GENE-ENVIRONMENT INTERACTIONS IN CORTISOL REACTIVITY: SEX, GENES, AND ADVERSITY PREDICT RESPONSES TO PSYCHOSOCIAL STRESS

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DISSERTATION ABSTRACT

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Extreme variations in cortisol reactivity are associated with multiple psychological and physiological diseases. These variations may be explained by sex, by genetic vulnerabilities, and by exposure to either recent life stressors (severe life events or ongoing difficulties) or early life adversity (e.g., antipathy; neglect; or psychological, physical, or sexual abuse). To explore interactions among these variables, a subset of 20-22 years-old individuals (N = 373) recruited for an ongoing longitudinal cohort-sequential study of substance abuse risk factors were assessed. These individuals were interviewed about early childhood abuse and recent stressful life experiences. They were also genotyped for multiple polymorphisms within genes associated with attenuated or exaggerated cortisol reactivity (5-HTTLPR and rs25532 in SERT, rs4680 in COMT, rs5522 in MR gene NR3C2, rs110402 and rs1876831 in CRHR1, rs1799971 in OPRM1, and rs1800497 in ANKK1), participated in a laboratory social stress task, and provided salivary cortisol samples throughout the task. Results indicate that cortisol reactivity may be shaped by both early and recent life experiences and genetic vulnerabilities; most interactions between these variables differed depending on an individual's sex. Specifically, carriers of two copies of minor alleles of ANKK1, COMT, and CRHR1

displayed dysregulated cortisol that varied according to sex and early life experiences. Male minor allele carriers who experienced more severe physical abuse displayed attenuated reactivity, and males who were not severely abused displayed exaggerated responses. Female minor allele carriers displayed the opposite pattern – abused females displayed exaggerated reactivity. Carriers of major alleles did not show these patterns. Attenuated cortisol reactivity was also observed in all individuals who experienced sexual abuse or neglect, and elevated responses were observed in individuals carrying two copies of minor alleles in both SERT polymorphisms and OPRM1. Together, results inform a developmental model of cortisol dysregulation. Cortisol reactivity may present a useful endophenotype for future studies of physiological and psychological disease processes and treatment outcomes.

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CHAPTER I

INTRODUCTION

Overview

Variations in the activity of the stress hormone cortisol are integral components of daily functioning for humans, altering our physiology upon waking each morning (diurnal regulation) and in response to stressful experiences (reactivity). Released via the hypothalamic-pituitary-adrenal (HPA) neuroendocrine system, cortisol allows for the maintenance of diurnal rhythm of key metabolic functions; and regulates gluconeogenic, anti-inflammatory, and immunosuppressive processes when the body mounts short-term responses to stressors (i.e., allostasis; Sterling and Eyer, 1988). Over time, many individuals who are repeatedly exposed to acute or chronic stressors do not adapt as effectively and exhibit dysregulation of the HPA system – a physiological consequence described as allostatic load (McEwen, 1998).

Extreme variations in cortisol release are regarded as endophenotypes of multiple psychological and physiological conditions. Dysregulation of cortisol functioning has been observed in adults with major depressive disorder (MDD; see de Kloet, Joels, and Holsboer, 2005), posttraumatic stress disorder (PTSD; e.g., Yehuda, 2000) most other anxiety disorders (see Vreeburg et al., 2010), and many externalizing disorders – e.g., substance use disorders (SUDs; see Rao, Hammen, and Poland, 2009) conduct disorder (Pajer, Gardner, Rubin, Perel, and Neal, 2001), or antisocial personality disorder (e.g., Shirtcliff, Granger, Booth, and Johnson, 2005). Cortisol dysregulation is also observed in survivors of childhood abuse or maltreatment (e.g., Fisher, Kim, Bruce, and Pears, 2012; Heim, Newport, Bonsall, Miller, and Nemeroff, 2001), in certain neurological conditions

(e.g., Huntington's disease; Aziz et al., 2009), and as symptomatic of and contributory toward a variety of cardiovascular and metabolic diseases (e.g., McEwen, 1998). In these diverse examples, the measure that defines the construct of "cortisol dysregulation" varies. When one examines cortisol functioning diurnally, individuals who display a flat pattern – an attenuated morning rise in cortisol and higher evening levels, compared to normal functioning – may be considered hypoactive; others may display hyperactivity, with abnormally elevated levels of cortisol throughout the day. When one examines cortisol release in response to psychosocial stress, hypo***re***activity refers to an attenuated cortisol release relative to normal functioning, and hyper***re***activity refers to exaggerated cortisol release (higher peak, longer time to return to typical functioning).

Cortisol dysregulation is dependent upon developmental experiences – e.g., early adversity or recent stressors – and biological characteristics – e.g., sex differences and other genetic variations linked to neurobiological functioning. The current study will consider the independent and interactive effects of these variables on cortisol responses to a laboratory psychosocial stress task.

Neurobiology of the Stress Response

The biological stress response in mammals is regulated by two interconnected neuroendocrine systems – the HPA system (also, HPA axis), and the SAM, or sympathetic-adrenal medullary system (Frankenhauser, 1986). A subcomponent of the sympathetic nervous system, the SAM system is responsible for releasing norepinephrine and epinephrine following exposure to threatening stimuli, allowing for the rapid "fight-or-flight" stress response (Cannon, 1929). In contrast, the HPA system produces steroid

hormones called glucocorticoids (in humans, cortisol; in other mammals such as rodents, corticosterone) that are also released in response to stressors, but are produced more slowly than epinephrine, and target the brain directly (de Kloet, Rots, and Cools, 1996).

HPA: The Long-Term Biological Stress Response

The HPA axis is regarded as the central regulatory and control system of most vertebrate organisms, activated in response to emotional-cognitive processing of environmental stressors (e.g., Gunnar and Quevedo, 2007). Norepinephrine released by the SAM system stimulates the release of CRH (corticotrophin releasing hormone) and AVP (arginine vasopression) in the paraventricular nucleus (PVN) of the hypothalamus. In concert with epinephrine, norepinephrine, and oxytocin, both CRH and AVP stimulate the release of ACTH (adrenocorticotropic hormone) from the anterior lobe of the pituitary gland, which in turn travels through the bloodstream and activates receptors in the adrenal cortex, leading to the production and release of glucocorticoids. Peaking 20-40 minutes after initial exposure to environmental threat and lasting for hours, glucocorticoid release effects systemic metabolic and epigenetic changes throughