta = 0.08$, se = 0.18, $p = 0.65$, 95% CI: -0.28 – 0.44, $N = 96$; Figure 2.9B).

I also found similar evidence for moderation in the *CRHBP* gene variant as well. The interaction term for *CRHBP* rs7728378 tSNP and CS was also significant (Table 2.8). I plotted levels of child depressive symptoms and CS for

the two allelic groups (Figure 2.9C). My analysis showed that children with at least one copy of the A allele showed an increasing levels of depressive symptoms as the life stress increased (β = 0.26, *se* = 0.29, *p* = 0.35, 95% CI: -0.30 – 0.82, *N* = 217); in contrast, the link between depressive symptoms and CS in children homozygous for the G allele was not significant (β = - 1.16, *se* = 0.33, *p* < 0.001, 95% CI: 0.51 – 1.79, *N* = 145).

Table 2.7. Summary of interactions between CRH system tSNPs and childhood stress (CS) predicting emerging anxiety symptoms. Significant model *p*-values are in bolded text.

Gene	SNP x CS	model	*β*	se	*p*-value
CRH					
	rs9694082	REC	0.23	0.35	0.51
	rs6999100	REC	0.40	0.29	0.17
	rs1870394	ADD	0.32	0.30	0.28
	rs11996294	REC	0.16	0.26	0.54
CRHR1					
	rs12944712	ADD	0.20	0.23	0.38
	rs16969853	ADD	-0.27	0.35	0.44
	rs242924*	REC	0.38	0.18	**0.03**
	rs171441	REC	-0.45	0.40	0.26
	rs1396862	ADD	0.02	0.27	0.93
	rs17763104	ADD	0.28	0.31	0.48
	rs17689966	REC	0.30	0.23	0.21
CRHBP					
	rs1715751	ADD	-0.18	0.27	0.50
	rs1715747	DOM	-0.13	0.27	0.62
	rs32897	REC	0.31	0.26	0.25
	rs7728378	ADD	0.08	0.19	0.66
	rs10062367	ADD	0.19	0.17	0.28
	rs10473984	ADD	0.32	0.37	0.17
	rs10514082	ADD	-0.06	0.30	0.85

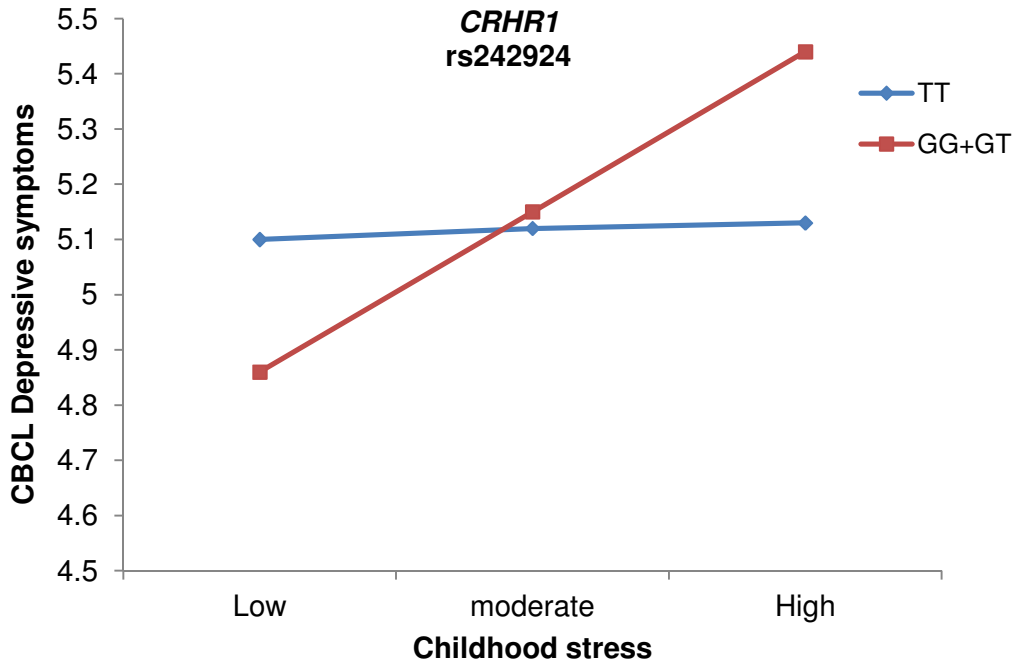
Note: SNP, single nucleotide polymorphism; ADD, additive model; REC, recessive model; DOM, dominant model; CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein. $^{*}p < 0.05$; $^{\dagger}p < 0.10$.

Table 2.8. Summary of interactions between CRH system tSNPs and childhood stress (CS) predicting emerging depressive symptoms. Significant model *p*-values are in bolded text.

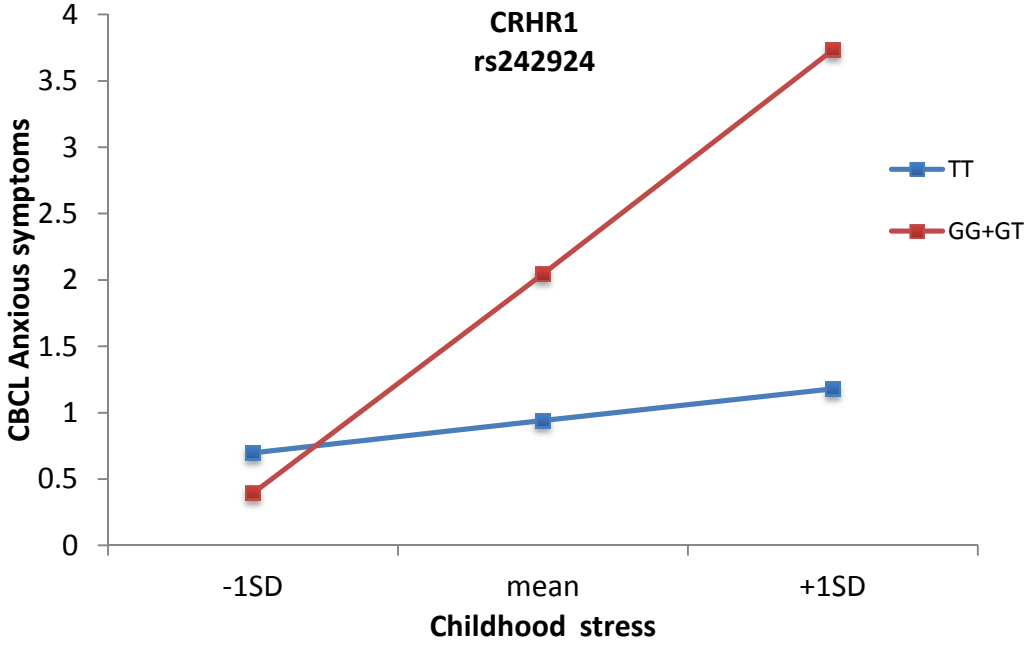
Gene	SNP x CS	model	*β*	*se*	*p-value*
CRH					
	rs9694082†	REC	0.43	0.25	0.09
	rs6999100	REC	0.20	0.28	0.48
	rs1870394†	ADD	0.51	0.29	0.08
	rs11996294	REC	0.38	0.34	0.26
CRHR1					
	rs12944712	ADD	0.12	0.20	0.54
	rs16969853	ADD	-0.36	0.34	0.29
	rs242924*	ADD	0.47	0.18	**0.01**
	rs171441†	REC	-0.66	0.39	0.09
	rs1396862	ADD	-0.13	0.26	0.62
	rs17763104	ADD	0.18	0.29	0.54
	rs17689966	REC	0.11	0.17	0.64
CRHBP					
	rs1715751	ADD	0.26	0.25	0.30
	rs1715747	DOM	-0.20	0.18	0.25
	rs32897	REC	-0.13	0.22	0.55
	rs7728378*	ADD	-0.42	0.18	**0.02**
	rs10062367	REC	0.58	0.26	0.58
	rs10473984	ADD	-0.55	0.36	0.13
	rs10514082	ADD	0.30	0.31	0.34

Note: SNP, single nucleotide polymorphism; ADD, additive; REC, recessive; DOM, dominant; CRH, Corticotrophin releasing hormone; CRHR1, Corticotrophin releasing hormone receptor-1; CRHBP, Corticotrophin releasing hormone binding protein. *$p < 0.05$; †$p < 0.10$.

A.


CRHR1
rs242924
CBCL Depressive symptoms
5.5
5.4
5.3
5.2
5.1
5
4.9
4.8
4.7
4.6
4.5
Low
moderate
High
Childhood stress
TT
GG+GT

B.


CRHR1
rs242924
CBCL Anxious symptoms
4
3.5
3
2.5
2
1.5
1
0.5
0
-1SD
mean
+1SD
Childhood stress
TT
GG+GT

C.

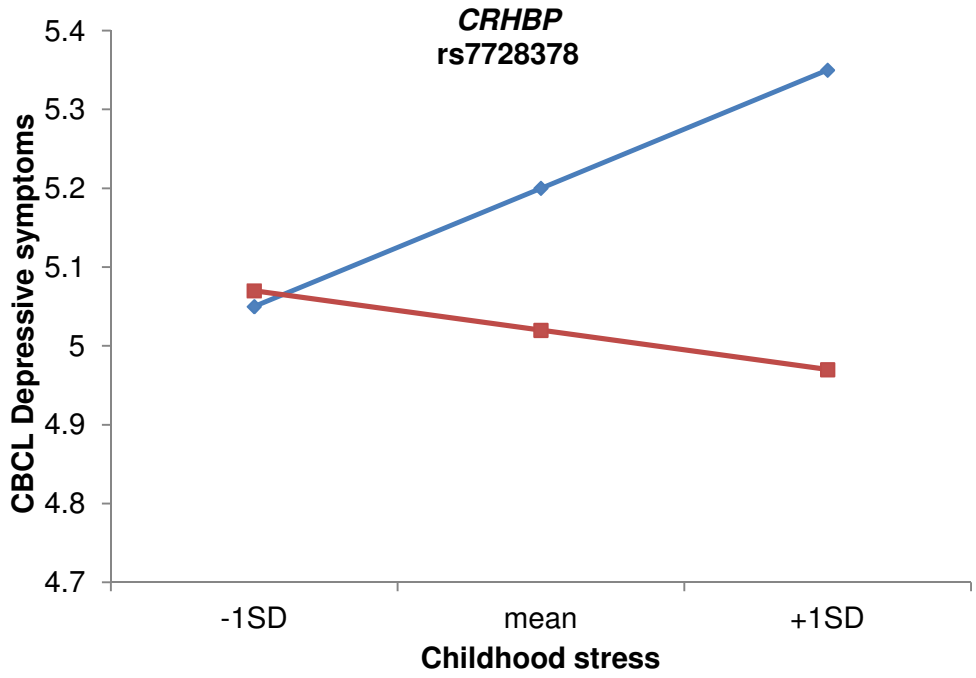


Figure 2.9. Relationship between levels of childhood stress and emerging symptoms by *CRHR1* tSNP rs242924 **(A, B)** and *CRHBP* tSNP rs7728378 **(C)** genotypes.

2.4 DISCUSSION

The first aim of this study was to explore links between genetic variation of the CRH system genes and children's cortisol responses to stress. Analyses found evidence for associations between CRH pathway gene variations and both the intensity (measured via AUC_i) and total hormonal response measured via AUC_g. Specifically, tSNPs of the *CRHR1* and *CRHBP* gene-coding regions were associated with preschoolers' cortisol responses to stress. The *CRHR1* tSNP, rs17736104 was associated with childhood AUC_i. Even though the *CRHR1* tSNP rs17763104 is intronic, it is likely that this SNP is flanked by nonsynonymous SNPs in *CRHR1* gene exons 6 through 8. Although speculative, it is possible that one of these SNPs may have functional effects on *CRHR1* gene transcription, thus influencing the number of functional CRH receptors on the pituitary gland leading to changes in the release of ACTH, and ultimately affecting cortisol release from the adrenal gland. Recent research indicates that most *CRHR1* isoforms are a result of splicing variation within the exon 6 through 10-coding regions (van Pett et al., 2000). Furthermore, intronic SNPs have also been shown to exert functional effects on gene expression when the intron is part of the gene's regulatory region. It is likely that this tSNP may be linked to such a variant, which may be regulating the *CRHR1* gene expression. Future research is needed to investigate the functional effects this SNP and either the regulatory nature or the alternatively spliced variants from this coding region on ACTH release from the pituitary and stress sensitivity.

I also found associations between *CRHR1* haplotypes and cortisol reactivity. Children with the GGTC and AAGC haplotypes showed significantly lower cortisol reactivity than those with the GAGT, GGGC and GAGC haplotypes. The linkage block that encompasses these haplotypes contain exons code for the transmembrane parts of the mature CRHR1 protein. It is plausible that the genetic variation in this region may lead to changes in the transmembrane protein structure, which initiates downstream signaling of this g-protein couple receptor. Differences in downstream activation could lead to altered release of ACTH and eventually cortisol release from the adrenal medulla. Animal knockout research lends some support to this hypothesis by showing that the *CRHR1* gene is critical for normative cortisol reactivity to stress. To my knowledge, my study is the first to show that common genetic variation of the *CRHR1* gene loci is also associated with differences in cortisol reactivity in children.

Association also existed between CRH system gene variants and emerging symptoms of depression and anxiety. Specifically, the *CRH* rs9494082 and *CRHBP* rs1715751 were associated with anxious symptoms, whereas the *CRHR1* tSNP, rs242924, was associated with depressive symptoms. I also found evidence for gene-environment interaction between CRH pathway genes and CS on emerging symptoms of depression, with the interaction between *CRHR1* tSNP rs242924 and CS predicting symptoms of depression. Although no main effect of these variants on depression or anxiety has been reported, a few studies have implicated these variants in gene-environment interactions REFS. For example,

polymorphisms within this region of the *CRHR1* gene, and rs110402 and rs242924 SNP genotypes in particular, have been shown to moderate the link between early childhood maltreatment and the development of depressive symptoms later in life (Bradley et al., 2008; Heim et al., 2009; Polanczyk et al., 2009; Wasserman et al., 2009; Grabe et al., 2010), suggesting genetic differences are shaped by the environment in CRH-dependent neurotransmission. Similarly, Tyrka and colleagues (2009) report that variation in the *CRHR1* gene, and rs242924 GG homozygotes in particular, moderated the effect of childhood maltreatment on cortisol responses to the dexamethasone/corticotrophin-releasing hormone test. Thus, pathways between CRH system genes and negative outcomes are clearly complex, with evidence for both moderation and main effects. These findings contribute to this literature, showing that the CRH system genetic variation is associated with early stress reactivity and could act as a moderator of CS' effects on emerging risk for psychopathology.

My analysis also showed that a tSNP in the *CRHBP*-coding region, rs10473984 was associated with intensity of cortisol reactivity, and that *CRHBP* rs10062367 genotype was associated with total cortisol response or AUC_g. Additionally, the interaction between *CRHBP* tSNP rs7728378 and CS also predicted childhood symptoms of depression. Once again, children homozygous for the major allele (A allele) showed greater depressive symptoms as a function of life stress; however, this relationship was nonsignificant for children with at least one copy of the minor allele or the G allele. Since 65–90% of total CRH is

bound to CRHBP (Jahn et al., 2005), the effectiveness of CRH in stimulating ACTH to release cortisol may be influenced by a variation in *CRHBP* expression.

A number of recent studies have focused on the *CRHBP* gene as a candidate gene for stress-related disorders such as major depression and suicidality (De Luca et al., 2010). A study by Binder and colleagues (2010) report that allelic variants of rs10473984 are associated with higher ACTH concentrations, and these data extend this finding by showing this SNP is associated with early childhood cortisol reactivity as well. Although this SNP is 30kb upstream of the gene-coding region, it is likely that it is in linkage with SNP(s) with functional consequences, resulting in decreased affinity for free CRH, thus increasing the bioavailability of CRH to activate its receptor, the CRHR1. Such a functional change could lead to an increase in subsequent ACTH release from the pituitary gland and downstream cortisol release from the adrenal glands. Recent work by Enoch et al. (2008) provides some support for this speculation, identifying a second CRHBP isoform in the brain in which the terminal exon (exon 7) is spliced out, resulting in a truncation of the messenger RNA, such that the C-terminus of ancestral 52 amino acids is truncated to only 18 amino acids. This change in peptide sequence might affect protein folding and stability, which might alter CRH binding affinity. Further research including ultra-deep sequencing of this region and molecular analyses are needed to explain the role of the genetic variation in this region and HPA axis function.

I also found evidence for gene-gene interactions predicting both AUC_i cortisol reactivity and total cortisol response. The interactions between *CRH*

rs9698042 and *CRHR1* rs171441 were such that children heterozygous for the *CRHR1* rs171441 showed lower cortisol reactivity than the homozygotes for these loci. The second interaction, between *CRH* rs11996294 and *CRHR1* rs12944712 also predicted lower cortisol reactivity for heterozygotes at both gene loci. Although I did not find any main effects of genetic variation in the CRH gene on cortisol reactivity, the interaction between the *CRH* and *CRHR1* tSNPs indicates that CRH gene variants are important in HPA axis function, but only by virtue of epistatic effects. An interaction between *CRHR1* rs171441 and *CRHBP* rs32897 also predicted cortisol reactivity such that children homozygous for the rs32897 GG genotype showed significantly higher cortisol reactivity than the A allele carriers. The rs32897 tSNP has been implicated in other studies examining the genetic bases of alcoholism (Enoch et al., 2008) and inflammation (Velez et al., 2008). However, the interaction I found between the loci on the *CRHR1* and *CRHBP* genes and cortisol reactivity is novel. Taken together, the presence of multiple interactions between genes that shape different levels of the HPA signaling cascade highlights the complex nature of the genetic bases of HPA function in humans.

Overall, these findings illustrate an important relationship between genetic variation of the various CRH system genes and early cortisol reactivity and the role of these variants in emerging risk for internalizing symptoms. Additionally, these findings support and extend recent adult literature where CRH system genes were found to moderate the early environment on psychopathological risk. These findings have the potential to shed light on the mechanisms involved in the

transmission of risk and the origins of stress sensitivity and resilience observed in disorders such as major depression and post-traumatic stress disorder. I demonstrated that cortisol reactivity to stress in humans has complex polygenic underpinnings.

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Chapter 3 - Catechol-*O*-Methyltransferase gene (*val*158*met*) polymorphisms and anxious symptoms in early childhood: The roles of hypothalamus-pituitary-adrenal axis reactivity and life stress

3.1 Introduction

Catechol-*O*-methyltransferase (COMT), a catabolic enzyme that degrades cortical catecholamines including dopamine and epinephrine, plays a vital role in regulating prefrontal cortex catecholamine levels (Meyer-Lindenberg & Weinberger, 2006). The gene encoding COMT (Gene ID: 1312) is mapped to chromosome 22p11, and contains four exons (Brahe et al., 1986). Further, a non-synonymous G→A single nucleotide polymorphism (rs4680) in exon four leads to a valine (*val*) to methionine (*met*) peptide change in the mature protein, and is called the *val*158*met* polymorphism. This substitution impacts the thermostability of the COMT protein and reduces the enzyme's catabolic function, thereby reducing dopamine degradation in carriers with at least one copy of the *met* allele by more than one-third compared to carriers homozygous for the *val* allele (Lotta et al. 1995; Chen et al. 2004). Thus, functional differences in catecholinergic activity due to this genetic variation appear to lead to individual differences in cortical dopamine availability, which may account for associations between this gene and various forms of psychopathology (Dickinson & Elvevag, 2009). For example, Hamilton and colleagues (2002) reported an association between the *COMT val*158*met* polymorphism and panic disorder in a family-based sample of 83 parent-offspring triads. Additionally, the *val* allele has been

associated with early-onset major depression (Massat et al., 2005), and other studies have shown that this variant may increase risk for panic disorders and anxiety in adults (Rothe et al., 2006; Domschke et al., 2007; Hosak, 2007). A recent study from our group reported associations between the *val*158*met* polymorphism and emerging symptoms of depression and anxiety in two large, independent community samples of preschoolers (Sheikh et al., 2013). Specifically, in both samples, *val* homozygous children exhibited significantly higher symptoms of depression and anxiety compared to children with at least one copy of the *met* allele.

In addition to main effects on the development of symptoms, a few studies have implicated this gene in gene-environment interactions. For example, Baumann and colleagues (2013) reported that an interaction between childhood adversity and the *COMT* gene locus predicted anxiety sensitivity and anxious apprehension. Similarly, an interaction between *COMT* genotype and childhood trauma predicted risk for psychotic symptoms in adolescents; specifically, *val* homozygotes who were also exposed to childhood trauma also reported more psychotic experiences compared to *met* allele carriers (Ramsay et al., 2013). Other studies have linked interactions between the *val*158*met* polymorphism and childhood maltreatment in shaping cognitive performance, neuroticism, and risk for alcohol dependence (Goldberg et al., 2013; Hoth et al., 2006; Schellekens et al., 2013). Taken together, recent literature suggests that the *val*158*met* polymorphism may moderate the effect of childhood adversity on a broad spectrum of psychological outcomes later in life, and could hold etiological

significance for internalizing disorders. However, research is still lacking on whether an interaction between *COMT* genotype and life stress predicts early symptoms of internalizing disorders.

In addition to gene-environment interactions, a parallel line of research has also focused on identifying the mechanistic processes (i.e., mediators) through which *COMT* functional variance may contribute to maladaptive outcomes. The hypothalamic–pituitary–adrenal (HPA) axis is widely posited to be one such mediator of links between neurotransmitter gene function and risk for future psychopathology (Charney, 2004; Krishnan & Nestler, 2008; McEwen, 1998; Mokrani et al., 1997; Pitchot et al., 2003). Dopaminergic metabolism is known to regulate endocrine stress reactivity primarily by affecting extra-hypothalamic brain structures that regulate HPA axis functioning, such as the prefrontal cortex, hippocampus and the amygdala (for in depth reviews see Locatelli et al., 2010; Vermetten & Bremner, 2002). For example, distribution of dopamine was found to alter HPA axis reactivity in rodents and non-human primates exposed to early life stressors paradigms such as maternal separation and glucocorticoid administration (Kofman, 2002; Macri et al., 2009; Posener et al., 1994, 1999). Additionally, the altered HPA axis functioning was linked to anhedonia-like symptoms and high anxiety-like traits. Taken together, the extant literature suggests a link between catecholamine metabolism and HPA axis function (Moghaddam, 2002), and it is plausible that genetic variations leading to functional differences within the catecholamine system, such as the *COMT* *val*158*met* polymorphism, may also be linked to HPA axis reactivity.

In a complementary line of research, studies have implicated *COMT val*158*met* variation in stress sensitivity. For example, in a community sample of adults, Jabbi and colleagues (2007) showed that the *COMT met*/*met* genotype was associated with increased adrenocorticotropic hormone responses, although the authors did not test cortisol function. Similarly, a recent study conducted in a large community sample of adolescents demonstrated an association between the *COMT val*158*met* polymorphism and cortisol reactivity to stress (Bouma et al., 2012). However, the link between genotype and stress reactivity was gender-specific, such that boys with the *met* homozygous genotype had higher cortisol levels than boys with the *val* homozygous genotype. Conversely, girls with the *val* homozygous genotype had stronger cortisol responses than the *met* allele carrier girls (Bouma et al., 2012). Recently, in a sample of 8-year-olds, *met* homozygotes were reported to have a higher cortisol response to psychosocial stress task when compared to heterozygotes and *val* homozygotes (Armbruster et al., 2011). A brief review of these studies suggests a link between the *COMT val*158*met* polymorphism and HPA axis function, but the literature is inconsistent on the allele associated with cortisol reactivity. Considering the importance of cortisol reactivity in the etiology of various mood disorders (McEwen, 2008), further research is needed to clarify the link between the *val*158*met* polymorphism and HPA axis response.

In sum, separate lines of research show links between *COMT val*158*met* polymorphism and emotional problems. Literature also suggests that interactions between the *COMT* functional polymorphism and early life stressors may predict

psychological outcomes later in life. Additionally, research suggests that the link between catecholamine signaling and risk for emerging internalizing symptoms may be influenced by psychophysiological response of the HPA axis (Bouma et al., 2012). However, whether these processes are at play during early childhood and contribute to early symptoms of internalizing disorders has not yet been explored. To address these gaps in knowledge, we investigated whether the *COMT val*158*met* polymorphism would be associated with cortisol responses to stress in a community sample of preschoolers. We also explored whether early cortisol function mediated links between COMT and emerging symptoms. Finally, in an effort to understand the process through which the gene-environment interactions may influence early behaviour, we developed an integrated pathway (Figure 3.1) where we tested the role of these variables on emerging symptoms using path analysis.

3.2 Methods

Detailed sample demographic are provided in Chapter 2, pg. 42. Sample demographics by *COMT* genotype are presented in Table 3.1. Children's mean age was 36.2 months (*SD* = 0.16). As in the previous chapter, population stratification was minimized by restricting all analyses to include Caucasian participants (*N* = 371) only.

3.2.1 Genotyping

Genomic DNA was purified from buccal swab cellular extracts and stored according to manufacturer instructions (Qiagen, Valencia, CA, USA). The rs4680 (*val*158*met*) SNP was genotyped using a TaqMan allelic discrimination assay

(Assay ID: C_25746809_50), developed for use on the StepOne® instrument (Applied Biosystems, Foster City, California). Polymerase chain reactions were performed in 10µL reaction volumes in 48-well plates and contained 10 ng of DNA. Thermal cycler conditions were 95 ℃ for 10 min and then 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The SDS 2.2 software (Applied Biosystems) was used for allelic discrimination. For quality control, 10.0% of the samples were randomly chosen and genotyped as duplicates across and within a 48-well plate. All genotyping was performed by technicians blind to other study data.

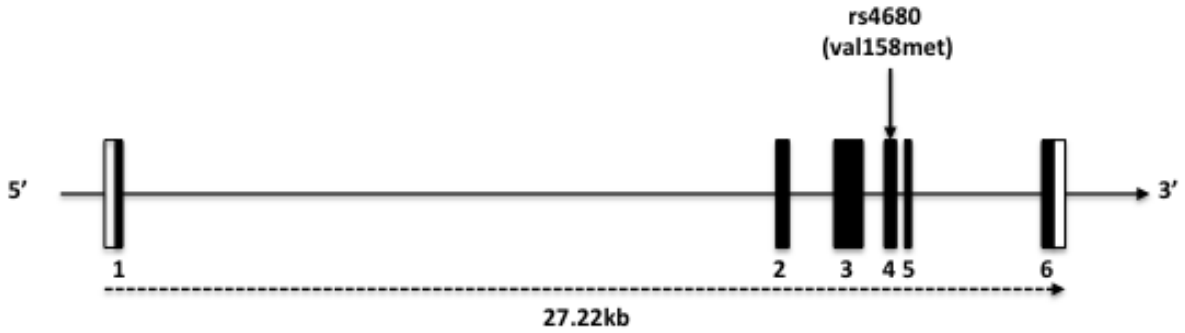


Figure 3.1. Schematic diagram of the human *COMT* gene. Exon positions are indicated by solid black blocks and introns are represented by lines. The location of the rs4680 *val*158*met* polymorphism in exon 4 is indicated (adapted from Brahe et al., 1986).

3.2.2 Behaviour and childhood stress measures

Similar to Chapter 2, we used the child's primary caregiver reports on the Child Behavior Checklist (CBCL; Achenbach, 2000). The stress task used to illicit children's cortisol responses to stress was described on page 54. As described in Chapter 2 (p. 58), the overall life stress aggregate score was used as a measure of stress during childhood in all analyses in this chapter and will be referred to as CS (i.e., childhood stress).

3.2.3 Data Analyses

To test the hypothesis that *COMT val*158*met* genotype moderated the influence of CS on emerging symptoms of anxiety and depression, I analyzed the interaction between life stress and genotypes using a macro for PASW developed by Hayes (2013) with parent reports of children's anxious and depressive symptoms as the dependent variables. This macro uses a regression-based framework for testing two-way interactions in moderation models along with simple slopes. All predictor values were centered as needed. This same macro was used to test the hypothesis that HPA axis reactivity mediates the link between *COMT* genotype and emerging symptoms. This method uses a bootstrapping procedure which yields mean direct and indirect (mediated) effects. The test also generates confidence intervals (CIs) for the means. The estimated effect is only significant at a *p*-value less than 0.05, when CIs do not contain a 'zero' within them. For further details on this method, see Hayes (2013). All analyses were performed using the PASW 20 (IBM Inc., USA). All statistical tests were two-tailed with alpha set at $p = 0.05$.

Finally, I tested the integrated pathway in Figure 1.3 using a path analysis framework using Mplus software. Path analysis is a form of structural equation modeling, which takes a confirmatory (i.e., hypothesis testing) approach to the multivariate analysis of a structural model (Byrne, 1998). Using this method, the causal associations under investigation are represented by a series of structural (i.e., regression) equations, and these structural equations are modeled pictorially (Byrne, 1998). The chi-square statistic is used as a measure of fit between the sample covariance and fitted covariance matrices (Byrne, 1998). The higher the probability associated with chi-square, the closer the fit between the hypothesized model and perfect fit. In addition, other indices are used to assess the appropriateness of the proposed model to the sample data. These indices include the goodness of fit index, the non-normed fit index, and the comparative fit index. Values for these indices, in the mid .90 range and above, are indicative of optimal fit (e.g., Hu & Bentler, 1995; Schumaker & Lomax, 1996). Furthermore, the Root Mean Square of Approximation (RMSEA) is yet another fit index which takes into account the error of approximation in the population (Byrne, 1998). Values less than .05 indicate good fit, and values of .08 or less indicate acceptable fit (Byrne, 1998), whereas values of .08 to .10 indicate mediocre fit, and values above .10 indicate poor fit (MacCullum, Browne, & Sugawara, 1996).

3.3 Results

3.3.1 Associations between COMT val158met polymorphism and major study variables

The genotype frequencies were as follows: 118 children (29.4%) were *val* homozygous, 190 (46.3%) were heterozygous, and 93 (23.2%) children were homozygous for the *met* substitution. This distribution was in Hardy-Weinberg

equilibrium ($\chi^2_{1, 400} = 0.95$, $p = 0.33$). Demographic variables such as child gender and family income were not associated with child genotype (all *ps* > 0.22). However, positive associations existed between child gender and symptoms of depression ($F_{1, 369} = 17.89$, $p < 0.01$) and anxiety ($F_{1, 369} = 13.50$, $p = 0.01$); therefore, child sex was used a covariate in subsequent analyses. No associations existed between *COMT* genotypes and child gender ($\chi^2_{4, 380} = 1.73$, $p = 0.23$) or family income ($F_{1, 368} = .005$, $p = 0.94$). No evidence for associations existed between COMT genotype and child depressive symptoms (mean difference: 0.13, $t = 1.22$, $p = 0.31$), but there was a significant association between *COMT* genotype and child anxious symptoms (mean difference: 0.25, $t = 6.74$, $p < .05$). Specifically, children homozygous for the *val* allele had significantly lower symptoms of anxiety when compared to children with at least one copy of the *met* allele.

Table 3.1 Demographic and study variables by child *COMT val*158*met* genotype.

	COMT genotypes					
	val/*val* (*N* = 113)			*val*/*met* + *met*/*met* (*N* = 287)		
Variable	Mean	*SD*	*N*	Mean	*SD*	*N*
Sex (N, boys)			61			134
PPVT	111.94	12.87		112.04	14.59	
Family income	3.69	1.19		3.75	1.12	
Cortisol response [peak – baseline]*	0.05	0.01		0.03	0.01	
AUC_i†	0.06	0.13		0.04	0.09	
AUC_g	0.20	0.16		0.18	0.12	
Depressive symptoms	1.38	1.62		1.25	1.62	
Anxious symptoms*	1.40	1.69		1.15	1.29	

Note: AUC = area under curve; PPVT = Peabody picture vocabulary test; *val* = valine; *met* = methionine. Family income coded as 1 = < $20,000, 2 = $20,001 – $40000, 3 = $40,001 - $70,000, 4 = $70,001 – $100,000, 5 = >$ 100, 001.

†$p \leq .10$, *$p < .05$.

Figure 3.2 shows the analysis of stress reactivity as a function of *COMT* genotype. I found significant group differences in individual cortisol change scores [peak cortisol response – baseline cortisol], such that children homozygous for the *val* allele had significantly higher cortisol reactivity compared to the children with at least one copy of the *met* allele ($F_{1, 356} = 3.80$, $p < 0.05$). Allelic group differences existed only at the level of a trend ($p = .08$) when AUC_i was used as the dependent variable. Total cortisol response (AUC_g) was not associated with the *COMT* genotype (Table 2).

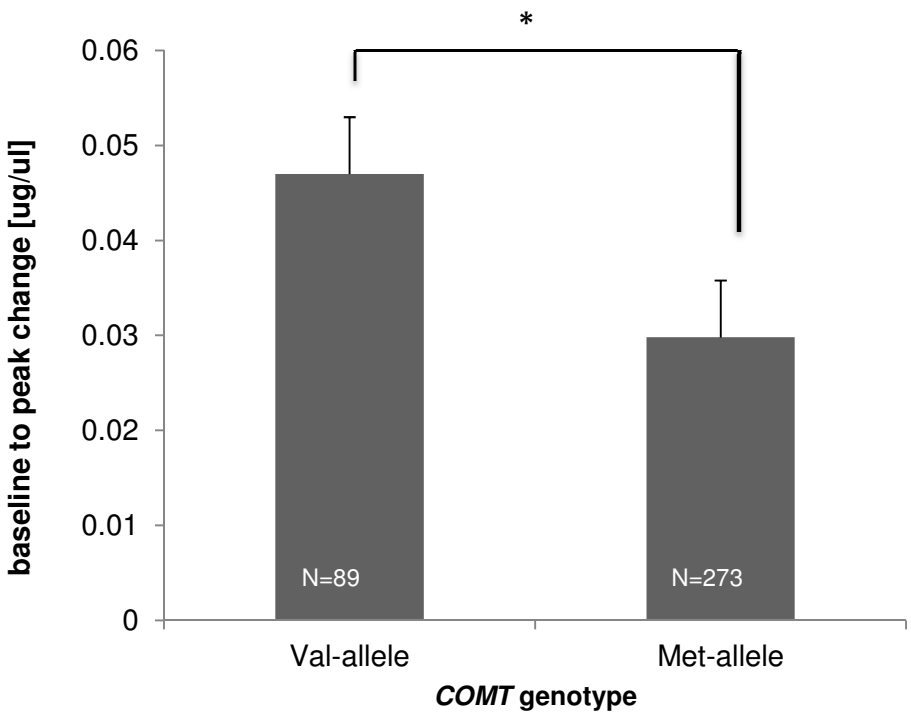


Figure 3.2. Main effects of *COMT val*158*met* genotype on child cortisol reactivity. *$p <$ 0.05.

Note: Child *COMT val*158*met* genotype coded as 0 = *val*/*val* genotype, 1 = *val*/*met* or *met*/*met* genotype; cortisol reactivity was measured via individualized cortisol change scores [baseline – each child's peak cortisol sample obtained post-stress task].

3.3.2 HPA axis reactivity mediates the link between COMT val158met polymorphism and child clinical symptoms

I also conducted mediation analyses to examine whether links between *COMT* genotype and emerging symptoms of depression or anxiety were mediated by child cortisol reactivity. As a precondition for testing mediation, associations must be present between the predictor and the outcome, the predictor and the hypothesized mediator, and the hypothesized mediator and the outcome (Baron and Kenny, 1986). Table 3.1 shows the associations between *COMT* genotype and anxiety symptoms, and *COMT* genotype and cortisol reactivity measured via baseline to peak cortisol change. After confirming the significant association between cortisol reactivity and anxiety symptoms ($r = 0.13$, $p < 0.01$), I proceeded with the mediation analysis. The bootstrapping procedure showed a significant estimate of the indirect effect of *COMT* genotype on anxiety symptoms mediated via child cortisol reactivity (B= -0.06, 95% CI: -0.17 to -0.01). The analysis also showed that the direct association between *COMT* genotype and anxiety symptoms was also significant, indicating that the genotype-symptoms link was only partially mediated by child cortisol reactivity (Figure 3.3).[3]

[3] I also modeled child depressive symptoms as the dependent variable in my mediation pathway but analysis showed that this model was not significant suggesting that the link between *COMT* genotype and depressive symptoms was not mediated by cortisol response to stress.

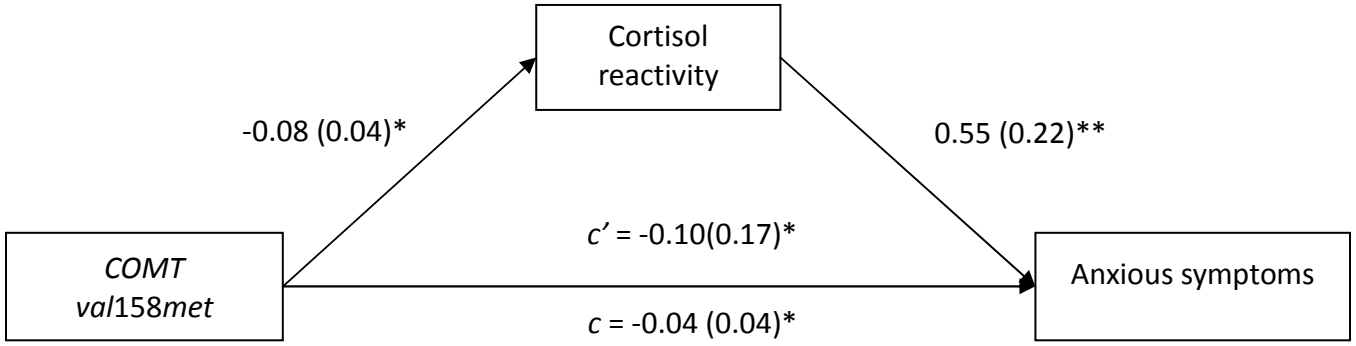


Figure 3.3. Cortisol reactivity in early childhood mediates the link between the *COMT* gene and anxious symptoms. * $p <$ 0.05, ** $p < 0.01$.

Note: Child *COMT val*158*met* genotype coded as 0 = *val*/*val* genotype, 1 = *val*/*met* or *met*/*met* genotype; cortisol reactivity was measured via cortisol reactivity [baseline – peak cortisol post-stress task]; c = coefficient for direct path between child *COMT* genotype and anxious symptoms; c' = coefficient for path between child *COMT* genotype and anxious symptoms, mediated by cortisol reactivity.

3.3.3 COMT val158met polymorphism and childhood life stress interact to predict emerging symptoms of anxiety but not depression

In an effort to replicate and extend findings where an interaction between *COMT val*158*met* and CS was associated with adult internalizing symptoms (Ramsey et al., 2013), I tested this interaction in this sample of preschoolers. The interaction term for *COMT* genotype and CS did not predict child depressive symptoms (β = 0.54, *se* = 1.03, p = 0.73), but the interaction term was significant for child anxiety symptoms (β = 4.38, *se* = 1.98, p = 0.03). Analyses showed that children homozygous for the *val* allele exhibited higher symptoms of anxiety with increasing life stress (β = 3.30, *se* = 1.14, $p <$ 0.01), but the interaction between genotype and life stress was nonsignificant in the *met* allele carriers (β = -1.08, *se* = 1.62, p = 0.51; Figure 3.4).

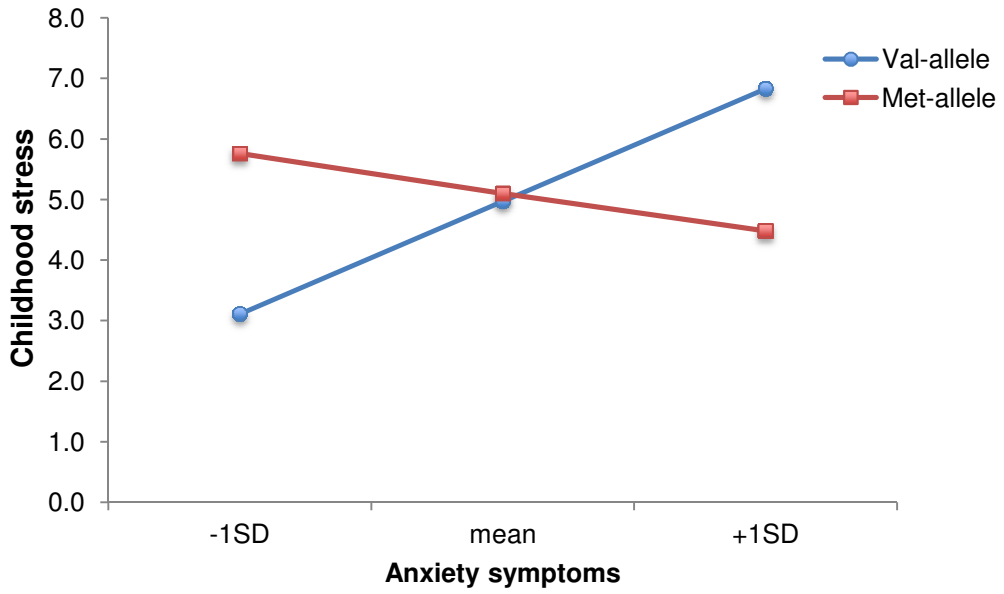


Figure 3.4. Moderation of the main effect of childhood stress on children's anxious symptoms by *COMT val*158*met* genotype. (*N* = 409).

3.3.4 Path analyses

Figure 3.5A shows the results of our path analyses, which aimed to test the integrated model of psychological risk based on Figure 1.3. CS was positively associated with baseline to peak cortisol change (β = 0.28, p = 0.001), and significant associations were found between genotype and cortisol response (β = -0.06, $p < 0.05$). Child cortisol reactivity was also positively associated with symptoms of anxiety (β = 0.61, $p < 0.01$). The overall model fit was very good as indexed by chi-square (χ^2 = 25.61, df = 7, $p < 0.001$), as well as the root mean square error of approximation (RMSEA=.00) and the normed fit index, which was 1.00.

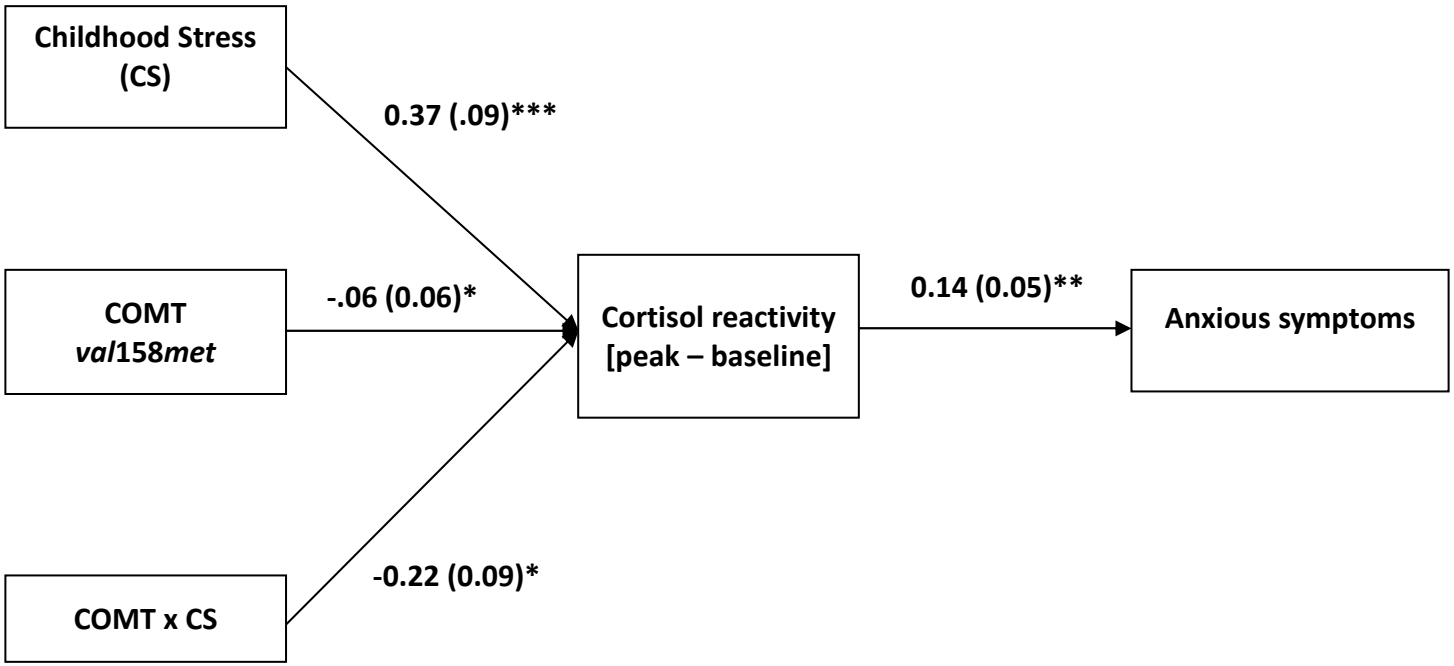
In the next path analysis (Figure 3.5B), we modeled AUC_i as an index of total cortisol produced post-stress task. Analysis showed that CS was positively associated with AUC_i (β = 0.19, p = 0.03), although nonsignificant associations were found between genotype and this index of cortisol response (i.e., AUC_i, β = -0.05, p = 0.30). Child AUC_i was positively correlated with symptoms of anxiety (β = 0.12, p = 0.01). The overall model fit was very good as indexed by chi-square (χ^2 = 20.46, df = 7, $p < 0.001$), as well as the root mean square error of approximation (RMSEA=.00) and the normed fit index, which was 1.00.

In our third model (Figure 3.5C), we modeled AUC_g as an index of total cortisol produced post-stress task. Childhood life stress was positively associated with AUC_g (β = 0.11, p = 0.001) and positive associations were also found between genotype and cortisol response measured via AUC_g (β = -0.04, $p < 0.05$). Child cortisol reactivity was also positively correlated with emerging symptoms of anxiety (β = 0.13, p = 0.01). The overall model fit was very good as indexed by chi-square (χ^2 = 23.70, df = 7, $p < 0.001$),

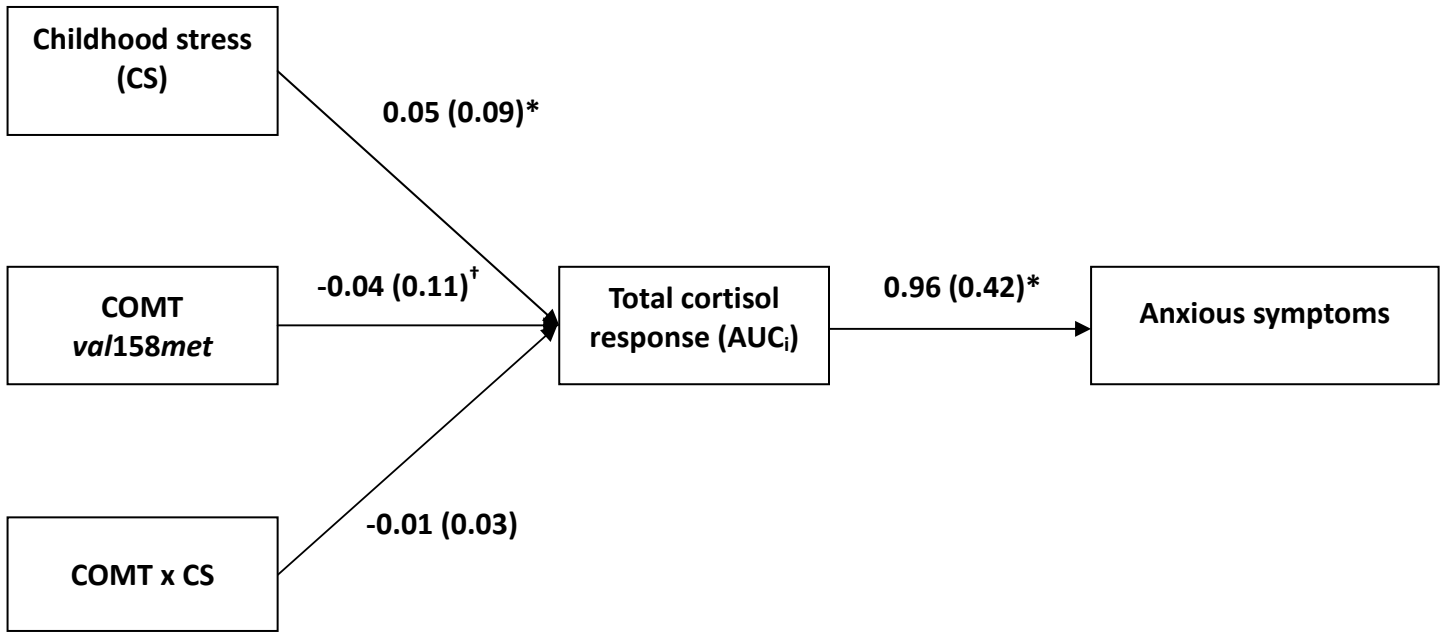
as well as the root mean square error of approximation (RMSEA=.00) and the normed fit index, which was 1.00.[4]

[4] We also modeled parent reports on child depressive symptoms as a dependent variable in our path analysis. However, the overall fit for these models were poor when baseline to peak cortisol change (RMSEA = 0.14, fit index = 0.31), AUC_i (RMSEA = 0.10, fit index = 0.55) and AUC_g (RMSEA = 0.11, fit index = 0.53) were used in the path analyses.

A.


Childhood Stress (CS)
0.37 (.09)***
COMT *val*158*met*
-.06 (0.06)*
Cortisol reactivity [peak – baseline]
0.14 (0.05)**
Anxious symptoms
COMT x CS
-0.22 (0.09)*

B.


Childhood stress (CS)
0.05 (0.09)*
COMT *val*158*met*
-0.04 (0.11)†
Total cortisol response (AUCi)
0.96 (0.42)*
Anxious symptoms
COMT x CS
-0.01 (0.03)

C.

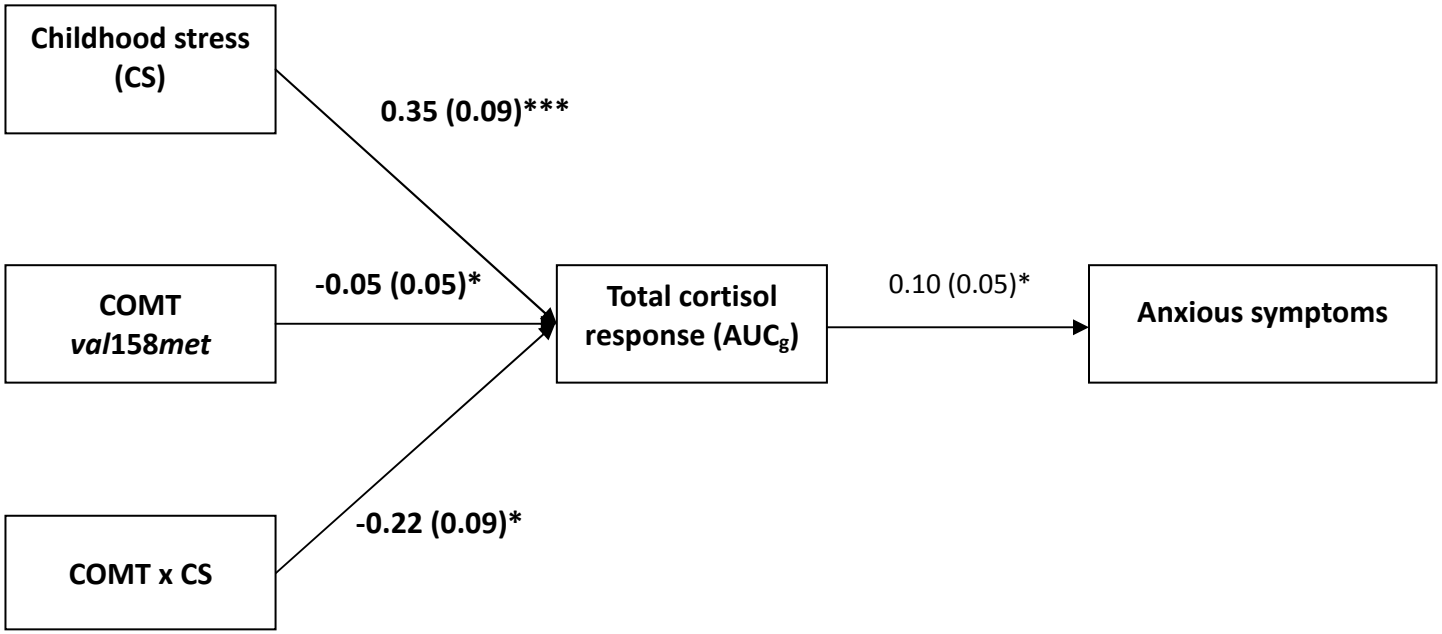


Figure 3.5. Pathway analysis. The path shows interaction between *COMT val*158*met* genotype and early life stress as predictor of child anxious symptoms via baseline to peak cortisol change (A), AUC_i (B) and AUC_g (C). Child anxious symptoms are based on parent reports from the Child Behavior Checklist (Achenbach et al., 1991). Standardized *β*-weights are shown for each path. $^{\dagger}p \leq 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Note: *val*, valine; *met*, methionine; AUC_g, Area under curve with respect to ground.

3.4 Discussion

In this study, I examined whether the *COMT val*158*met* genotype and CS were linked to emerging symptoms, and whether cortisol reactivity in children played a role in this pathway. Based on previous literature linking neurotransmitter gene polymorphisms and cortisol reactivity to stress (Derijk et al., 2009; Wust et al., 2009), my first set of analyses examined whether the *COMT val*158*met* genotype predicted early childhood cortisol reactivity to stress. I found significant associations between the *COMT val*158*met* genotype and cortisol reactivity such that children with two copies of the *val* allele had significantly higher cortisol reactivity compared to children with at least one copy of the *met* allele. These findings are consistent with previous studies in adults that have found a link between the *val* allele and higher cortisol reactivity (Jabbi et al., 2007; Ramsey et al., 2013). However, a recent study in 8-12-year-olds found that the *met* allele was linked to higher cortisol reactivity to the Trier Social Stress Task or TSST (Armbruster et al., 2012). The inconsistency in findings could be due to a few reasons. The TSST-C is not only a social stressor but also a cognitive challenge compared to the stress task used in the current study, which is based on the social evaluative threat model (e.g., threat of social rejection and evaluation). Although speculative, it is possible that the nature of the stressor is differentially related to catecholamine activity. Supporting this notion, studies have documented that *met* allele carriers perform better at cognitive tasks compared to *val* allele carriers (Dumontheil et al., 2011; Egan et al., 2001; Mier et al., 2010). However, additional research is needed to clarify

whether associations between *COMT val*158*met* polymorphism differ depending on stressor paradigms. Also, when compared to previous studies such as Armbruster and colleagues (2012), additional factors such as the younger age of this sample and a larger sample size may have contributed towards the differences in findings.[5]

In addition to the association between the *COMT val*158*met* polymorphism and cortisol reactivity in children, we also found evidence for mediation of links between *COMT* genotype and emerging anxious symptoms by cortisol reactivity. Extant literature is scarce on findings where HPA axis function is demonstrated to mediate the link between a dopamine gene functional polymorphism and emotional problems such as anxiety or depression. A recent study from Burghy and colleagues (2013) demonstrated that HPA axis function is linked to anxiety symptoms in adolescents via reduced activation of the amygdala-ventromedial prefrontal cortex (vmPFC) functional connectivity. Although speculative, its is possible that increased dopaminergic clearance in *val* allele carriers may be one of the causes of reduced amygdala-vmPFC activation, which in turn may lead to individual differences in evaluation of stress stimuli and HPA axis activation.

I found evidence for a gene-environment interaction, such that *COMT* genotype moderated the association between childhood stress on child anxiety

[5] Based on previous work by Bouma and colleagues (2012) where girls with the *val* homozygous genotype had stronger cortisol responses than the *met* allele carrier girls, I also conducted these analyses in my sample based on gender, However, I did not find any associations between *COMT val*158*met* genotype and cortisol response when the sample was stratified by gender ($p = 0.25$).

symptoms. Specifically, my analysis showed that under high levels of life stress, *val* allele carriers had significantly more symptoms of anxiety. A number of studies have reported on interactions between monoaminergic gene variants and childhood maltreatment as predictors of mood disorders. In a large study of over 5000 participants, significant interaction between *COMT* gene region haplotypes (including the *val*158*met* polymorphism) and high early CS predicted depressive symptoms during adulthood (Nyman et al., 2011). Similarly, in a study by Klauke and colleagues (2012), *COMT* genotype moderated the effect of childhood trauma on startle response, which is widely considered an endophenotype for anxiety disorders. Specifically, the *val* homozygotes showed a higher increase in startle response as a function of childhood traumatic events when compared to the *met* allele carriers. Additionally, in a study by Kolassa and colleagues (2010), an interaction between *COMT* genotype and recent traumatic events predicted an increased risk for posttraumatic stress disorder but only in *val* homozygotes. Thus, our findings are broadly consistent with research implicating the *val* allele in anxiety disorder risk.

I did not find evidence for moderation of life stress by the *val*158*met* polymorphism on emerging depressive symptoms. Depressive symptoms are rare at preschool age and emerge during middle to late childhood (Kovacs, 1996). Therefore, it is possible that any gene-environment effect on depressive symptoms may emerge in later childhood years when depressive symptoms are more common. Overall, my findings extend existing literature and show that the

moderation of CS by *COMT* genotype contributes to emerging anxiety symptoms from an early age.

In order to understand the pathways by which *COMT val*158*met* genotype, life stress, and their interaction may be linked to emerging symptoms, and the possible role cortisol response may play in this pathway, we tested an integrated model of emerging risk (Figure 3.5). My analyses showed that the *COMT* genotype and CS are linked to emerging anxiety symptoms via early age cortisol response. A few studies have reported on links between the *COMT val*158*met* polymorphism and endophenotypes of anxiety. For example, carriers of the *COMT val* allele showed an allele-dose effect on increased left amygdala activity in response to fearful/angry facial stimuli (Domschke et al., 2012) and amygdala activity has been linked to increases in neuroendocrine reactivity to stress (Jankord & Herman, 2008). These findings compliment and extend this literature and present a biological pathway in which interaction between catecholamine metabolism and CS is linked to emergence of psychological risk. The path analysis also demonstrates that the individual differences in neuroendocrine stress response are an important part of this pathway and the interaction between genotype and childhood stress contributes to childhood anxious symptoms via physiological stress response.

In conclusion, I found an important link between life stress during early childhood and emerging symptoms of anxiety in a community sample of preschoolers. My findings suggest that functional variant of the *COMT* gene moderated the link of life stress on emerging symptoms in early childhood and

that the components of HPA axis function play a mechanistic role in this pathway. To my knowledge this is the first study to demonstrate the role of these variables in a relatively large community sample of young children. Thus, these findings add to the knowledge of underlying pathways involved in emerging risk for emotional problems and aid in our understanding of the complex etiology of mood disorders.

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Chapter 4 - Dopamine pathway gene variants and early-emerging internalizing symptoms: The roles of childhood stress and psychophysiological reactivity

4.1 Introduction

As discussed in Chapter 1, dopamine (DA) receptors regulate a number of neurological processes including cognition, memory, learning, and motor control, as well as modulation of neuroendocrine signaling (Money & Stanwood, 2013); thus, individual differences in DA receptor density are potentially relevant to many psychiatric disorders (Nemoda, Szekely & Sasvari-Szekely, 2011). In particular, evidence suggests that the limbic DA system may be involved in a number of stress-related pathologies such as depression and anxiety (Willner, 1991). For example, DA may shape behaviour in the context of "learned helplessness," a mental state in which an organism forced to endure aversive stimuli, or stimuli that are painful or otherwise unpleasant, becomes unable or unwilling to avoid subsequent encounters with those stimuli, even if they are escapable, presumably because it has learned that it cannot control the situation (Colelli et al., 2010). Animals trained in the learned helplessness paradigm commonly exhibit widespread DA depletion in stress-regulating regions such as the prefrontal cortex, the caudate nucleus, and the nucleus accumbens compared to control animals (Charney, 2004). Similarly, prior treatment with a DA agonist prevents the development of learned helplessness and associated DA depletion in the prefrontal cortex (Boyce-Rustay, Janos & Holmes, 2008; Colelli et al., 2010). Conversely, DA antagonists exacerbate learned

helplessness and reduce the improvement produced by antidepressant treatment (Mozhui et al., 2010).

Behavioural despair, which is often assessed using the forced swim test in animals, is a model in which animals are forced to swim in a confined space. After initial attempts to escape they assume an immobile posture. Animals prone to high behavioural despair (i.e., those which do not attempt to escape or swim) also show significantly lower levels of DA in the limbic structures of the brain, regions commonly associated with stress regulation (Touma et al., 2008). Similar to their effects on behaviour during learned helplessness paradigms, antidepressants exert an anti-immobility effect whereas DA agonists augment the anti-immobility effect (Cabib & Puglisi-Allegra, 2012; Orsini et al., 2002). The most consistent finding in human studies is decreased turnover of DA in patients with depressive disorders. Homovanillic acid (HVA) is the major metabolite of DA, and almost all cerebrospinal fluid (CSF) HVA originates from the brain. Thus, CSF HVA reflects CNS DA turnover. Studies have used probenecid to block the transport of HVA from the CSF, to increase the validity of CSF HVA as a measure of CNS DA turnover (Mineur, Belzung & Crusio, 2007). Most studies have found a decrease in the CSF HVA of patients with depression (Nemeroff & Dunlop, 2007). In sum, based on animal and human literature there is evidence for an important role of DA in behaviours commonly related to depression and anxiety; some of this risk may emerge based on genetic variation that influences dopaminergic activity in the brain. DA pathway candidate genes commonly

implicated in cortisol function and psychopathology are discussed in the following sections.

4.1.1 DRD4

A number of dopaminergic candidate genes have been examined as candidates for depression and anxiety, although most research has focused on the major DA receptors and synaptic DA transporter (DRD2, DRD4 & DAT) due to their widespread expression in brain regions commonly linked to emotion and cognition. The human DA receptor D4 (*DRD4*) gene (Figure 4.1), located near the telomere of chromosome 11p, exhibits an unusual amount of variation, including single nucleotide polymorphisms (SNPs) and variable number tandem repeats (VNTR). One of the common *DRD4* polymorphism implicated in psychiatric literature is a 48-bp VNTR in the third exon (Schoots & Van Tol, 2003). The VNTR consists of 2-11 repeats, with the most common versions being 2 (2R), 4 (4R) and 7 (7R) repeats. Amongst these the 4R allele is the most common, whereas 2R and 7R allele frequencies vary widely in Caucasians (Zalsman et al., 2004). The 48-bp VNTR is part of the third cytoplasmic loop of the receptor protein and molecular genetics studies have shown to affect the function of the D4 receptor. Specifically, *in vitro* studies suggest that the 7R variant exhibits decreased signal transduction efficiency relative to the 4R variant (Asghari et al., 1995), and may have decreased RNA stability or translational efficiency (Schoots & Van Tol, 2003). Furthermore, there are robust differences between receptor variants in folding efficiency when shaping the final protein product, such that the mRNA transcript of the *DRD4* 2R allele folds more quickly

into a protein product than the transcripts of longer alleles, thus increasing DRD4 transmission (van Craenenbroeck et al., 2005). Cumulatively, these effects are likely to have a significant impact on the signaling and functioning of neural circuits involved in stress regulation.

Findings of main effects of *DRD4* on internalizing problems are less consistent. One study reported an association between the 2R of *DRD4* and unipolar depression (Leon et al., 2005). However, a recent study reported no association between the *DRD4* VNTR genotype and depression (Kang et al., 2008) or suicide attempts (Persson et al., 1999; Zalsman et al., 2004). A few studies have looked at GxE involving the *DRD4* VNTR polymorphism and early adversity predicting cortisol response to stress and internalizing outcomes during adulthood. For example, Armbruster and colleagues (2011) found that adolescent carriers of the 7R allele exhibited lower cortisol stress responses in presence of early adversity such as parental loss. The *DRD4* genotype also moderated associations between stressors during early childhood such as parental depression and depressive symptoms during adolescence and early adulthood (Adkins et al., 2012). However, conflicting results were published by Dragan and colleagues (2009), who reported that participants with at least 1 copy of the *DRD4* 7R allele had more intense post-traumatic stress disorder (PTSD) symptoms. Thus, although a number of studies have looked at the role of *DRD4* gene variation and psychopathological outcomes, further research is needed to clarify which *DRD4* variant is associated with psychopathological symptoms. Additionally, it remains unknown whether *DRD4* variants are associated with

early childhood cortisol response. Finally, whether the environment moderates genetic influences on these outcomes merits further exploration.

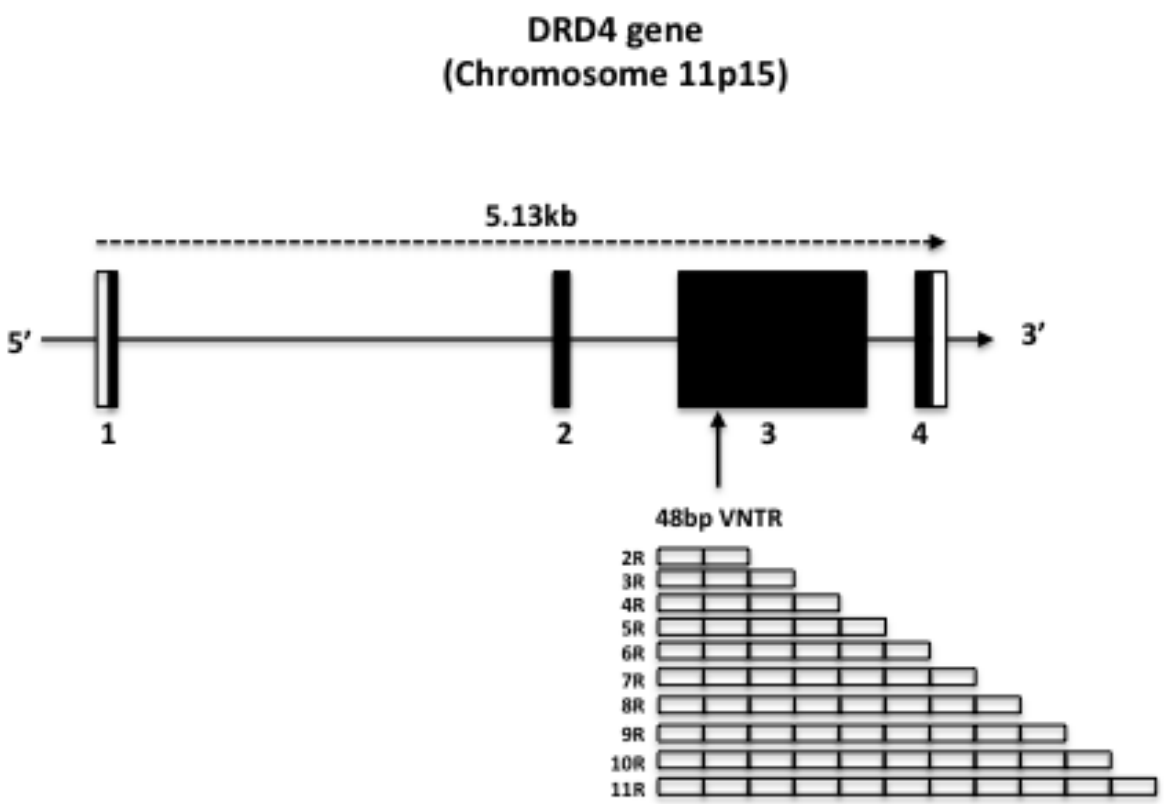


Figure 4.1. Diagrammatic representation of the human *DRD4* gene. Exon positions are indicated by solid black blocks and introns by lines. The location of the 48bp exon 3 variable number tandem repeat (VNTR) is indicated. The 2-repeat to 11-repeat variants of the VNTR are indicated below exon 3 (Adapted from Van Tol et al., 1992).

4.1.2 DRD2

The *DRD2* gene, first described by Grandy et al. (1989), is on 11q22 (Figure 4.2), and consists of six exons spanning almost 14kb. The gene has a restriction fragment length polymorphism downstream of the 3'UTR, called Taq1A (rs1800497) with two alleles referred to as A1 and A2 (Eisenberg et al., 2007). Using radioactively labeled agonists to detect D2 ligand binding, autoradiography of the caudate, putamen, and nucleus accumbens in tissue from normal middle-aged and elderly individuals without histories of substance abuse, neurological disorders, or psychopathology, it was shown that one or two A1 alleles was associated with reduced receptor binding throughout the striatum, with decreases found in the ventral caudate and putamen, brain regions implicated in emotion regulation. This supports findings from a few studies linking the Taq1A polymorphism with stress-related mood disorders. For example, a study of Chinese participants with bipolar disorder reported an association with Taq1A polymorphism (Li et al., 1999). A European multicenter study (Massat et al., 2002) reported an association of the (AC)- repeat polymorphism and bipolar, but not unipolar disorder. Additionally, studies have implicated the *DRD2* A1 allele in posttraumatic stress disorder or PTSD (Comings & Blum, 2000; Noble, 2000). The findings of one study (Broekman, Olff & Boer, 2007) studied Vietnam veterans who had been exposed to severe combat conditions. The authors examined the prevalence of the *DRD2* TaqI A1 allele in those who developed PTSD versus veterans without PTSD. The prevalence of the A1 allele was 60% in those with PTSD compared to 5% in those without PTSD. These findings were replicated in Caucasians where the frequency of A1 allele was significantly higher in PTSD combat veterans than controls (Young et al., 2000). In

addition to PTSD, a recent study has linked the Taq1A polymorphism with major depressive disorders as well (Savitz et al., 2013). Additionally, research from our group shows an association between the A1 allele and higher emerging symptoms of depression and anxiety in preschoolers (Hayden et al., 2010), suggesting a role of this variant in young children's symptoms.

In addition to main effects on symptoms, a handful of studies have also examined gene-environment interaction involving the Taq1A polymorphism. For example, in a large study of almost 2500 participants, *DRD2* Taq1A variants moderated the role of violent victimization experiences on depression (Vaske et al., 2009). The results suggest that females who carry the A1 allele of *DRD2* may be more vulnerable to the negative effects of violent victimization than females who do not carry at least one copy of the A1 allele. Similarly, data from our laboratory shows that the interaction between Taq1A genotype and early negative parenting, such as parental intrusion, predicted children's symptoms of depression and anxiety (Hayden et al., 2010). In sum, literature suggests that the *DRD2* polymorphism could have implications for emerging symptoms and that this gene may act as moderator of the impact of early stress on negative outcomes. However, to date no research exists in literature that has examined links between the *DRD2* Taq1A variant and cortisol responses to stress.

Furthermore, a gap in knowledge still remains as to whether this variant also moderates the effect of normative aspects of early stress, which are much more common for a majority of children, as compared to extreme forms of stress such as violent victimization in adults. Exploring these questions is an important addition to research examining the role of this variant in the etiology of depression and anxiety.

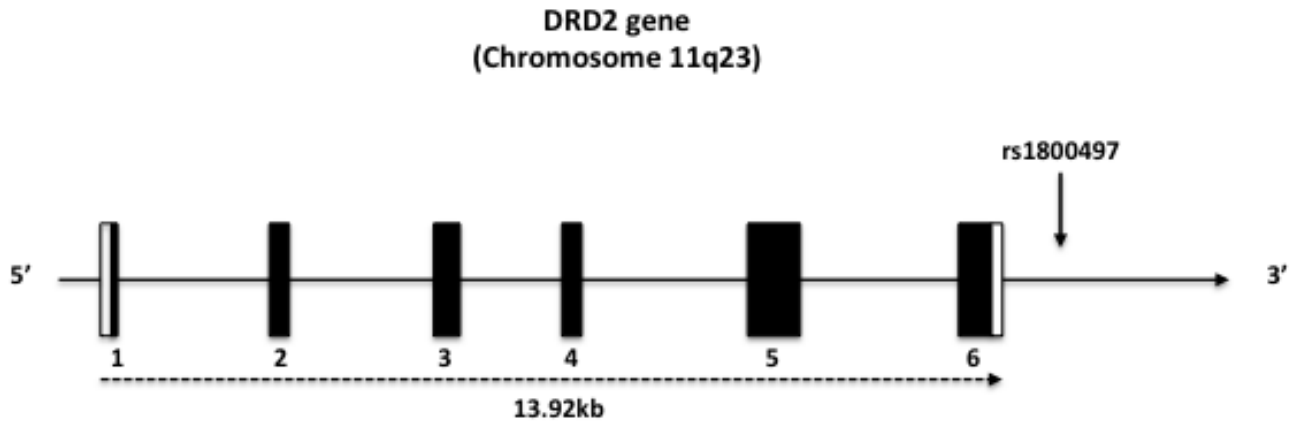


Figure 4.2. A schematic showing *DRD2* gene structure, and physical location of the rs1800497 polymorphism. Exons are indicated with solid black boxes and connecting black lines indicate introns. White boxes represent the 5'- and 3' - untranslated regions (Adapted from Eubanks et al., 1992).

4.1.3 DAT1

The DA transporter (*DAT1*) gene (Figure 4.3), located on chromosome 5p15.3, is heavily expressed in the human striatum, where it acts as the primary means of DA re-uptake (Sesack et al., 1998). The most studied polymorphism is a 40bp VNTR in the 3' untranslated region (UTR) of the *DAT1* gene (Mitchell et al., 2000). Alleles from 3–13 repeats have been described, but alleles with 9- and 10- repeats are the most frequently reported (Mitchell et al., 2000). Since this VNTR is not in the coding region of the gene, it does not affect the protein sequence of the DA transporter. However, it is thought to have functional significance, with *in vitro* studies indicating altered expression of the transporter as a function of VNTR alleles (Fuke et al., 2001; Miller & Madras, 2002), such that carrying 1 or 2 copies of the 10-repeat (10R) allele is associated with increased DAT1 expression. In addition, adults who are 10/10 homozygotes have significantly reduced DA transporter binding in the striatum relative to those having at least one 9-repeat (9R) allele (Jacobsen et al., 2000). In this way, the *DAT1* genotype is thought to effect DA levels indirectly by altering translational efficiency and the amount of protein expressed.

Given the central role of DA in the regulation of mood, it is unsurprising that association studies of *DAT1* have linked this gene to various stress-related psychiatric problems including depression (Dunlop & Nemeroff, 2007). Literature indicates links between *DAT1* 30bp VNTR and childhood disorders, such as attention-deficit hyperactivity disorder/ADHD (Cook et al., 1995; Durston et al., 2008) and conduct disorder (Lahey et al., 2011). There is also evidence for interactions between this genotype and psychosocial adversity predicts ADHD (Laucht et al., 2007; Neuman et

al., 2007). For example, adolescents homozygous for the 10R allele of the *DAT1* 40bp VNTR polymorphism who grew up in greater psychosocial adversity exhibited significantly more inattention and hyperactivity-impulsivity than adolescents with other genotypes or who lived in less adverse family conditions; a finding recently replicated by Li and colleagues (2013) in a sample of six- to nine-year-olds. Furthermore, *DAT1* has also been implicated in the etiology of depression and anxiety more generally, with inconsistencies regarding which genotype confers risk. Some have implicated the 9R allele rather than the 10R allele as being the risk allele (Lee et al., 2007; Young et al., 2002), while others have suggested that it is the 10R. Given this pattern of results from previous studies, I will examine links between the 9R and 10R allele and children's cortisol responses and internalizing symptoms.

Based on this brief review, it seems possible that the DA gene variants discussed in the sections above are linked to both cortisol response and stress-related disorders in adolescents and adults. However, a gap in knowledge remains concerning whether links are evident at an early age, which could have implications for early intervention. Therefore, I examined links between variants in DA candidate gene variants (*DRD2*, *DRD4*, *DAT1*) and both cortisol reactivity to stress and emerging symptoms of depression and anxiety in early childhood. Specifically, I examined the following questions: First, given the role of cortisol reactivity to stress in the etiology of stress-related mood disorders, I examined links between DA gene variants and children's cortisol responses. Second, I examined whether associations existed between DA gene variants and children's symptoms of depression and anxiety. Third, given the evidence for moderation of early adversity by DA genes on physiological stress responses and

internalizing symptoms, I examined the evidence for gene-environment interaction in this community sample of preschoolers.

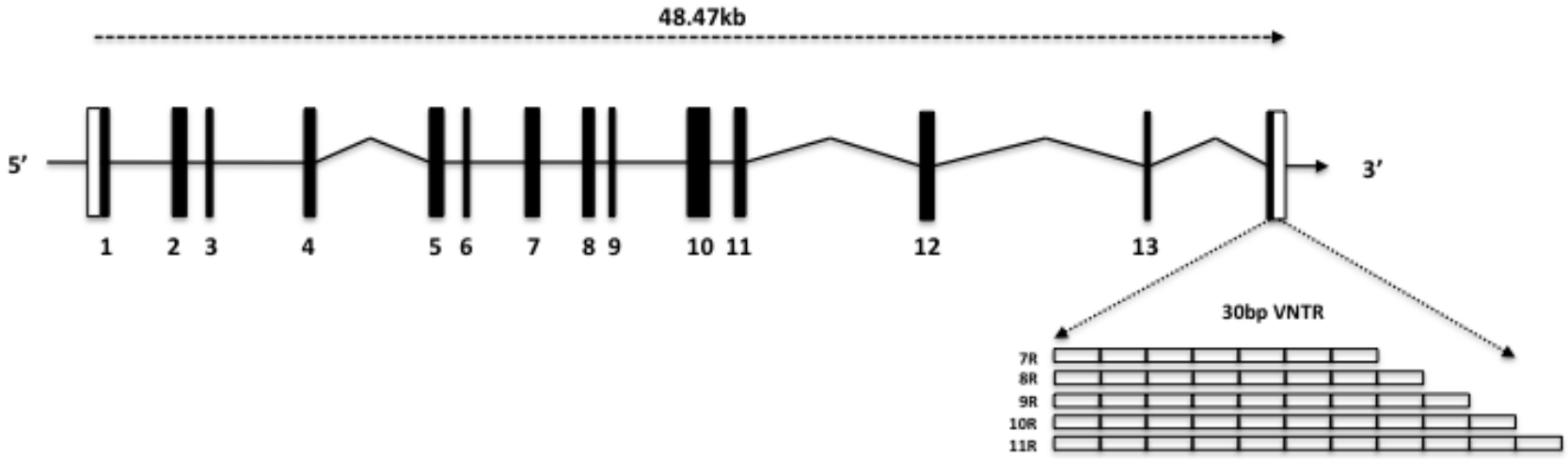


Figure 4.3. Diagrammatic representation of the human *SLC6A3* (*DAT1*) gene. Exons are indicated by solid black blocks and introns by lines. The location of the 30bp exon 14 variable number tandem repeat (VNTR) is indicated. The 7- to 11-repeat variants of the VNTR are indicated by dashed arrows (Vandenberg et al., 1992).

4.2 Methods

Detailed sample demographic are provided in Chapter 2, pg. 44. Similar to Chapter 2, I used the child's primary caregiver reports on the Child Behavior Checklist (CBCL; Achenbach, 2000). As described in Chapter 2, the overall life stress aggregate score was used as a measure of life stress in all analyses in this chapter. The AUC_i, baseline to peak cortisol change, and AUC_g were used as measures of cortisol response as previously described in detail on page 57. Finally, population stratification was minimized by restricting all analyses to include Caucasian participants ($N = 371$) only.

4.2.1 Genotyping methods

Participant DNA was extracted as described previously in Chapter 2. The 48-base pair VNTR located in the third exon of the *DRD4* gene was amplified using a 25 µl reaction containing 25 ng of genomic DNA template with forward primer 5'-CGCGACTACGTGGTCTACTCG-3' and reverse primer 5'-AGGACCCTCATGGCCTTG-3', and 1 U of NovaTaq polymerase (Novagen, Gibbstown, New Jersey, USA). The reaction also included 2 mM each of dATP, dCTP and dTTP, 1mM each of dGTP, dITP, with 10% DMSO and 1X PCR amplification buffer (20 mmol/l Tris-HCL pH 8.4, 50 mmol/L KCL). PCR amplification was carried out in a GeneAmp PCR System 9700 (ABI Biosystems, Foster City, California, USA). Following an initial denaturation at 95°C for 5 minutes, thirty cycles of amplification were run with each cycle consisting of denaturation at 95°C for 20 sec, annealing at 54°C for 20 sec, and extension at 72°C for 40 sec, ending with a final extension step of 5 min at 72°C. The PCR amplicons were

then resolved on a 2% agarose gel, stained with ethidium bromide (Sigma, Oakville, Ontario, Canada) and documented on the Bio-Rad 1300 Gel documentation system (Mississauga, ON, Canada). Product sizes were determined against a 100 bp molecular weight standard (Invitrogen, Carlsbad, California, USA).

The *DAT1* exon 3 VNTR was genotyped using the primers: 5'-TGTGGTGTAGGGAACGGCCTGAG-3' (forward) and 5'-CTTCCTGGAGGTCACGG CTCAAGG-3' (reverse). The PCR conditions were as follows: 5 min initial denaturation at 95 ℃ and 30 cycles of 30 s initial denaturation at 94 ℃, 45 s annealing at 67.5 ℃, 45 s extension at 72 ℃, followed by 5 min of final extension at 72 ℃. The 9R and 10R products yield a 440 bp and 480 bp fragment, respectively.

For the detection of the polymorphism in the Taq1A site, oligonucleotide primers 5′-CACGGCTGGCCAAGTTGT CTA-3′ (forward) and 5′-CACCTTCCTGAGTGTCATCAA-3′ (reverse) were used to amplify a 300-bp region comprising the Taq1A site (Grandy et al., 1993). The PCR conditions used were initial denaturation for 5 min at 95℃ followed by 30 cycles of 30 s denaturation at 94℃, 30 s annealing at 58℃, and a 30 s extension at 72℃, followed by a 5 min final extension at 72℃. The 300 bp PCR product was digested overnight with 1U of TaqαI restriction enzyme (New England BioLabs, Massachusetts, USA).

4.2.2 Data Analysis

To test the hypothesis that DA candidate gene variants moderated the influence of childhood stress (CS) on emerging symptoms of anxiety and depression, similar to Chapters 2 and 3 I analyzed the interaction between life stress and genotypes using a

macro for PASW developed by Hayes (2013) with parent reports of children's anxious and depressive symptoms as the dependent variables. This macro uses a regression-based path analytical framework for estimating direct and indirect effects in simple and multiple mediator models, along with simple slopes for probing interactions. All predictor values were centered as needed. Additionally, I once again tested the integrated pathway in Figure 1.3 using a path analysis framework. Details of the path analysis and guidelines for model interpretation are provided in Chapter 3, page 120.

4.3 Results

The *DRD4* VNTR polymorphism, like other VNTRs, has many possible variants (Wang et al., 2004), ranging from 2- to 11-repeat copies reported in the literature to date. In our sample, the following variants were present: 2/2 (N = 10, 2.4%), 2/4 (N = 67,16.3%), 2/5 (N = 1, 0.2%), 2/7 (N = 8, 2.0%), 2/8 (N = 2, .5%), 3/3 (N = 3, .7%), 3/4 (N = 9, 2.2%), 3/5 (N = 7, 1.7%), 3/7 (N = 2, .5%), 3/11 (N = 1, .2%), 4/4 (N = 157, 38.3%), 4/5 (N = 4, 1.0%), 4/7 (N = 96, 23.4%), 4/8 (N = 3, .7%), 5/5 (N =1, .2%), 7/7 (N = 21, 5.1%), and 7/11 (N = 1, .2%). This distribution is not consistent with Hardy-Weinberg equilibrium (Pearson X^2 (45) = 163.31, $p < 0.05$), but is comparable to recently reported frequencies (Ding et al., 2002). Consistent with the majority of published research (e.g., Faraone et al., 2001; Sheese et al., 2007), groups for data analysis were formed based on whether children had (N = 128) or did not have (N = 266) a 7R allele.

Although genotypes were successfully obtained for 371 children, for the purposes of our analyses, six participants with rare variants of the *DAT1* were excluded.

The genotypes of the remaining 365 children were distributed as follows: 177 (48%) children had the 10/10 genotype, 153 (42%) had the 9/10 genotype, and 35 (10%) had the 9/9 genotype. This distribution is in Hardy-Weinberg equilibrium, $X^2 = 0.05$, $p = 0.82$. For the *DRD2* Taq1A polymorphism, 369 children were genotyped successfully and the distributed as follows: 12 (3.2%) children had the A1A1 genotype, 114 (30.7%) had the A1A2 genotype, and 243 (65.5%) had the A2A2 genotype. This distribution is in Hardy-Weinberg equilibrium, $X^2 = 0.10$, $p = 0.76$.

4.3.1 Main effects

Table 4.1 shows associations between study variables and DA pathway gene polymorphisms stratified by genotypes. One-way analysis of variance revealed no associations existed between DA gene polymorphisms and symptoms of anxiety and depression measured via the CBCL. I also did not find any associations between DA gene variants and our three indices of cortisol reactivity. The DA gene polymorphisms were also not associated with either the child's gender or ethnicity (all $ps > 0.21$).

Table 4.1. Study variables stratified by DA pathway gene polymorphism genotypes.

	Genotype					
	DRD2		*DRD4*		*DAT1*	
Variable	A1A1+A1A2 (*N*=143)	A2A2 (*N*=260)	<7-repeat (*N*=262)	≥7-repeat (*N*=136)	9/9 + 9/10 (*N*=207)	10/10 (*N*=183)
Sex (N, boys)	64	134	125	69	98	94
Family income	3.67 (1.18)	3.77 (1.12)	3.69 (1.13)	3.80 (1.17)	3.60 (1.16)	3.84 (1.11)
PPVT	110.35 (15.11)	112.96 (13.45)	112.2 (14.21)	111.70 (13.69)	112.36 (13.68)	111.41 (14.57)
Baseline to peak cortisol change	-1.11 (0.35)	-1.08 (0.37)	-1.09 (0.37)	-1.09 (0.36)	-1.11 (0.38)	-1.06 (0.35)
AUC_i	0.05 (0.09)	0.05 (0.11)	0.05 (0.09)	0.06 (0.10)	0.05 (0.08)	0.05 (0.12)
AUC_g	0.18 (0.13)	0.18 (0.13)	0.18 (0.13)	0.19 (0.15)	0.18 (0.13)	0.18 (0.13)
CBCL Depression	1.35 (1.46)	1.24 (1.62)	1.39 (1.64)	1.16 (1.58)	1.25 (1.42)	1.37 (1.82)
CBCL Anxiety	1.17 (1.27)	1.40 (1.71)	1.36 (1.61)	1.25 (1.50)	1.39 (1.51)	1.26 (1.65)

Note: DRD2, DA receptor-D2; DRD4, DA receptor-D4; DAT1, DA Transporter-1; AUC_i, Area Under Curve with respect to baseline; AUC_g, Area Under Curve with respect to ground; CBCL, Child Behavior Checklist; Family income was coded as 1 = < $20,000; 2= $20,000-$40,000; 3= $40,001-$70,000; 4 = $70,001-$100,000; 5 = > $100,001.

4.3.2 Moderation analyses

Based on a large body of literature implicating DA pathway genes in gene-environment interactions, I tested whether DA gene variants moderated the effect of life stress on symptoms of depression and anxiety. Details for moderation analyses have been discussed in Chapters 2 & 3.

4.3.2.1 DRD2

Multiple regression analysis showed evidence for moderation of the association between CS and children's cortisol responses, measured via AUC_i by *DRD2* genotype ($\beta = 0.04$, $se = 0.21$, $p = 0.03$).[6] Analyses showed that children carrying at least one copy of the A1 allele exhibited higher cortisol reactivity as function of increasing CS ($\beta = 1.03$, $se = 0.09$, $p = 0.001$), but for children homozygous for the A2 allele, CS was not associated with cortisol response ($\beta = -0.03$, $se = 0.13$, $p = 0.77$; Figure 4.4A). Similarly, the interaction term predicting AUC_g was significant as well ($\beta = 0.04$, $se = 0.02$, $p = 0.03$). Analyses showed that children carrying at least one copy of the A1 allele exhibited higher AUC_g as function of increasing CS ($\beta = 1.05$, $se = 0.13$, $p < 0.001$), but for children homozygous for the A2 allele, CS was not associated with cortisol response ($\beta = 0.00$, $se = 0.18$, $p = 0.86$; Figure 4.4B). There was no evidence

[6] Since AUCi and change score analyses yielded virtually identical results, only analyses predicting AUCi are reported here. However, the interaction term between *DRD2* genotype and CS also predicted cortisol response measured via baseline to peak cortisol change ($p = 0.02$). As both baseline to peak cortisol change and AUC_i represent the intensity of cortisol response after stress, only one of these DVs is reported.

for interaction between *DRD2* genotype and CS in predicting children's symptoms of depression (β = 0.03, *se* = 0.27, *p* = 0.89) or anxiety (β = -0.03, *se* = 0.26, *p* = 0.92).

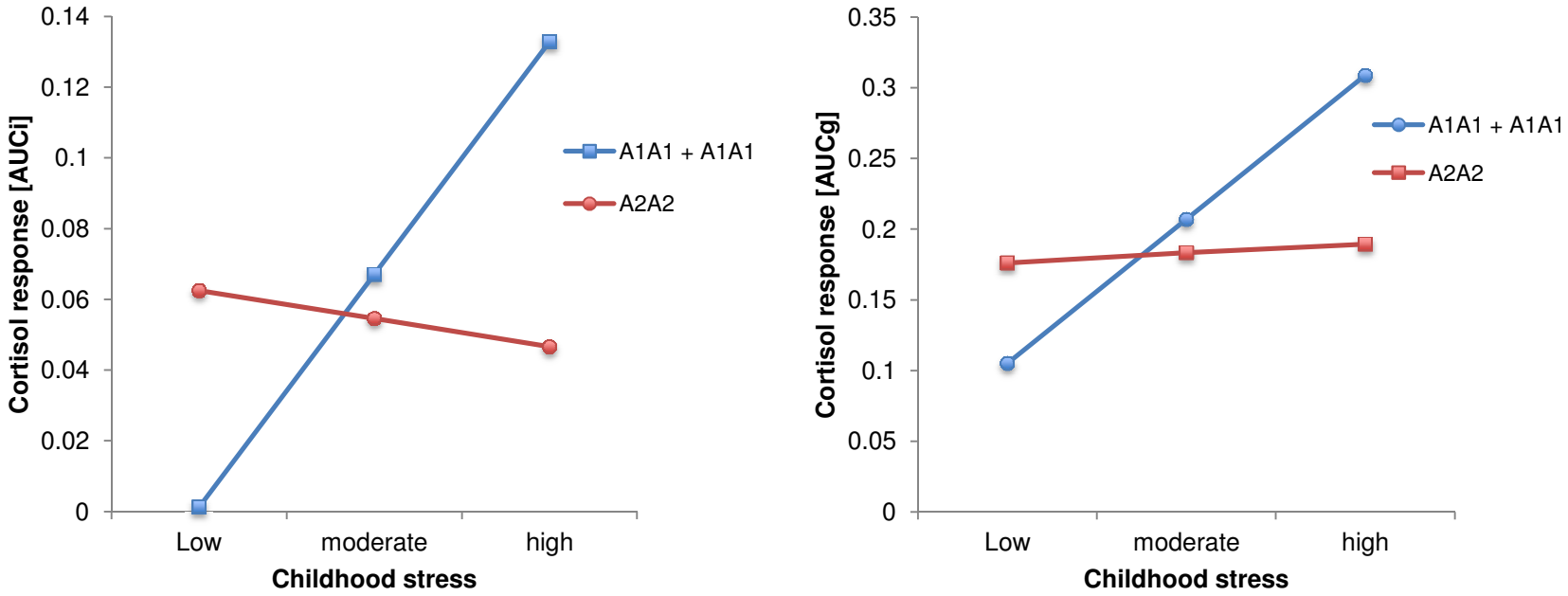


Figure 4.4. Relationship between childhood stress and AUC_i **(A)** and AUC_g **(B)** by *DRD2* Taq1A (rs1870493) allelic groups.

4.3.2.2 DRD4

Although I found no main effects of DRD4 genotype on either cortisol response or emerging anxious and depressive symptoms, based on previous reports of evidence for GxE involving this genotype (Adkins et al., 2012), I proceeded to conduct interaction analyses to explore the possibility of GxE in this sample. Using multiple regression analyses, I examined whether the *DRD4* genotype moderated the effect of early stress on cortisol response. The interaction terms were nonsignificant for AUC_i (β = -0.03, *se* = 0.02, *p* = 0.08) and AUC_g (β = -0.04, *se* = 0.03, *p* = 0.10).[7] Similarly, in the next set of analyses, I examined whether the *DRD4* genotype moderated the effect of early stress on emerging symptoms of depression or anxiety. Once again, the interaction term for *DRD4* genotype and CS did not predict child depressive symptoms (β = 0.02, *se* = 0.28, *p* = 0.93) or child anxiety symptoms (β = -0.14, *se* = 0.27, *p* = 0.61).

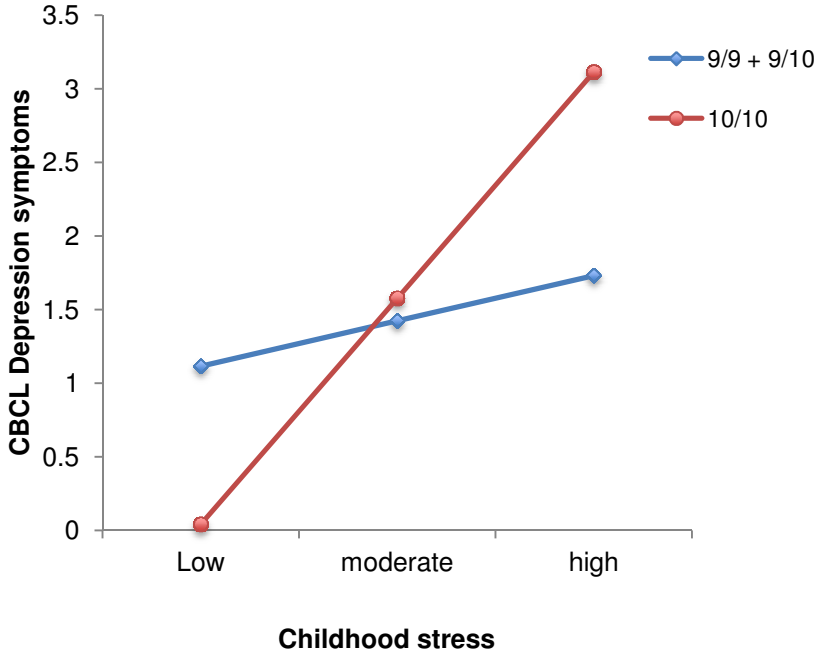
4.3.2.3 DAT1

Once again, multiple regression analyses showed that the interaction between *DAT1* genotype and CS did not predict AUC_i (β = 0.00, *se* = 0.16, *p* = 0.87) and AUC_g (β = -0.02, *se* = 0.02, *p* = 0.42). However, the interaction term for *DAT1* genotype and CS was a significant predictor of children's depressive symptoms (β = 1.04, *se* = 0.26, *p* < 0.001). Analyses showed that children homozygous for the 10R allele exhibited higher symptoms of depression as a function of CS (β = 1.29, *se* = 0.20, *p* = 0.001). However,

[7] The interaction term between *DRD4* genotype and CS did not predict cortisol response measured via baseline to peak cortisol change or internalizing symptoms (all *ps* > 0.05).

CS was not associated with childhood depressive symptoms in children who had at least one copy of the DAT1 9R allele ($\beta = 0.25$, $se = 0.16$, $p = 0.13$; Figure 4.5A).

Similarly, the interaction between *DAT1* genotype and CS also predicted child anxiety symptoms ($\beta = 0.59$, $se = 0.26$, $p = 0.02$). Analyses showed that children homozygous for the 10R allele exhibited higher symptoms of depression with increasing life stress ($\beta = 0.74$, $se = 0.20$, $p < 0.001$), but CS was not associated with anxious symptoms in children with at least one copy of the 9R allele ($\beta = 0.14$, $se = 0.16$, $p = 0.36$; Figure 4.5B).


3.5
3
2.5
2
1.5
1
0.5
0
CBCL Depression symptoms
9/9 + 9/10
10/10
Low
moderate
high
Childhood stress

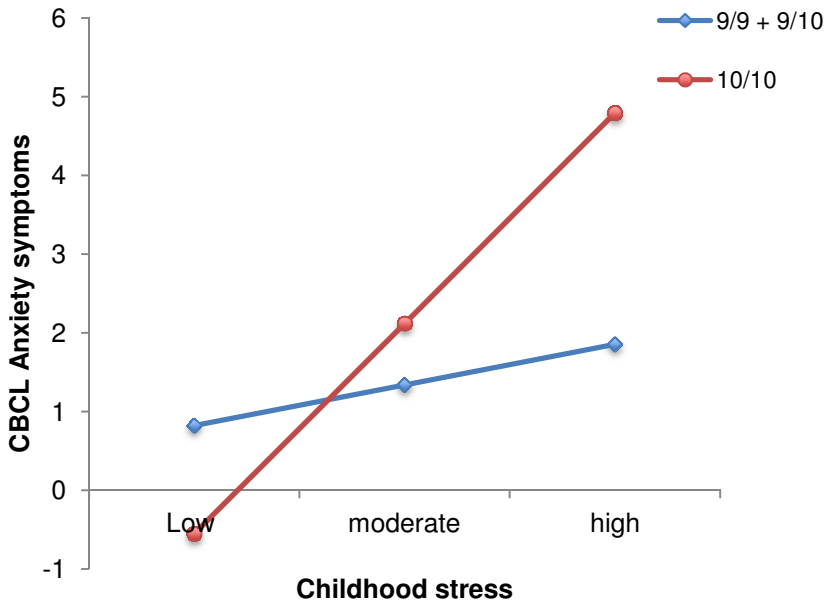


Figure 4.5. Relationship between childhood stress and CBCL depression **(A)** and CBCL anxiety **(B)** by *DAT1* 30bpVNTR allelic groups.

4.3.3 Path analyses

In previous sections, I examined associations between DA genetic variants and children's cortisol responses to stress. Second, I examined the moderating role of DA gene polymorphisms on the association between CS and children's depressive and anxious symptoms. In the final set of analyses, I examined an integrative model where I tested the moderating role of cortisol response on the effect DA gene polymorphisms and CS on emerging symptoms of depression and anxiety in a combined analytical framework.

Figure 4.6A shows the results of the path analyses. The CS measure was positively associated with cortisol responses to stress ($\beta = 0.22$, $p = 0.01$), but the associations between genotype and cortisol were non-significant ($\beta = 0.06$, $p = 0.21$). Child cortisol reactivity, AUC_i, was also positively correlated with emerging symptoms of anxiety ($\beta = 0.14$, $p < 0.01$). The *DRD2*-CS interaction path predicting AUC_i was also non-significant ($\beta = -0.04$, $p = 0.67$). The overall model fit was very good ($\chi^2 = 26.28$, $df = 7$, $p < 0.001$; root mean square error of approximation [RMSEA]= 0.02; normed fit index = 0.97 (guidelines for interpreting path analyses are provided on page 118).

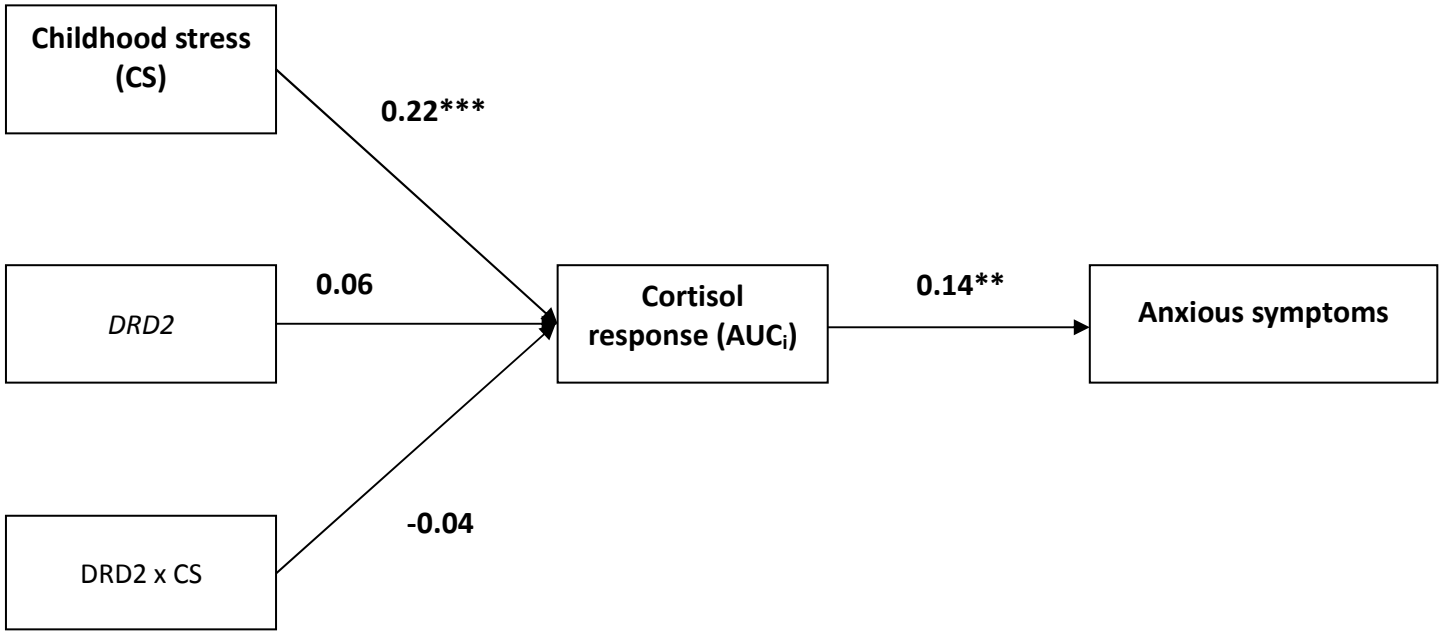
Figure 4.6B shows the results of the path analyses. The CS measure was positively associated with cortisol responses to stress ($\beta = 0.18$, $p < 0.01$), but the associations between genotype and cortisol were non-significant ($\beta = 0.01$ $p = 0.87$). Child cortisol reactivity, AUC_i, was also positively correlated with emerging symptoms of anxiety ($\beta = 0.14$, $p < 0.01$). The *DRD4*-CS interaction path predicting AUC_i was also non-significant ($\beta = -0.04$, $p = 0.67$). The overall model fit was very good ($\chi^2 = 21.25$, *df*

= 7, $p < 0.01$; root mean square error of approximation [RMSEA]= 0.00; normed fit index = 1.00.

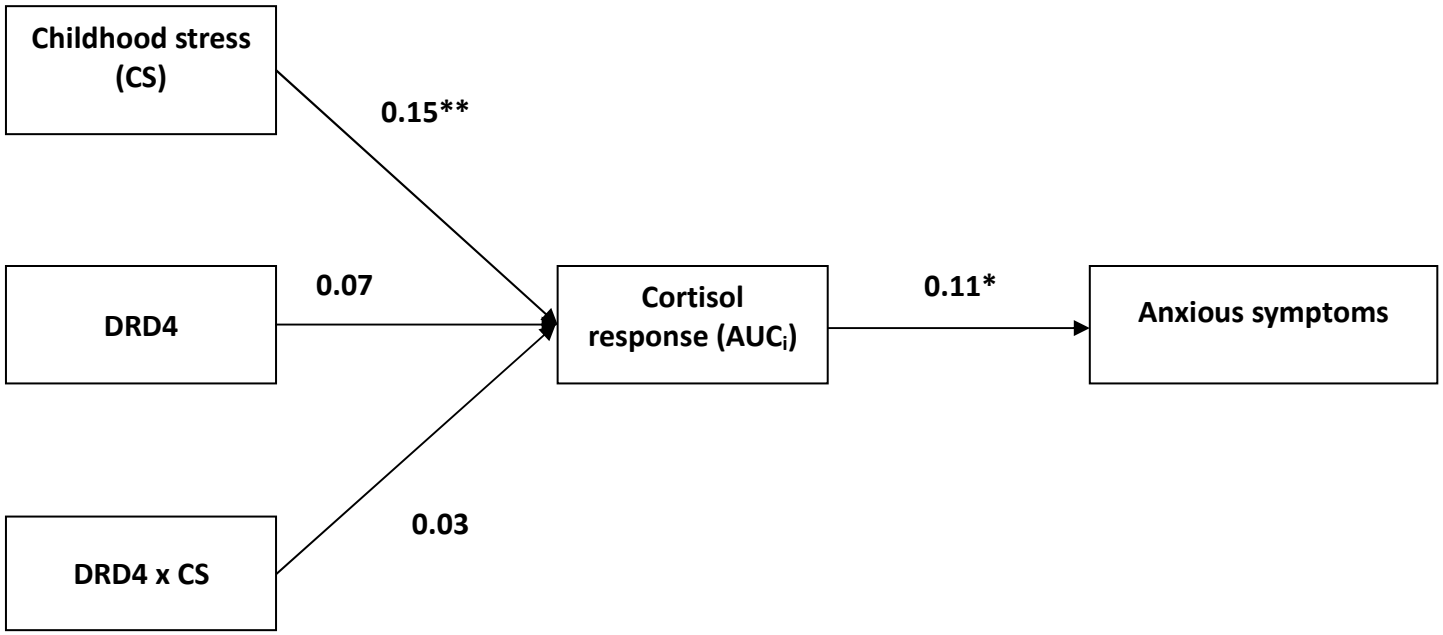
Figure 4.6C shows the results of the path analyses. CS was positively associated with cortisol responses to stress ($\beta = 0.13$, $p = 0.04$), but the associations between genotype and cortisol were non-significant ($\beta = 0.07$ $p = 0.25$). Child cortisol reactivity, AUC_i, was also positively correlated with emerging symptoms of anxiety ($\beta = 0.14$, $p < 0.01$). The *DAT1*-CS interaction path predicting AUC_i was also non-significant ($\beta = -0.04$, $p = 0.04$). However, the overall model fit was mediocre as indexed by the RMSEA and normed fit index analyses ($\chi^2 = 28.39$, $df = 7$, $p < 0.001$; root mean square error of approximation [RMSEA]= 0.08; normed fit index = 0.65).[8]

[8] Similar to AUC_i, I also conducted pathway analyses where AUC_g was modeled as an index of total cortisol produced. The results of these analyses were identical to the models presented in section 4.3.4 (all *ps* < 0.001). Therefore, these pathways were not included in the main text.

A.


Childhood stress (CS)
0.22***
DRD2
0.06
Cortisol response (AUCi)
0.14**
Anxious symptoms
DRD2 x CS
-0.04

B.


Childhood stress (CS)
0.15**
DRD4
0.07
Cortisol response (AUCi)
0.11*
Anxious symptoms
DRD4 x CS
0.03

C.

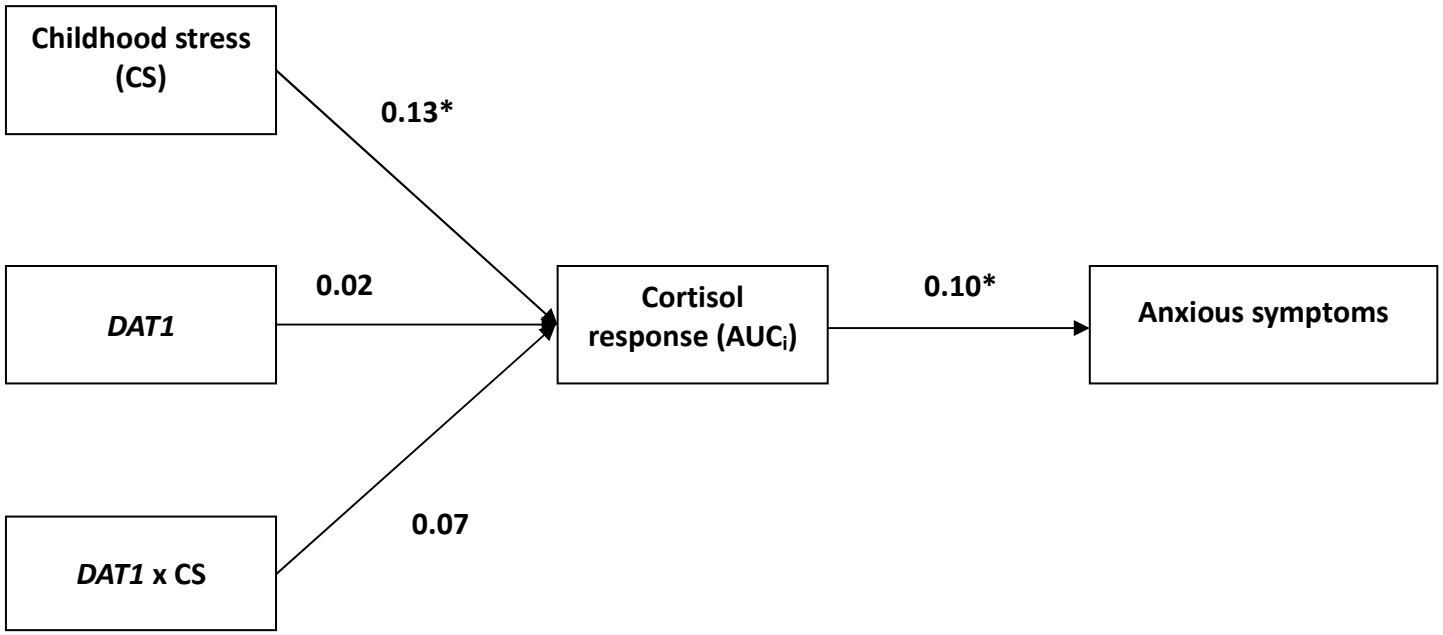


Figure 4.6. Pathway analysis. The path shows interaction between *DRD2* rs1804973 genotype and childhood stress as predictor of child anxious symptoms via AUC_i (A) and AUC_g (B). Child anxious symptoms are based on parent reports from the Child Behavior Checklist (Achenbach et al., 2000). Standardized β-weights are shown for each path. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.4 Discussion

In this chapter, I conducted an analysis of the links between dopaminergic gene variants, cortisol reactivity and emerging symptoms of depression and anxiety in preschoolers. In the first set of analyses, I found that the *DRD2* rs1800473 SNP was associated with children's internalizing symptoms. Analyses did not show associations between this gene variant and either emerging symptoms of depression and anxiety or cortisol reactivity to stress in preschoolers. These findings are consistent with a number of studies that have also reported no associations between *DRD2* Taq1A variant and symptoms (Rot, Mathew & Charney, 2009). However, a few studies have reported positive associations between DA receptor genes and internalizing problems, including a previous study from our group that reported an association between the *DRD2* genotype and symptoms of depression and anxiety (Hayden et al., 2010; Lucht et al., 2006). Similarly, there were no associations between the *DRD2* genotype and cortisol reactivity. However, the interaction between the *DRD2* Taq1A polymorphism and CS predicted both cortisol intensity (AUC_i) and total cortisol response over time (AUC_g). Specifically, the cortisol response increased as a function of CS in carriers with at least one copy of the A1 allele, while childhood stress was not associated with cortisol response in A2 homozygous children. My findings are the first to report moderation of the association between CS and cortisol reactivity by this polymorphism.

There was also evidence for moderation of the association between CS and symptoms of anxiety and depression by the *DAT1* VNTR polymorphism. Specifically, the 9R allele carriers had increasing symptoms as a function of CS, whereas no association between CS and symptoms was present in children with at least one copy of the 10R allele. Previous work from our lab and others has shown that genetic influences on children's psychopathology risk may be moderated by contextual factors (Hayden et al., 2013). Other studies have reported that interactions between *DAT1* and life stress predicted the development of conduct disorder and attention-deficit hyperactivity disorder in children (Bidwell et al., 2011; Lahey et al., 2011). Specifically, the children carriers for the *DAT1* 9R allele had higher risk as a function of increasing negative early life contexts such as negative parenting. My findings complement this literature by showing that interaction between CS and *DAT1* genotype may also contribute to early symptoms of depression and anxiety.

I also conducted path analyses in which I examined the role of DA gene variants, CS and cortisol reactivity in predicting children's symptoms of depression and anxiety. Analyses showed that the path models were only significant for anxiety as an outcome. To my knowledge, no research in literature to date has explored these variables in a path analysis framework, although some research has linked contextual risk exposure to differential DA secretion in brain's limbic regions. Specifically, excessive mesocortical DA released upon exposure to varying levels of stressful life events (such as childhood maltreatment) may increase vulnerability to depression through an inhibition of

subcortical DA transmission (Ventura et al., 2002; Cabib et al., 2002). While directly establishing that regional DA levels are different in children exposed varying levels of stress is not possible, the analyses in this chapter point to a positive link between functional DA gene polymorphisms, levels of CS and emerging symptoms of anxiety.

Taken together, the analyses presented in this chapter suggest that the *DRD2* and *DAT1* gene polymorphisms contribute to emerging risk for anxiety in young children. However, there were no associations between *DRD4* VNTR and cortisol reactivity and emerging symptoms of depression and anxiety. Current GxE research involving the *DRD4* gene polymorphism suggests that adults with the 7R allele are more likely to be affected by life stress than are those with carriers of less than 7R allele. Additionally, a large amount of GxE literature involving *DRD4* comes from cohorts suffering from substance abuse disorders where carriers the 2R and 4R allele did not relapse back to drug use when they experienced high levels of life stress when compared to 7R carriers, suggesting some implication for this gene in stress resilience. Due to the role of DA circuits in reward processing in the brain, it is plausible to assume that some of the DA receptor polymorphisms may be linked to individual differences towards stress contexts. However, future research should examine the role of the *DRD4* polymorphism in contextual processes at various levels of analysis (family, peer, school, or neighborhood) over middle and late childhood.

Some limitations of the research in this chapter should be noted. Only one genetic polymorphism for each gene was examined; this does not represent all of

the genetic variation important to shaping cortisol reactivity or emerging symptoms. As discussed previously, many genetic variants may alter risk, the expression of which may emerge only under particular contextual conditions. These limitations notwithstanding, the present chapter offers several novel points for stress research and examines common DA pathway candidate genes implicated in adult psychopathology. Analyses suggest that DA gene polymorphisms contribute to early childhood development and likely contribute to future behaviour outcomes.

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Chapter 5 - The serotonin candidate genes and emerging symptoms of psychopathology: The roles of cortisol reactivity and childhood stress

Along with the dopamine pathway, the serotonin pathway has been widely implicated in shaping early neurodevelopment. Among the multiple neurochemical processes involved, reduced brain serotonin (5-HT) seems to be feature of depression and anxiety (Van Praag, 2004). Although there are questions about the exact role of 5-HT in the onset and course of depression, 5-HT dysfunction is commonly found in depressed patients as captured by lower brain availability of tryptophan and 5-hydroxytryptophan (Agren & Reibring, 1994; Maes et al, 1990). Specifically, impaired reuptake and degradation in the synapse have been implicated in stress-related mood disorders such as depression (Arango et al. 2002; Markus & Firk, 2011; Malison et al, 1998; Sargent et al, 2000) and antidepressant drugs act by improving brain 5-HT function (van Praag, 2004). Candidate genes with functional effects on the 5-HT reuptake and its degradation, and their links to the stress response, are discussed in the following sections.

5.1.1 5-Hydroxytryptophan transporter (SLC6A4) *gene polymorphism*

Probably the most widely-studied and controversial candidate genes for depression is the serotonin transporter gene (5-HTT). The *5-HTT* (*SLC6A4*) gene spans 31kb and contains 14 exons (Lesch et al., 1994). A common functional polymorphism has been described in the promoter region this gene known as the 5-HTTLPR (Figure 5.1). Variants of the 5-HTTLPR consist of a long (L) allele,

comprised of 16 copies of an approximately 22 base pair (bp) repeat unit, and a short (S) allele, consisting of 14 copies (for a review, see Hariri & Holmes, 2006). Compared to the L allele, the S allele is associated with decreased transcriptional efficiency of the promoter (Lesch et al., 1994). Reduced density of the 5-HT transporter has been found in subjects with depression and in the postmortem brain tissue of suicide victims (Arango et al. 2002, Drevets et al., 1992). In addition to associations between 5-HTTLPR and psychopathology, a large body of research suggests that the 5-HTTLPR moderates the effects of adverse life experiences on the probability and severity of a diverse array of mental health related conditions and constructs, including depression (Taylor et al., 2006; Caspi et al., 2003), suicide (Roy et al., 2007; Retz et al., 2008) and anxiety (Gunthert et al., 2007; Stein et al., 2007). Across these diverse stress-related psychological disorders, the majority of studies associate the 5-HTTLPR S-allele, and particularly the S/S genotype, with greater psychological sensitivity to stress (Uher et al., 2007). However, there is variability in the success of replicating such findings as some meta-analysis studies have found no evidence that the 5-HTTLPR genotype alone or in interaction with stressful life events is associated with elevated depression risk (Risch et al., 2009). However, these meta-analyses have been criticized on the grounds that they are heavily influenced by large studies that typically have the poorest measurement of life events (i.e., self-report checklists) and, as a consequence, are poorly equipped to detect conditional effects of the environment (Caspi et al., 2010; Karg et al., 2011). However, it is also possible that the 5-HTTLPR may be more closely associated with the

regulation of the hypothalamic-pituitary-adrenal (HPA) axis response to stress than it is with specific psychological states that are influenced by stress (Markus & Firk, 2011). Thus, the cortisol response to stressful life events may represent an intermediate phenotype between the 5-HTTLPR and psychological disorder. Literature shows that serotonin fibers activate the hypothalamic CRH neurons (Heisler et al., 2007; Liposits, Phelix & Paul, 1987) that initiate the hormonal cascade leading to cortisol release. The S/S genotype of the 5-HTTLPR is associated with greater cortisol reactivity to psychosocial stress in adults (Way & Taylor, 2010). Additionally, a study by Wust and colleagues (2009) linked this polymorphism to basal cortisol secretion over the course of the day, showing a link between HPA axis function and this gene. However, two recent independent studies in adults have failed to find positive associations between this polymorphism and cortisol reactivity in adults (Markus & Firk, 2009; Vinberg et al., 2010). Taken together, the present literature suggests a need for further research to ascertain the relationship between this polymorphism and cortisol responses to stress, as well as links to emerging symptoms of depression and anxiety.

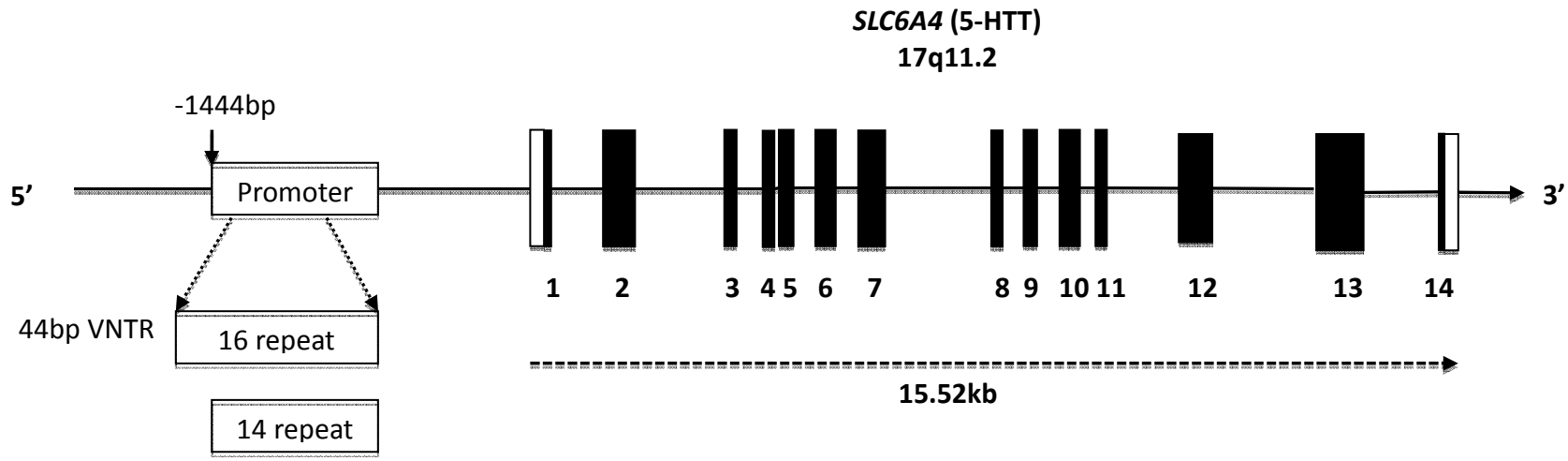

Figure 5.1. Diagrammatic representation of the human *SLC6A4* (5-HTT) gene. Exons are indicated by solid black blocks and introns by lines. The location of the 44bp promoter region variable number tandem repeat (VNTR) are indicated. The 16-repeats (Long) and 14-repeats (Short) variants of the VNTR are indicated by dashed arrows (Adapted from Lesch et al., 1996).

5.1.2 Monoamine Oxidase-A

Along with the 5-HTTLPR and its possible role in the etiology of mood disorders, the MAOA enzyme also regulates availability of serotonin in the synaptic cleft and therefore regulates serotonergic tone in response to external stimuli (Jacob et al., 2005). MAOA is an enzyme involved in the metabolism of biological amines, including the monoaminergic neurotransmitters serotonin and norepinephrine (Jacob et al., 2005). MAO contributes to controlling amine availability by oxidative deamination of neuronal serotonin (Shih et al., 1999). MAOA enzyme expression is dependent on the *MAOA* gene (Gene ID: 4128), which is located on chromosome Xp11.3 and contains 16 exons, spanning 60 kilobases (Figure 5.2). A naturally occurring sequence polymorphism exists in the *MAOA* promoter region with functional effects on MAOA transcription. This VNTR polymorphism consists of 30-bp repeat elements with 3, 3.5, 4, 5 or 6 copies (Sabol et al., 1998). Relative to the longer alleles, the 3-repeat allele results in a significantly decreased expression of *MAOA* gene, and is therefore commonly referred to as the low activity variant compared to the longer or high activity alleles (Deckert et al., 1999). The latter allelic variant also results in higher cerebrospinal fluid (CSF) homovanillic acid levels, a byproduct of catecholamine catabolysis, indicating functional effects of this polymorphism in humans (Zalsman et al, 2005). As the *MAOA* gene is X linked, males (XY) carry only a single copy of the *MAOA* allele, whereas females (XX) carry two, one of which is subject to random X-chromosome inactivation in the cell. Thus, high activity boys have a single long or “high activity” allele, whereas girls must have the high/high genotype in order to be unequivocally established as having the high activity allele for

the purposes of research, as one copy will be randomly inactivated due to X-linked inactivation (Deckert et al., 1999).

Given the functional nature of *MAOA* VNTR and its effect on serotonin signaling (or serotonergic tone) in the brain, it is plausible that this polymorphism may be linked to individual differences in cortisol response to stress. Only a single study so far has examined associations between the *MAOA* VNTR polymorphism and cortisol reactivity, and found a positive association between the low activity MAOA allele and elevated cortisol reactivity to psychosocial stress task (Bouma et al., 2012). In addition to this recent study, a few studies have also examined the main effects of *MAOA* genotype on stress-related disorders such as depression and anxiety, but the findings in this literature have been mixed. Some have found positive links between the *MAOA* VNTR polymorphism and anxiety (Gutierrez et al., 2004; Tochigi et al., 2006) but others have failed to replicate such findings (Arbelle et al., 2003; Eley et al., 2003; Jacob et al., 2005; Syaglio et al., 2001), suggesting a need for further work to clarify the link between this polymorphism and anxiety. There are similar mixed results for associations with depression, with depression associated with *MAOA* VNTR in some studies (Tochigi et al., 2006) and not in others (Gutierrez et al., 2004).

In addition to main effects, this gene has been implicated in GxE. For example, GxE has been found for *MAOA* and adversity (Prom-Wormley et al., 2009), life trauma (Frazzetto et al., 2007), and childhood maltreatment (Taylor & Kim-Cohen, 2007) in predicting both internalizing and externalizing outcomes. More complex interactions have also been noted, with high MAOA activity buffering the effects of early adversity on later antisocial behaviour in Caucasian participants (Widom & Brzustowicz, 2006).

Similarly, Cicchetti and colleagues (2007) found that depressive symptoms were elevated in maltreated children only if they had the low-activity *MAOA* variant. These findings were also replicated in adults by Kinnally and colleagues (2009), who reported that adults having lower activity MAOA and exposed to early family stressors showed higher impulsivity/aggression than girls with good parental care. Other data (Kim-Cohen et al., 2006) indicate that adolescents with the low MAOA activity allele who were exposed to physical abuse had more mental health problems. Taken together, these studies suggest that MAOA plays a role in moderating the effects of the early environment on psychopathology.

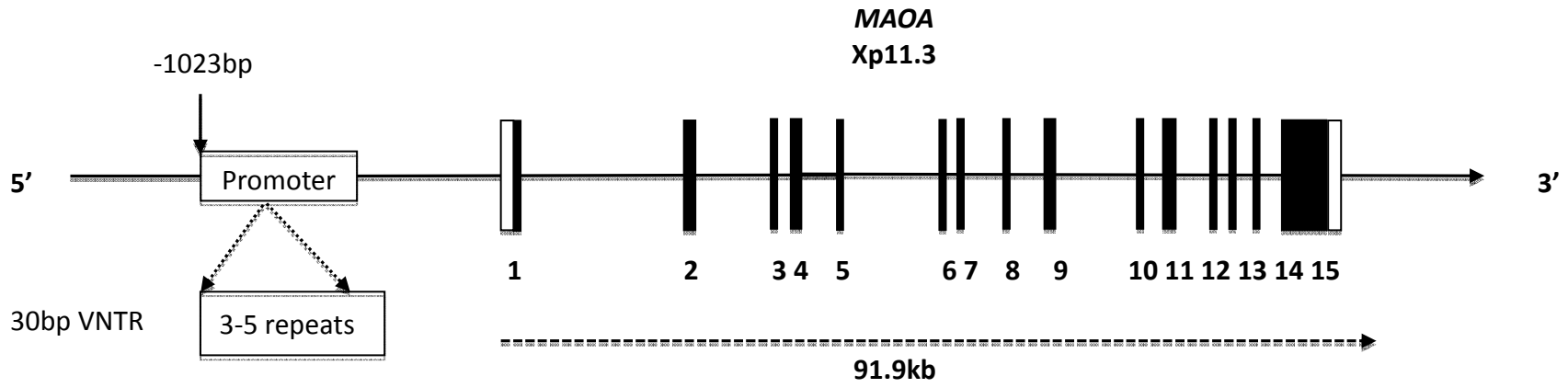

Figure 5.2. Diagrammatic representation of the human *MAOA* gene. Exons are indicated by solid black blocks and introns by lines. The location of the 30bp promoter region variable number tandem repeat (VNTR) are indicated. The approximate location of 3-5 repeats VNTR is indicated by dashed arrows (Adapted from Sabol et al., 1998).

In addition to gene-environment interaction in influencing symptoms of depression and anxiety, a limited literature has also examined the effect of interaction between *MAOA* and 5-HTTLPR gene polymorphisms (gene-gene interaction; GxG) on symptoms of anxiety and depression. For example, interactions between *MAOA* VNTR and 5-HTTLPR have been linked to cognitive performance in a large, population-based sample of 6000 children (Barnett et al., 2011). Specifically, an epistatic interaction between the two loci was associated with better working memory in children with the long alleles of both MAOA and SLC6A4 genes. However, studies to date have not reported epistasis between the *MAOA* and 5-HTTLPR loci in predicting risk for psychopathology in humans. In additions to GxG, a report by Eley and colleagues (2004) reported that the interaction between *MAOA* and 5-HTTLPR moderated the effect of life stress on anxious and depressive symptoms. Specifically, adults homozygous for the low activity alleles of *MAOA* and 5-HTTLPR VNTR polymorphisms interacted with low socio-economic status to predict higher number of anxious and depressive symptoms. Taken together, this limited literature suggests GxG are linked to emotional problems in adults. However, whether GxG predicts emerging symptoms of depression or anxiety remains unexplored.

In sum, the aim of this chapter is to explore main effects of 5-HTTLPR and MAOA polymorphisms on children's cortisol responses to stress and emerging symptoms of depression and anxiety. Based on research reporting positive associations between cortisol function and 5-HTTLPR and *MAOA* genes (Bouma et al., 2012; Way & Taylor, 2010), and the possible role of cortisol reactivity as a vulnerability marker for later psychopathology, I examined these links in a community sample of preschoolers.

Second, based on previous evidence for moderation of early environment risk contexts by serotonin genes on symptoms of internalizing psychopathology risk, I predicted that these links would also exist at an early age as well. The final set of analyses in this chapter would aim to examine and extend the evidence for epistasis as moderator of childhood stress (CS) on cortisol response and emerging symptoms.

5.2 Methods

Participant DNA was extracted as described previously in chapter 2. Also, as described in Chapters 2 and 3, parent reports on the Child Behavior Checklist (Achenbach et al., 2000) were used to ascertain levels of depression and anxiety. AUC_i, baseline to cortisol change, and AUC_g were used as measures of cortisol response as previously described in detail on page 54. Finally, details of the CS variable are detailed in Chapter 2 as well.

5.5.1 Genotyping

The 5-HTTLPR polymorphisms were assayed using methods described by Chorbov et al. (2007). Briefly, the forward primer was 5-GGCGTTGCCGCTCTGAATGC-3', and the reverse primer was 5-GAGGGACTGAGCTGGACAACCAC-3, which yielded 486-bp (short) and 529-bp (long) amplicons. Polymerase chain reaction (PCR) was performed in a total volume of 25µl, containing 100 ng of DNA, 160 nM of each primer, 1mM Tris-HCL (pH 8.3), 5mM KCl, 1.5 mM MgCl2, 2% DMSO (v/v), 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 200 µM of dATP, dCTP, dTTP, and 100 µM of dGTP, and 7-deaza-2-dGTP. After an initial denaturation at 94° C for 5 min, 35 cycles of

denaturation (94°C for 30 sec), annealing (63°C for 30 sec), and extension (72°C for 1 min) was performed followed by a final extension at 72°C for 20 min. Amplicons were separated on 6% polyacrylamide gels, visualized using SYBR-Green and documented using the Bio-Rad 2000 gel documentation system (Bio-Rad Laboratories, Mississauga, ON, Canada).

M*AOA* promoter VNTR was amplified using the primer sequences forward 5'-CCCAGGCTGCTCCAGAAACATG-3' and reverse 5'-GTTCGGGACCTGGGCAGTTGTG-3'. Conditions used for amplification were one cycle at 94°C for 5 min followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 30s, and a final 7 min extension at 72°C (Sabol et al., 1998). To improve reaction fidelity, we used the Invitrogen PCRx Enhancer (Invitrogen, Carlsbad, CA, USA) as an adjuvant in PCR amplifications. Amplicons were separated on 6% polyacrylamide gels, visualized using SYBR-Green and documented using the Bio-Rad 2000 gel documentation system (Bio-Rad Laboratories, Mississauga, ON, Canada).

5.3 Results

5.3.1 Main effects

In this sample, 119 children (32.1%) were homozygous for the long (L) allele of the 5-HTTLPR, 171 (46.1%) were heterozygous, and 76 (20.5%) were homozygous for the short (S) allele. I was unable to genotype five children due to non-specificity of PCR amplification. This distribution is in Hardy-Weinberg equilibrium, $\chi^2 = 0.31$, $p > 0.52$. Analysis of variance was used to test the associations between genotype and study variables including cortisol response and behaviour measures (Table 5.1). The 5-

HTTLPR polymorphism was not associated with child gender ($\chi^2 = 4.78$, $p > 0.09$) or family income ($\chi^2 = 41.25$, $p = 0.59$). I found a main effect of the 5-HTTLPR polymorphism on emerging anxiety symptoms such that children with at least one copy of the S-allele had higher symptoms of anxiety compared to their L-allele counterparts (t (365)= 2.14, $p = 0.03$).

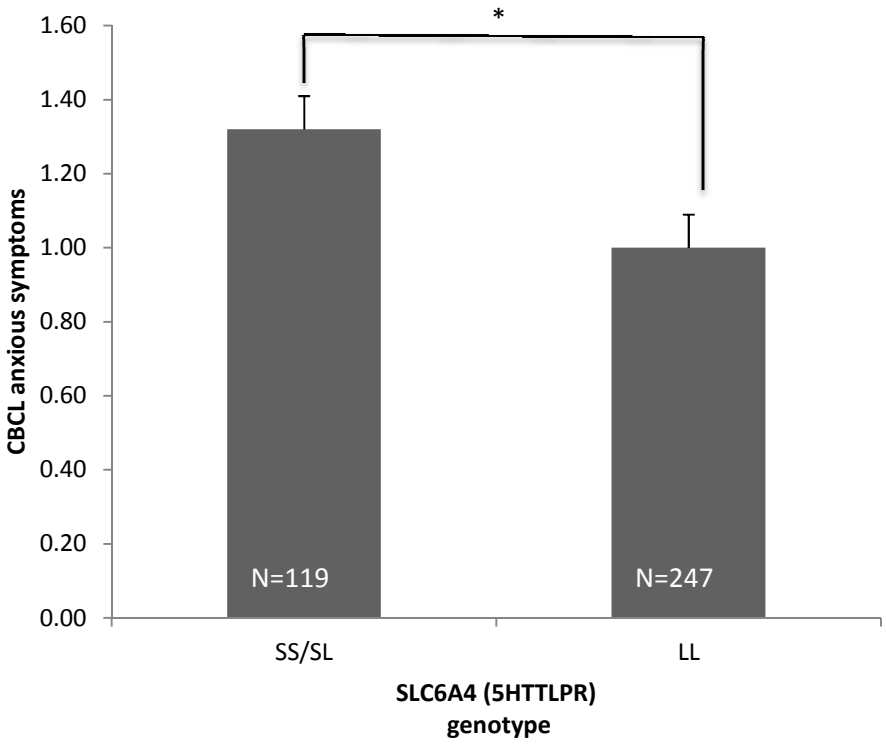

Figure 5.3. Relationship between the 5HTTLPR 44bp VNTR polymorphism and symptoms of anxiety in preschoolers (N=366).

Table 5.1. Demographic and study variables by child 5-HTTLPR 44bp VNTR genotype.

	5-HTTLPR genotype					
	s/s + s/l (short allele) (N=241)			l/l (long allele) (N=114)		
Variable	*M*	*SD*	*N*	*M*	*SD*	*N*
Sex (N, boys)			114			69
Family income	3.80	1.05		3.70	1.2	
PPVT	112.19	13.80		111.22	14.89	
Baseline to peak change	-1.10	0.35		-1.06	0.36	
AUC_i	0.05	0.10		0.05	0.08	
AUC_g	0.17	0.12		0.18	0.12	
CBCL Depression	1.36	1.62		1.22	1.48	
CBCL Anxiety*	1.32	1.09		1.00	1.33	

Note: PPVT = Peabody Picture Vocabulary Test; Family income coded as 1 = < $20,000; 2= $20,000-$40,000; 3= $40,001-$70,000; 4 = $70,001-$100,000; 5 = > $100,001; AUC_i, Area Under Curve with respect to increase; AUC_g, Area Under Curve with respect to ground; CBCL, Child Behavior Checklist.

* $p < 0.05$ $^{\dagger}p < 0.10$.

I was unable to genotype three children for the *MAOA* polymorphism due to lack of PCR amplification. For boys, the *MAOA* hemizygous frequencies were: 3R (N = 74, 19.8%), 3.5R (N = 8, 2.2%), 4R (N = 101, 27.2%) and 5R (N = 4, 1.1%). *MAOA* genotypes in girls were, 3/3 (N = 54, 13.7%), 3.5/4 (N = 1, 0.3%), 3/4 (N = 52, 14.0%), 3/5 (N = 4, 1.1%), 4/4 (N = 65, 17.5%) and 4/5 (N = 5, 1.1%). This distribution is not in Hardy-Weinberg equilibrium (Pearson X^2 (38) = 91.02, $p < 0.05$), but is comparable to recently reported frequencies (Sabol et al., 1998). Consistent with the majority of published research (e.g., Zalsman et al., 2005; Ducci et al., 2006), groups for data analysis were formed based on whether children had 3R polymorphism (N = 183) or did not have (N = 185) a 3R allele. Following previous studies (Zalsman et al., 2005), heterozygous females (N = 62) were excluded from analyses, as it is not possible to know which X-chromosome escaped inactivation. Furthermore, four boys homozygous for the *MAOA* 5R allele were excluded from analyses as the literature is unclear with respect to functionality of the translated protein associated with this allele. This meant that the final sample size available for analyses in the chapter was 301.

Table 5.2. Demographic and study variables by child *MAOA* 30bp VNTR repeat status.

	Child *MAOA* genotypes					
	3-repeats (N = 131)			≥ 3.5 repeats (N = 171)		
Variable	*M*	*SD*	*N*	*M*	*SD*	*N*
Sex (N, boys)			80			100
Family income	3.73	1.11		3.8	1.11	
PPVT	113.11	15.46		111.01	13.03	
Baseline to peak change	-1.12	0.38		-1.07	0.35	
AUC_i	0.05	0.08		0.05	0.10	
AUC_g	0.18	0.13		0.18	0.13	
CBCL Depressive symptoms	1.19	1.45		1.25	1.48	
CBCL Anxious symptoms	1.14	1.27		1.22	1.46	

Note: PPVT = Peabody Picture Vocabulary Test; Family income coded as 1 = < $20,000; 2= $20,000-$40,000; 3= $40,001-$70,000; 4 = $70,001-$100,000; 5 = > $100,001; AUCi, Area Under Curve with respect to baseline; AUCg, Area Under Curve with respect to ground; CBCL, Child Behavior Checklist; ODD, Oppositional Defiance Disorder; ADHD, Attention-Deficit Hyperactivity Disorder.

5.3.2 Moderation analyses

5.3.2.1 5-HTTLPR

Using multiple regression, in my next set of analyses I examined whether GxE predicted children's cortisol response and symptoms of depression and anxiety. Analyses showed no evidence for moderation of CS by 5-HTTLPR in predicting any index of cortisol reactivity or symptoms of depression and anxiety. More specifically, the interaction between CS and 5-HTTLPR did not predict baseline to peak cortisol change (β = -.03, *se* = 0.05, *p* = 0.58), AUC_i (β = 0.01, *se* = 0.02, *p* = 0.77) or AUC_g (β = -0.01, *se* = 0.03, *p* = 0.59). Similarly, there was no evidence for moderation by 5HTTLPR of CS association with emerging symptoms of depression (β = 0.03, *se* = 0.26, *p* = 0.88) and anxiety (β = 0.40, *se* = 0.30, *p* = 0.18).

5.3.2.2 MAOA

Similarly, I conducted GxE analysis with *MAOA* as a moderator variable. Regression analyses showed that the interaction between MAOA and CS did not predict individual baseline to peak cortisol change (β = 0.02, *se* = 0.05, *p* = 0.56), AUC_i (β = 0.01, *se* = 0.02, *p* = 0.72) or AUC_g (β = 0.01, *se* = 0.02, *p* = 0.61). However, analyses showed significant moderation of CS by *MAOA* genotype on child anxious symptoms (β = 0.77, *se* = 0.27, *p* = 0.004). Further analysis showed that children without the 3-repeat allele exhibited higher anxiety symptoms as a function of increasing CS (β = 0.63, *se* = 0.17, *p* = 0.001), but CS was not associated with symptoms of anxiety in children with the 3-repeat allele (β = -0.14, *se* = 0.20, *p* = 0.50; Figure 5.4). I did not find evidence of

moderation of CS by *MAOA* when child depressive symptoms were the dependent variable (β = 0.08, *se* = 0.28, *p* = 0.79).

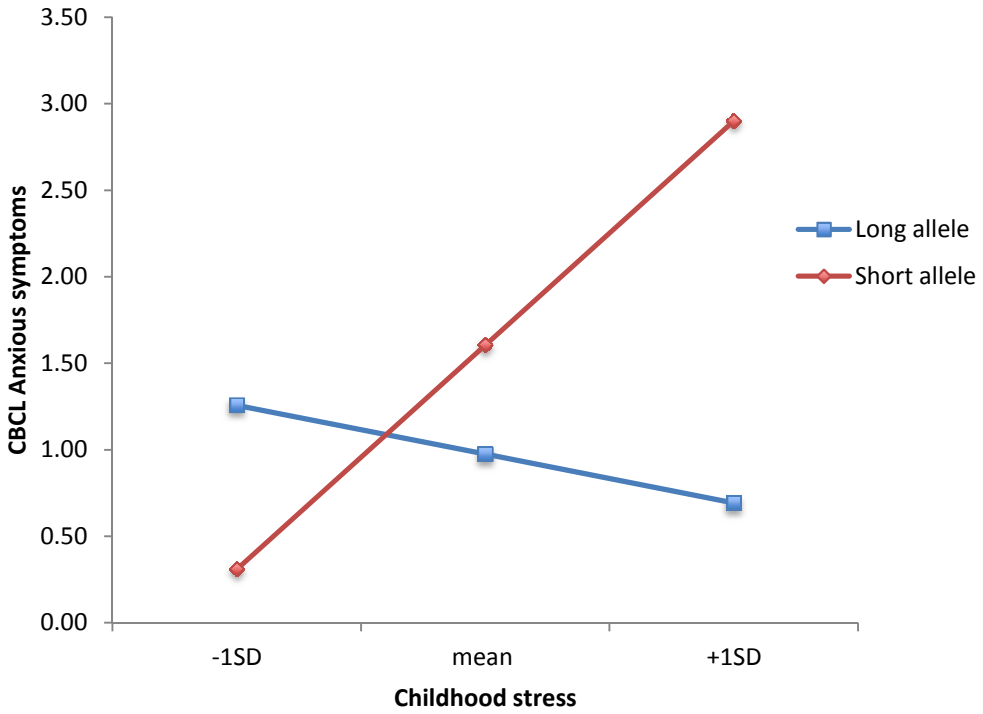

Figure 5.4. Relationship between childhood stress and child anxious symptoms by *MAOA* 3-repeat (short allele) status.

5.3.3 Gene-Gene interactions

Previous literature has also implicated an interactive effect of the 5-HTTLPR VNTR and *MAOA* VNTR on developmental risk for psychopathology (Barnett et al., 2011; Roiser et al., 2007). Therefore, I also investigated whether such a gene-gene interaction (GxG) also predicted symptoms of depression and anxiety in preschoolers. Analysis showed that the GxG terms were nonsignificant and did predict either cortisol response or emerging risk for internalizing problems (all *ps* > 0.16; figure not presented).

5.4 Discussion

The aim of this chapter was to examine links between serotogenic gene functional polymorphisms and symptoms of depression and anxiety in preschoolers. The first finding was a positive association between the 5-HTTLPR polymorphism and child anxious symptoms. Specifically, children with at least one copy of the S-allele had higher levels of anxiety than children homozygous for the L-allele. This finding extends and compliments extant literature linking this polymorphism and internalizing symptoms. For example, literature has reported that the S-allele of the 5-HTTLPR was associated with anxiety traits (Katsuragi et al., 1999; Lesch et al., 1996; Ohara et al., 1998; Gonda et al., 2007) and with anxiety-related personality traits such as neuroticism and impulsivity (Gorwood, 2004; Sen et al., 2004). The presence of associations between emerging anxiety symptoms in three-year-olds and this polymorphism is a novel finding and suggests a possible role of this variant in the early development of anxiety vulnerability. Along with anxiety traits, research has also linked this polymorphism with depression in adults (Arya et al., 2009), but such associations did not exist in this sample. The lack of associations could be due to a few possibilities including the young age and the non-clinical nature of this sample. As discussed in Chapter 3 and 4, depressive symptoms are rare during preschool years; it is possible that associations between this gene and depressive symptoms may emerge later when such symptoms are more common and variable across children. A number of prior studies have detected an interaction between 5-HTTLPR and stressful life experiences such as

childhood abuse (Caspi et al. 2003; Araya et al., 2009) in predicting depressive symptoms. However, I did not find evidence for such an interaction in this sample. As discussed in earlier chapters, the lack of an extreme stressor context could be one of the reasons for the lack of replication in this sample (see this discussion in Chapter 4). Additionally, a reason for the failure to find a significant association between 5-HTTLPR and emerging symptoms (and cortisol reactivity) may reflect insufficient power to detect such associations.

In addition to 5HTTLPR polymorphism, previous research on GxE in adults has implicated the *MAOA* 30bp VNTR in moderating the effect of childhood maltreatment on psychiatric symptoms (Kraft et al., 2006; Wendland et al., 2006); therefore, I tested this model in the current sample. My analyses complemented this earlier literature in adults by showing that the interaction between *MAOA* genotype and CS predicts children's anxious symptoms. In addition to anxiety problems, previous GxE studies in literature have linked *MAOA* VNTR polymorphism with other psychiatric phenotypes in the presence of early adversity. For example, in their seminal work, Caspi et al. (2002) found that individuals possessing the 3-repeat genotype of *MAOA* showed increased risk for antisocial behaviour, but only if they had experienced an adverse early environment. Other studies have found similar interactions between *MAOA* gene polymorphism and early maltreatment for behaviours reflecting conduct disorder (Foley et al., 2004), antisocial behaviour (Kim-Cohen et al., 2006), antisocial alcoholism (Ducci et al., 2007), and physical aggression (Frazzetto et al., 2007). These findings add to this literature by showing the moderation of symptoms by

MAOA VNTR genotype in the presence of more subtle forms of life stress. As most research has reported the moderation effect of this genotype on extreme forms of adverse experience (such as physical and sexual abuse), the findings in this chapter suggests that this variant may moderate a broad contexts of early life stressors, and is a novel finding in literature with implications on early childhood development.

In my final set of analyses, I looked at the effect of MAOA and SLC6A4 (5-HTTLPR) on either cortisol response or emerging symptoms at preschool age and found no evidence for GxG. My analyses did not support literature from previous adult studies, which reported that GxG predicted symptoms of psychopathology (Roiser et al., 2007). However, my findings are in line with literature from young samples such as adolescents, which show that the interaction between *MAOA* and 5HTTLPR variants did not predict symptoms of anxiety (Armbruster et al., 2011). Although speculative, these findings suggest that the interaction between these two variants may be evident at a later age rather than in early childhood.

In sum, I present evidence that functional polymorphisms in 5-HTTLPR and *MAOA*, two gene involved in the functional deactivation of serotonin, are linked to emerging symptoms of anxiety in young children. While neither gene has been unquestionably linked to psychiatric symptoms, the data in this chapter suggests that serotonergic polymorphisms linked to normal variation in brain function are at play in influencing early-emerging symptoms of depression and anxiety in the presence of life stress. The lack of links between these genes and

cortisol reactivity suggests that such links may possible be influenced by other hormone and/or components of the HPA axis pathway or more evident at a later age.

5.5. References

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Chapter 6 - Discussion

The current thesis is an examination of genetic markers of children's cortisol responses to stress and their possible role in contributing to emerging internalizing disorder symptoms. The implications of my findings for our understanding of the biological and environmental bases of children's internalizing disorders risk are discussed in the following sections.

6.1 Context of study findings

6.1.1 Chapter 2 – The CRH system genes

Chapter 2 expanded on the hypothesis that variation of the CRH system genes would be linked to individual differences in cortisol responses to stress. Specifically, single nucleotide polymorphisms (SNPs) spanning the coding and regulatory regions of *CRH*, *CRHR1* and *CRHBP* genes were examined. I identified significant main effects of gene variants of *CRHR1* and *CRHBP* coding regions on childhood cortisol reactivity. In addition to main effects, I identified GxE with multiple individual SNPs, as well as with common variants of the *CRHR1* and *CRHBP* locus, that were associated with children's internalizing symptoms in the presence of stress (Figures 2.7 & 2.9). The associations between SNPs of the *CRHR1* and *CRHBP* coding region and cortisol reactivity are the first reported in the literature. Additionally, the GxEs I found are an extension of previous work suggesting that CRH system gene variant SNPs moderate the effect of early adversity on future psychopathology (Bradley et al., 2008). The SNP variants involved in these studies were the same as those

described previously in two separate studies by Bradley and colleagues (2008) and Polanczyk and colleagues (2009). However, the stress contexts in the present study and previous studies by Bradley and colleagues (2008) were different. Specifically, the CS variable used in this study included family income, dyadic adjustment and family stressors such as illness, job loss, etc. over the past 12 months, and were in some cases less extreme forms of stress than those typically examined in the field. For example, Bradley and colleagues (2008) examined severe or extreme forms of stressors such as physical and sexual abuse. Taken together, findings in Chapter 2 and previous findings (Bradley et al., 2008; Cicchetti et al., 2009; Heim et al., 2009; Polancyzk et al., 2009) suggest that the CRH gene variants interact with a broad range of forms of early adversity to influence adjustment in childhood and later in life.

6.1.2 Chapter 3 - The Catechol-O-Methyltransferase (COMT) gene

As an important regulator of dopaminergic signaling in the prefrontal and limbic systems of the brain, the functional polymorphism of the *COMT* gene (*val*158*met*) has been extensively studied in psychiatric literature (Meyer-Lindenberg & Weinberger, 2006). Based on previous findings in animal models where *COMT* gene knockouts exhibited abnormal HPA axis function, I examined the association between the *val*158*met* polymorphism and cortisol reactivity in preschoolers. The analyses showed a main effect of the *val*158*met* polymorphism on children's cortisol reactivity, supporting previous work by Jabbi and colleagues (2005), who showed that adolescents who were *val* homozygotes showed heightened cortisol responses to psychosocial stress tasks.

In addition to evidence for direct associations, I also investigated GxE. Analyses showed that the *val*158*met* polymorphism moderated the link between CS and emerging symptoms of anxiety; expanding on recent work by Klauke and colleagues (2012) who found that *COMT* genotype moderated the effect of childhood trauma on startle response, which is considered an endophenotype for anxiety disorders. Additionally, in a study by Kolassa and colleagues (2010), the interaction between *COMT* genotype and recent traumatic events predicted an increased risk for posttraumatic stress disorder, such that *val* homozygotes who experienced trauma were more likely to develop posttraumatic stress disorder. In sum, analyses in Chapter 3 along with recent studies in the literature suggest that *val* homozygous carriers are especially vulnerable to stress during early childhood.

6.1.3 Chapter 4 - The dopamine pathway candidate genes

In Chapter 4 of this dissertation, I looked at common variants of the dopamine (DA) pathway and their links to cortisol reactivity to stress and children's symptoms of depression and anxiety. Analyses did not yield evidence for main effects of DA candidate genes on any of these outcomes; however, I did find evidence for GxE in my analyses. Specifically, the *DRD2* genotype moderated the effect of CS on children's cortisol responses to stress. Although this is the first study to report such an effect, these findings expand on a previous study from our group that reported a positive association between the *DRD2* genotype and symptoms of depression and anxiety (Hayden et al., 2010). In Chapter 4, I also reported evidence for moderation of the association between

CS and symptoms of anxiety and depression in preschoolers by the *DAT1* VNTR polymorphism. Specifically, the 9R allele carriers had increasing symptoms as a function of CS, whereas there was no significant association between CS and symptoms in children with at least one copy of the 10R allele. These findings extend previous work from our lab showing that genetic influences on children's psychopathology risk were moderated by contextual factors (Hayden et al., 2013). Other studies have reported an interaction between *DAT1* and life stress in shaping the development of conduct disorder and attention-deficit hyperactivity disorder in children (Bidwell et al., 2011; Lahey et al., 2011). Specifically, the children carriers for the *DAT1* 9R allele had higher risk as a function of increasing negative early life contexts such as negative parenting. Taken together, these studies combined with my findings suggest that this polymorphisms may play a role in etiology of mood and emotion problems.

I did not find associations between the *DRD4* VNTR genotype and cortisol reactivity or internalizing symptoms. The lack of associations could be due to several factors. Recent work from our lab suggests that *DRD4* genotype moderated the association between parenting and child effortful control, a temperament trait associated with both internalizing and externalizing psychopathology (Smith et al., 2013). Specifically, the association between children's effortful control and positive parenting was moderated by children's *DRD4* 7R status, such that children with at least one 7R allele displayed both better and worse effortful control than children without this allele, depending on the degree of positive parenting. These findings suggest that this gene may be

linked to other endophenotypes, such as child temperament, rather than children's psychophysiological stress responses.

6.1.4 Chapter 5 - The serotonin pathway candidate genes

In Chapter 5, I reported analyses examining links between serotonin pathway candidate gene variants and cortisol reactivity. There was no evidence for associations between *MAOA* and 5-HTTLPR and measures of children's cortisol responses to stress. However, I did find evidence for a main effect of 5-HTTLPR genotype on symptoms of anxiety in preschoolers. This finding extends previous work showing that the S-allele of the 5-HTTLPR is associated with anxiety traits (Katsuragi et al., 1999; Lesch et al., 1996; Ohara et al., 1998; Gonda et al., 2007) and with anxiety traits such as neuroticism and impulsivity (Gorwood, 2004; Sen et al., 2004). In addition to main effects, I also tested gene-environment interactions and found significant evidence for moderation of the effect of CS on child anxiety symptoms by *MAOA* genotype. Specifically, children without the 3-repeat allele exhibited higher anxious symptoms as a function of increasing CS. These results extended previous findings where *MAOA* genotype moderated the link between childhood adversity and risk for conduct disorder and antisocial behaviour (Foley et al., 2004; Kim-Cohen et al., 2006), although this report of GxE predicting symptoms of anxiety in preschoolers is the first to be reported, to my knowledge.

In addition to GxE, literature has suggested that interactions between these serotonin candidate gene variants may also moderate the effect of life

stress on psychopathology in adults (Roiser et al., 2007). My analyses extended the work by Roiser and colleagues (2007) and recent work by Barnett and colleagues (2013), suggesting that interaction between *MAOA* and 5-HTTLPR moderated the effect of childhood stress also predicted symptoms of anxiety.

6.2 Implications for risk and resilience

Epidemiological and clinical research studies have consistently identified early exposure to stress as a major risk factor for mood and anxiety disorders (Chapman et al., 2004; Dube et al., 2001; Felitti et al., 1998; Gladstone et al., 2004). The parallel study of individuals who emerge from such adverse environments without significant mood or anxiety disorder (Rutter, 2006) has resulted in the identification of psychosocial and biological variables associated with psychological resilience (Feder et al., 2009). As with environmental variables, predisposing genetic factors also influence vulnerability (Stein et al., 2002; Sullivan, Neale & Kendler, 2000) and resilience (Rijsdijk et al., 2003) in terms of risk for mood and anxiety disorders. However, the relationship between individual genetic variability and exposure to various forms of early life stress and how these interactions translate to early risk remains largely unclear and was the main focus of this dissertation.

Analyses in this thesis show the importance of gene-environment interactions in predicting cortisol reactivity and symptoms of depression and anxiety. More specifically, in Chapters 2, 3 and 4, I report findings that confirm gene-environment interactions as predictors of depressive and anxious

symptoms. These findings extend a literature which has documented the role of gene-environment interaction in conferring risk. This line of work started over a decade ago with the publication of seminal studies by Caspi and colleagues (2002a, 2002b), where gene-environment interaction, predict risk for conduct disorders and risk for depression. In both studies, specific risk alleles drawn from prior biological research on candidate genes were shown to predict behaviour problems, but only under specific environmental contexts, such as childhood maltreatment. Caspi and colleagues (2010) recently provided a cogent review of studies based on their initial discovery that variation in the promoter region of the serotonin transporter gene (*5-HTT*/*SLC6A4*) moderated the link between stressful life events and depression. The present work may not have replicated the exact finding for this variant, but my analyses nonetheless point to the importance of functional monoaminergic variation in moderating the role of more subtle contexts of stress faced by a vast majority of children during early development.

Taken together, what's especially interesting from these studies is that some gene variants contribute to vulnerability by virtue of direct and indirect pathways. Moreover, the data confirm and extend Caspi and colleagues (2010) assertion that risk alleles may carry risk for behavioural disorders because they moderate reactions to the environment. The implication, then, is that specific gene markers are influential but only in combination with environmental triggers, not as main effects on behavioural dimensions and disorders.

6.3 Methodological considerations

6.3.1 Study strengths

The study had a number of strengths. First, I chose to examine genes that are an integral part of the HPA axis pathways activation and inhibition. The gene and pathway-based association analysis considers a gene or a pathway as the basic unit of analysis, and aim to study simultaneously the association of a group of genetic variants in the same biological pathway, in this case, the CRH system pathway (Neale & Sham, 2004). This can help unravel the complex genetic structure of phenotypes in order to gain insight into the biological processes and developmental mechanisms (Curtis, Oresic & Vidal-Puig, 2005). Additionally, in case of the CRH system genes (*CRH*, *CRHR1* and *CRHBP*) the haplotyping of tag-SNPs captured entire genetic variation in the coding and regulatory regions of these genes, making the analyses comprehensive. Similarly, monoaminergic candidate genes polymorphisms examined in this thesis were also functional in nature, with well-documented functional effects on brain neurotransmitter systems. Furthermore, in Chapter 2, I also controlled for Type I error by using permutation-based procedures (Schmidt et al., 2002, 2003) that randomly assigned the sample AUC_i scores to subjects (sampled without replacement) while holding each subject's genotype fixed. This permutation method is preferred as it accounts for linkage disequilibrium among SNPs in a haplotype block; therefore conserving power compared to commonly used correction techniques such as the Bonferroni method (Schmidt et al., 2003). Moreover, I tried to keep population stratification to a minimum by conducting all analyses in Caucasians only.

In addition to precautions taken during data analyses, conducting this research in a young sample should be considered a major strength. Specifically, as mood disorders are rare in preschoolers, our data was not confounded by previous or current psychopathology. Additionally, literature has linked sex hormone status such as estrogen during the menstrual cycle with cortisol reactivity to stress. However, sex hormones are not a confounder in my study due the young nature of this sample. Similarly, precautions were taken during cortisol sampling as well to obtain an accurate index of children's stress reactivity. For example, the stress task was conducted during the same time of the day to control for diurnal variance of cortisol in humans (Gunnar, Talge & Herrera), and parents were instructed not to feed their children immediately prior to cortisol sampling to address the influence of certain foods on cortisol levels (Gunnar, Talge & Herrera). The stress task was conducted after an acclimatization period of 30 minutes to capture normalized baseline cortisol and was conducted by trained study personnel. Additionally, all cortisol samples were measured in duplicates in a single batch for a given participant usually within 24 hours of sample collection. All measurements were repeated if cortisol variance within the duplicates or even different plates exceeded >5-6 % variance, a very stringent criteria for ELISA assays. Furthermore, 10 % of all genotyping was repeated and all probes were sequenced for accuracy. Of the ~13000 genotypes, 99.8 % were in concordance.

6.3.2 Limitations

Some limitations of this thesis should be considered as well. Even though the sample size of this study was relatively large compared other studies in developmental literature, it is small for a genetic study. However, it may be logistically challenging and not financially viable to perform stress tasks for cortisol assessment on a very large sample. Additionally, due to the sample size, all the variants in this thesis are common genetic variants in the Caucasian population. Biological interactions that involve rare genetic variants or rare environment exposures are unlikely to detect as significant statistical interactions (Uher, 2008). In this case, therefore, even a very large sample size will only be powerful enough to detect moderately strong GXE. Therefore, replication of findings in independent samples is very important. For some studies, I examined a limited number of markers at each gene, and I did not correct for multiple statistical tests in some analyses due to the hypothesis-driven nature of the work.

The study also used parent-reported measures of child behaviour. The difference between objective interview and self-report or parent-report measures of behaviour is a question of specificity and objective validity (Monroe, 2008). For example, parental psychopathology may “color” descriptions of child problems as may occur when abusive or depressed mothers provide negative or exaggerated descriptions of their children (Gotlib & Hammen, 1992). Similarly, dismissive/avoidant adult informants deny the presence of emotional problems at the same time that professionals observe a high level of symptoms (Dozier & Lee, 1995), or parents downplay the importance of a given behaviour or sometimes individuals simply try to avoid thinking of past traumatic events (Uher

et al., 2011). In some cases, some parents take the instruction too literally, which may lead to omission of data that could otherwise be an important stressful life event. Additionally, parents could misunderstand and misinterpret questions; the opportunity to clarify symptoms is an important asset of interview measures. However, it should be noted that, due to the young age of the sample, parent-reports were more logistically feasible. Further, the CBCL is the most widely-used measure of children's symptoms, and has been shown to be a reliable indicator of current symptomatology.

In addition to the limitations described above, I conducted most analyses in candidate genes previously described in psychopathology research. However, one of the limitation of the current analyses is that I did not utilize the current knowledge from ENCODE or HaploReg databases to predict a possible regulatory effect of polymorphisms genotyped in this study on remote genes. It is likely that some of the variants genotyped in this study may code for DNA regulatory elements with functional effects on genes not investigated here but may be linked to early risk for psychopathology. Additionally, complex traits being polygenic in nature are also influenced by other gene variants and extant literature has documented their associations with stress-related problems such as depressive and anxious symptoms. Of importance amongst these are the glucocorticoid receptor complex genes such as *NR3C1* and *FKBP5* (Binder et al., 2009; Roy et al., 2012). Also widely implicated are the oxytocin receptor gene and the brain neurotrophins, which directly influence synaptic plasticity and therefore regulate the effects of stress and HPA axis response at the neuronal

level (Zheng et al., 2014). Below, some of the current knowledge is discussed to add to our understanding of the complex underpinning of the developmental risk.

6.3.2.1 The glucocorticoid complex genes

The regulating effects of cortisol on brain and behaviour are mediated by binding of the hormone to specific receptors. Glucocorticoid receptors (GR) are widely distributed throughout the PFC, amygdala, hippocampus, and brainstem, and are critical to regulating responses during conditions of elevated cortisol secretion or stress (Myers, McKlveen & Herman, 2014; Timmermans et al., 2013). Relative GR resistance is a core feature of depression, perhaps leading to disinhibition of central CRH secretion and HPA axis hyperactivity (Silverman & Sternberg, 2012). For example, Bet and colleagues (2009) demonstrated that, in a longitudinal aging study, SNPs in the GR gene interacted with adverse life events during youth, including war experiences, sexual abuse, parental loss, or physical illness in the prediction of major depressive disorder (MDD).

However, the sensitivity of the GR is fine-tuned by a co-chaperone chaperone protein, FKBP5. When bound to the GR complex, FKBP5 decreases GR affinity for cortisol and prevents translocation of the GR to the nucleus (Binder et al., 2009). Given the important role of GR in regulating stress responses and the evidence for GR resistance in depression, variation of the *FKBP5* gene likely is a critical modulator of the relationship between childhood maltreatment and depression (Binder et al., 2009). In a recent population-representative sample of more than 2000 Caucasians, Appel et al., (2011)

reported an interaction between the rs1360780 of the *FKBP5* gene and maltreatment in predicting both depressive symptoms and diagnoses of depression. Another recent study in 884 adolescent and young adult individuals confirmed an interaction of different *FKBP5* variants and traumatic life events in predicting the onset of MDD (Zimmermann et al., 2011). These results, taken together, strongly implicate the GR and related pathway gene variants in the pathogenesis of stress-related depression. My future work will investigate the role of these genes and emerging symptoms to inform whether these variants are involved in developmental risk pathways.

6.3.2.2 Oxytocin Receptor

The human oxytocin (OXT) system mediates social attachment, including mother–infant bonding, and demonstrated to buffer emotional and physiological responses to stress (Meyer-Lindenberg et al., 2011). For example, in patients with MDD, plasma oxytocin concentrations are inversely correlated with symptom severity (Scantamburlo et al., 2007). Additionally, women reporting exposure to childhood maltreatment exhibit deceases in cerebrospinal fluid oxytocin concentrations, which in turn were associated with increased anxiety (Heim et al., 2009). Similarly, interaction of between oxytocin receptor gene (*OXTR*) SNP rs2254298 and adverse parental environment predicted symptoms of depression in 9–14 years old girls (Thompson et al., 2011). Other studies have also reported interactions between OXTR variants and childhood stress predicting cortisol reactivity in adults (Chen et al., 2011). Future work from our group will investigate these links in our sample as well.

6.3.2.3 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophin in the brain that is implicated in neuronal growth, synaptic plasticity, and neuronal survival. In animal models, prolonged stress exposure and elevated glucocorticoid levels down-regulate BDNF expression, whereas administration of antidepressant drugs induces BDNF expression; antidepressant treatment normalizes hippocampal volume in depressed patients (Nestler et al., 2002). The *BDNF* gene contains a functional polymorphism (rs6265), which is associated with a valine to methionine substitution (*val*66*met*) and leads to reduced BDNF expression in the brain. Literature reports on interaction between this polymorphism and childhood sexual abuse in the prediction of adult depression (Aguilera et al., 2009). A report from our group demonstrated found that *BDNF* val66met polymorphism moderated the interactive effects between 5-HTTLPR and stress on HPA axis reactivity in a sample of preschool children (Dougherty et al., 2010). Furthermore, the *BDNF val*66*met* × 5-HTTLPR × childhood maltreatment interaction also predicted depression (Wichers et al., 2008).

In addition to DNA sequence variants that may contribute to risk for later psychopathology, structural DNA variation also regulates gene expression in response to external stress stimuli. The biology of gene regulation via DNA structural variation is the emerging field of epigenetics that may provide a promising avenue of research, which may aid in our understanding of biological bases of psychopathology and briefly discussed in this section. The influence of genetic variations in the DNA sequences of HPA axis related genes on mood

disorders is clear, but may only be one of the many factors contributing to our understanding of mood disorders. One of the most promising pathways of future research is the investigation of epigenetic processes modulating the relationship between Gene × CS interactions and depression. Epigenetic changes are changes in gene expression, which remain stable during cell divisions but do not affect the DNA sequence itself. Such changes are heritable and can be caused by changes such as those found in DNA methylation, the modeling of chromatin, and the deacetylation of histones in the DNA (Spijker & van Rossum, 2012). Epigenetic control of gene regulation has been implicated as influencing early development. For example, quality of maternal care was found to influence HPA function in rats through epigenetic programing of GR expression (Meaney, 2001). Poor quality of maternal care was associated with changes in the promoter region methylation of the GR gene. These rodents also exhibited enhanced hormonal and anxiety-like behavioural responses to stress, suggesting a role of epigenetic status in early neurodevelopment. In humans, recent work showed that the brain tissue of human suicide victims who were also exposed to childhood abuse had considerably higher methylation in the promoter region of the GR gene when compared to controls (McGowan et al., 2009). Additionally poor maternal rearing conditions led to differential DNA methylation in the prefrontal cortex glial cells of rhesus macaques (Provencal et al., 2012). In a recent study by Klengel and colleagues (2013), methylation pattern differences were observed in *FKBP5* gene following childhood trauma. However, a large gap

in knowledge still remains when it comes to epigenetic mechanisms that play a role in early childhood.

6.5 Future directions

6.5.1 Additional stress contexts that contribute to risk: The role of parenting

Due to the complexity and multifactorial nature of depressive and anxious disorders, this thesis focused on well-studied dimensions of life stress such as family income, dyadic adjustment and stressful life events in the child's family in the past 12 months. However, along with genetic factors which may be at play and described in the previous sections, an equally important contextual risk in the child's life is parenting. Parental effects on children's emotional development are hypothesized to be due, in part, to the influence of early childhood parental care on stress reactivity (Leuken et al., 2004; Loman & Gunnar, 2010). In addition, by shaping the nature of the response to stress and challenge, such an influence is also likely to determine the impact a broad range of environmental factors across development (Ellis & Boyce, 2008) and have a broad and lasting influence across time. However, despite the importance of the hypothesis that early parenting influences stress reactivity, support for this notion is indirect. For example, animal research has demonstrated lasting effects of parenting on stress reactivity later in life. In rodents, the offspring of mothers who exhibit high levels of licking, grooming and arched-back nursing (which facilitates pups' access to milk) show increased hippocampal glucocorticoid receptor expression, enhanced negative feedback regulation, decreased hypothalamic CRH expression, more modest

HPA-axis responses to stress, and less fearful behaviour (Levine, 2005; Zhang et al., 2006; Zhang & Meaney, 2010).

In addition to the research in rodents, studies in nonhuman primates demonstrate that in the presence of low maternal caregiving led to higher levels of plasma CRH in the offspring (Coplan et al., 2001). Parenting behaviours may alter cortisol levels, potentially representing a link in the overall pathway by which low income and cumulative risk eventually disrupts children's cortisol levels. Research has examined the relation of parenting to HPA axis functioning both in high-risk samples, such as low maternal education and high maternal stress, and low risk samples. In general, parenting that is lower in sensitivity, higher in intrusiveness and harshness is associated with lower morning cortisol levels (Roisman et al., 2009), flatter diurnal slope (Papp, Pendry & Adam, 2009), greater reactivity (Bugental et al., 2003), and slower cortisol recovery after a stressor (Albers et al., 2008). Studies typically examine one or two parenting variables at a time, with more emphasis on affective (i.e. sensitivity, harshness) parenting behaviours compared to control-related parenting behaviours (i.e. limit setting). This study sought to examine several parenting behaviours simultaneously to see which specific parenting behaviours, if any, accounted for the relations of income and cumulative family risk to disrupted cortisol patterns.

Parenting has been posited to mediate the relationship between other early risks and child cortisol levels. Specifically, two studies have examined environmental risk and parenting as mediators of the relation between poverty and disruptions to children's cortisol. In a sample of preschool-aged children

representing the full range of income, there was a trend toward an association between maternal negative affect and children's diurnal cortisol pattern, with negative affect trending toward mediating the relation between poverty status and a low, flat diurnal pattern (Zalewski et al., 2012). Another study found that positive parenting, but not negative parenting, explained a small but significant portion of higher basal cortisol levels in young children, over and above environmental risk factors (Blair et al., 2011). These two studies point to the possibility that parenting may mediate the relations of poverty and cumulative risk to disruptions in children's cortisol levels. In sum, a large amount of literature has suggested the importance of parenting in shaping neuroendocrine responsivity to childhood stress and will be a focus of future research from our group.

6.5.2 Differential susceptibility to the environment

The GxE model described above has historical roots in diathesis-stress models of psychopathology. Diathesis-stress models propose that clinically relevant phenotypes are the product of both high-risk genes and high-risk environments typically to lead to a psychological disorder (Caspi et al., 2010). Some of the data in this thesis supports this model by showing risk alleles interaction with high early life stressor to predict either high cortisol response to stress or higher depressive and/or anxious symptoms. However, my analyses also suggest that in some cases the supposed “risk allele” was associated with either lower cortisol response to stress or lower symptoms when compares to the advantageous or “resilience” gene in the presence of low life stress. For example, in Figure 3.4 the *COMT val*-allele carriers had lower anxiety symptoms

than their met-allele counterparts under low stress conditions, even though *val*-allele is widely considered the risk allele. This finding, along with other examples in this thesis (Figure 5.6B), may not entirely fit the notion that risk alleles only lend to vulnerability traits and specifically under high environmental stress. Based on similar findings in extant literature, theorists have posited that the "risk" alleles may also confer a particular ability to respond positively to environment based on the context. The key idea is that specific genes do not confer only vulnerability in the face of environmental adversity but they may also sensitize individuals to positive experiences and influences. This notion of dual action of a given allele is now commonly known as "differential susceptibility" to the environment (Belsky & Pluess, 2009; Ellis & Boyce, 2008).

Theorists suggest that specific genes may function more like "plasticity factors" rather than "vulnerability factors." Data from our group and others support this concept. For example, Hankin and colleagues (2011) examined interactions between 5-HTTLPR genotype and degree of positive and supportive parenting (ranging from low to high) in adolescents and young adults. Other studies replicate these findings and show the same effect, that youth homozygous for the short allele of 5-HTTLPR were more responsive to parenting, whether it was positive or negative. Youth at high genetic risk, or homozygous for the short allele, had low-levels of positive affect if their parents were unsupportive. However, the same group also had high levels of positive affect if their parents were supportive. Thus, the short allele group was not only vulnerable to negative parenting but also benefited from positive parenting as

well. In sum, based on these findings, the ontogenetic origins of differential susceptibility and, more specifically, identifying specific genetic variants linked to heightened susceptibility to environmental influence, need further attention in the future.

6.5.3 Beyond GxE: The role of gene-environment correlations

In large part, studies of gene–environment interplay (including this thesis) have been motivated by the search for gene–environment interactions, following recent demonstrations of genetic sensitivity to environmental effects on human phenotypes. By the late 1970s, behavioural geneticists had amassed a large body of research on twins and adoptees that attested to the importance of genetic influences on individual differences in personality, cognitive abilities and liability to disease (Plomin et al., 1994). These studies demonstrated that genetic factors influencing an individual's exposure to particular environments could make those environments themselves heritable. This phenomenon is referred to as gene-environment correlation, or rGE (Plomin, DeFries & Eaves, 1977).

rGEs reflect genetic differences in exposure to particular environments (Kendler & Eaves, 1986). Three separate types of rGE have been described: passive, evocative/ reactive, and active (Plomin, DeFries & Fulker, 1977). In passive rGE, parents pass on genes and also provide an environment, both of which influence the child’s development. For example, depressed parents may pass on depressogenic genes to their children, and also provide low levels of warmth and emotional support. These correlated genetic and environmental

influences increase the risk of depressive symptoms in the child. In evocative rGE, heritable traits influence the reaction of others and hence the environment provided by others. For example, a shy child may be perceived as less fun by other children, making other children less likely to spend time with him or her. In active rGE, a child's heritable traits influence his or her choice of environment. For example, a shy child may have reduced motivation to engage with peers. This reduced exposure to positive environments may increase depressive symptoms. The presence of significant correlation between genetic and environmental factors can make interpretation of results difficult, because it is difficult to distinguish whether it is the genes, the environment, or both that influence an outcome of interest (Jaffee & Price, 2013).

Developmental studies looking at evidence for rGE are rare (only 5 publications), but some studies from our group (Hayden et al., 2010, 2013) and others (Dick et al., 2006; Lucht et al., 2006) reported rGE in community samples. For example, Lucht et al. (2006) reported an association between perceived negative paternal parenting (reported retrospectively by adult offspring) and offspring variants of both *DRD2* and *GABRA6.* Similarly, *DAT1* 9-repeat variant was associated with child negative affect expressed toward the parent during parent-child interactions, and parents of children with a *DAT1* 9-repeat allele exhibited more hostility and lower guidance/engagement during the tasks than parents of children without a copy of the *DAT1* 9-repeat polymorphism. These gene-environment associations were partially mediated by child negative affect toward the parent, suggesting that evocative associations play a role in elevating

children's psychopathology risk (Hayden et al., 2013). Even though examining rGEs was beyond the scope of this thesis, the implications of this phenomenon on emerging risk for mood and anxiety disorders will receive further attention in my future research.

6.6 Conclusions

This thesis set out to examine the role of neurotransmitter systems commonly implicated in stress reactivity and pathogenesis of stress-related disorders. The findings support a role for CRH system gene variants in early age cortisol response and internalizing symptoms. Along with direct associations, common variants of CRH and monoaminergic system genes also acted as moderators of early environment's effect on developmental risk in young preschool age children. This research further supports a large body of GxE literature implicating the role of both genes and environment in etiology of mood disorders. Identifying the genetic underpinnings of stress response and emerging risk may ultimately aid in public health prevention efforts and help identifying at-risk individuals or subgroups of the population. This knowledge could eventually guide intervention efforts and may help to reduce the effects of chronic life stress on at-risk children. Since many of the genes studied here are targets of drug development for stress-related disorders, it may be possible to pharmacologically treat intermediate phenotypes that share genetic etiology with internalizing problems. In closing, the better understanding of nature and nurture will help reduce the tremendous toll mental health disorders exact of individuals and society.

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Appendices

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HAROON I. SHEIKH

CURRICULUM VITAE

EDUCATION

2010 - present	Ph.D. Candidate, Department of Biology, University of Western Ontario
2009 - 2010	M.Sc. Candidate, Department of Biology, University of Western Ontario
2008	B.Sc. (Hons.) in Biology, University of Western Ontario

RESEARCH EXPERIENCE

2006 – 2008	Research Associate, Dept. of Biology & Dept. of Psychology, University of Western Ontario
2004 – 2006	Laboratory Manager, Dept. of Microbiology and Immunology, University of Western Ontario
2002 – 2004	Research Technician, Lawson Health Research Institute & Child Health Research Institute

ACADEMIC HONORS AND AWARDS

2013	Robert and Ruth Lumsden Fellowship in Science, $1000
2013	University of Western Ontario, Graduate Teaching Award, $500
2013	Ontario Graduate Scholarship, $15000
2012	Ruth Horner Arnold Fellowship, $1,500
2012	Ontario Graduate Scholarship, $15,000
2012	Graduate Teaching Assistants Excellence Award, $500
2011	Robert and Ruth Lumsden Fellowship in Science, $1000
2010	Ruth Horner Arnold Fellowship, $1,000
2010-2013	Ontario Mental Health Foundation Studentship Award ($48,000)
1998-1999	University of Western Ontario Entrance Scholarship, $1500

RESEARCH AND TRAVEL GRANTS AWARDED

2013	Ontario Mental Health Foundation Travel Award, $450
2013	Department of Biology Travel Award, UWO, $500
2011	Ontario Mental Health Foundation Travel Award, $450
2011	Behaviour Genetics Association Travel Award, $200
2011	Department of Biology Travel Award, UWO, $300

2010 Joint Fund for Graduate Research, UWO, $1000
2003 Lawson Health Research Institute Research Award, $15000
2002 Department of National Defense Research Travel Award, $500

PUBLISHED AND IN-PRESS MANUSCRIPTS

1. Kryski, K.R., **Sheikh, H.I.**, Smith, H.J., Singh, S.M., Hayden, E.P. (*in press*) Evidence for evocative gene-environment correlation between child oxytocin receptor (*OXTR*) genotype and caregiver behaviour. *Personality & Individual Differences*

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20. Kim, S.O., **Sheikh, H.I.**, Ha, S.D., Martins, A., Reid, G. (2006) G-CSF-mediated inhibition of JNK is a key mechanism for *Lactobacillus rhamnosus*-induced suppression of TNF production in macrophages. *Cell Microbiology*. 8:1958-71.

MANUSCRIPTS SUBMITTED

1. **Sheikh, H.I.**, Joanisse, M.F., Mackrell, S.V.M., Kryski, K.R., Smith, H.J., Singh, S.M. Hayden, E.P. Associations between brain white matter microstructure and early age cortisol response to stress: Evidence for moderation by parenting. *Neuroimage* (Submission # NIMG-14-942)

POSTERS & COLLOQUIA

1. **Sheikh, H.I.**, Kryski, K.R., Smith, H.J., Hayden, E.P. & Singh, S.M. (May, 2013) *Interactions between chronic early life stress and HPA axis*

reactivity moderates the link between GLP-1R gene polymorphism (Leu260Phe) and early-emerging internalizing symptoms. Invited presentation at the 68th Annual meeting of the Society of Biological Psychiatry, San Francisco, CA.

2. **Sheikh, H.I.**, Joanisse, M.F., Mackrell, S.V., Kryski, K.R., Smith, H.J., Singh, S.M., Hayden, E.P. (June, 2013) *White matter microstructure is associated with the Hypothalamic-Pituitary-Adrenal axis reactivity in young girls*. Brain Plasticity, Learning and Education symposium, London, ON.

3. Kryski, K.R., Smith, H.J., **Sheikh, H.I.**, Singh, S.M., & Hayden, E.P. (2013, April). *Child oxytocin receptor (OXTR) genotype and observed parenting behaviour: Mediation by negative child behaviour.* Poster presented at the 2013 Biennial Meeting of the Society for Research in Child Development, Seattle, WA.

4. Mackrell, S. V. M., **Sheikh, H. I.**, Kotelnikova, Y., Jordan, P. L., Singh, S. M., & Hayden, E. P. (2013, April). *Child Temperament and Parental History of Internalizing Disorders: Associations with HPA Axis Reactivity in Middle Childhood*. Poster to be presented at the Biennial Meeting of the Society for Research in Child Development, Seattle, WA, USA.

5. **Sheikh, H.I.**, Kryski, K.R., Smith, H.J., Hayden, E.P. & Singh, S.M. *Interaction Between Gene Polymorphisms of the Hypothalamic-Pituitary-Adrenal Axis and Early Parenting Predicts Cortisol Reactivity in Preschoolers*. Invited presenter, University of Pittsburg, August 7th, 2012.

6. Mackrell, S. V. M., **Sheikh, H. I.**, Singh, S. M., Kryski, K. R., & Hayden, E. P. (2012, October). *Cognitive Vulnerability and Stressful Life Events Predicting HPA Axis Reactivity in Middle Childhood.* Poster to be presented at the Society for Research in Psychopathology annual meeting, Ann Arbor, MI.

7. Kryski, K. R., Smith, H. J., **Sheikh, H. I.**, Singh, S. M., & Hayden, E. P. (2012, October). *Life Stress and Parenting: Associations with HPA Axis Reactivity in Early Childhood.* Poster accepted for presentation at the 2012 Annual Meeting of the Society for Research in Psychopathology, Ann Arbor, MI.

8. **Sheikh, H.I.**, Kryski, K.R., Smith, H.J., Hayden, E.P. & Singh, S.M. *Genetic Variants of The Corticotropin-Releasing Hormone System Related*

Genes are Associated with HPA Axis Reactivity in Early-Childhood. Invited presentation at the 67th Annual meeting of the Society of Biological Psychiatry, Philadelphia, PA, May 3rd-5th, 2012.

9. **Sheikh, H.I.**, Kryski, K.R., Smith, H.J., Hayden, E.P. & Singh, S.M. *Genetic Variants of The Corticotropin-Releasing Hormone System Related Genes are Associated with HPA Axis Reactivity in Early-Childhood.* Poster presentation at the 102nd American PsychoPathogical Association, New York, NY, March 1st - 3rd.

10. Kryski, K. R., Smith, H. J., **Sheikh, H. I.**, Singh, S. M., & Hayden, E. P. (2012, March). Maternal Depression and Parenting: Associations with HPA Axis Reactivity in Early Childhood. Poster to be presented at the 2012 Annual Meeting of the American PsychoPathogical Association, New York, NY.

11. **Sheikh, H.I.**, Kryski, K.R., Smith, H.J., Hayden, E.P., Singh, S.M. (2011, October). *Neurogenetic correlates of early-age physiological stress response and its link to early emerging psychopathological risk: A prospective study*. Poster presented at the 12th International Congress of Human Genetics, Montreal, QC.

12. **Sheikh, H.I.**, Hayden, E.P., Dougherty, L.R., Laptook, R.S., Olino, T.M., Klein, D.N., Singh, S.M. (2011, October). *Monoamine oxidase-A gene promoter polymorphism is associated with early-emerging externalizing symptoms in male preschoolers: Evidence for externalizing symptom mediation via child temperament*. Invited Presentation, 2nd Biology Graduate Research Forum, UWO, London, ON.

13. **Sheikh, H.I.**, Hayden, E.P., Dougherty, L.R., Laptook, R.S., Olino, T.M., Klein, D.N., Singh, S.M. *Associations between COMT gene functional polymorphism and early-childhood internalizing symptoms: Evidence for mediation by child temperament*. Poster presented at the 41st Behaviour Genetics Association meeting, Newport, RI. June 5th – 9th, 2011.

14. Hanna, B., **Sheikh, H. I**., Laptook, R. S., Kim, J., Hayden, E. P., Singh, S. M., & Klein, D. N. (2011, September). *Associations between DAT1, parent-child interactions, and child negative emotionality.* Poster to be presented at the 25th Annual meeting of the Society for Research in Psychopathology, Boston, MA.

15. Kryski, K.R., **Sheikh, H.I.**, Smith, H.J. Hayden, E.P., Singh, S.M. *Child BDNF Genotype and Parenting Style: Associations with HPA Axis Reactivity in Early Childhood*. Society for Research in Child Development, Montreal, QC. March 31- April 2, 2011.

16. Smith, H.J., **Sheikh, H.I.**, Kryski, K.R., Hayden, E.P., Singh, S.M. *Genetic and Contextual Determinants of Early-Emerging Effortful Control.* Society for Research in Child Development, Montreal, QC. March 31- April 2, 2011.

17. Mackrell, S., **Sheikh, H.I.**, Hayden, E.P., Singh, S.M. *Longitudinal Associations Between Temperament, Social Adjustment and Internalizing Disorders Risk in Middle Childhood.* Society for Research in Child Development, Montreal, QC. March 31- April 2, 2011.

18. **Sheikh HI**, Dougherty LR, Hayden EP, Klein DN, Singh SM. *Glucagon-Like Peptide-1 Receptor Gene Polymorphism (Leu260Phe) Is Associated With Morning Cortisol in Preschoolers.* Genetic Society of Canada Conference, June 17 – 20, 2010, Hamilton.

19. Kryski, K.R., Smith, H.J., **Sheikh, H.I.**, Hayden, E.P., & Singh, S. *HPA Axis Reactivity Patterns and Internalizing and Externalizing Symptoms in Early Childhood*. Association for Psychological Science meeting, Boston, MA, May 27-30, 2010.

20. **Sheikh, H.I.**, Martins, A., Kim, S.O. (2006) *Probiotic Treated Macrophages Release Cytokines Capable of Influencing the Maturation of Dendritic Cells: A role for G-CSF.* Western Microbiology and Immunology Annual Research Forum.

21. Yuen, D.E., **Sheikh, H.I**. (2003). *Vitamin A activation of transforming growth factor-beta1 enhances porcine ileum wound healing in vitro.* 4th Toronto Critical Care Medicine Symposium.

DEPARTMENTAL SERVICE

2012-2014 Member, Biology Graduate Education Committee, UWO
2009-2012 Chair, Society of Biology Graduate Students, UWO (Elected)

2009-2012 Chair, Bursaries and Subsidies Committee, UWO (Elected)
2010-2011 Chair, Organizational Committee, Biology Graduate Research Forum
2009-2010 Member, Environmental Sustainability Committee, Society of Graduate Students

AD-HOC REVIEWER

American Journal of Medical Genetics Part B: Neuropsychiatric Genetics
Biological Psychology
BMC Neuroscience
Cognition & Emotion
Genes, Brain, & Behaviour
Neuroscience Letters
Journal of Clinical Child and Adolescent Psychology
Psychiatric Genetics
Progress in Neuro-Psychopharmacology & Biological Psychiatry

TEACHING EXPERIENCE

2010 - 2012 Human Molecular Genetics (BIO 4560). This course offers an up-to-date examination of the current status of human genetics with emphasis on the genetics of complex traits. Class Size: 50

2009 - 2013 Principles of Human Genetics (BIO 3592). The course emphasizes on genetic variation in humans, mutations, mechanisms of gene expression, and mapping the human genome. Class Size: 75

2009 - 2010 Introductory Genetics. (BIO 2581) An introductory course in genetics covering a wide range of genetic principles from patterns of Mendelian inheritance to molecular genetics. Class size: 950

2004 – 2006 Laboratory Demonstrator, Laboratory Techniques in Microbiology and Immunology (MNI 3600). This course consists of a series of laboratory exercises designed to familiarize students with techniques in immunology, bacterial genetics, virology and molecular biology. Class size: 50.

AFFILIATIONS

2012 - present	The Endocrine Society
2011 - present	Society of Biological Psychiatry
2009 - present	The American Society of Human Genetics
2009 - 2011	Behaviour Genetics Association

8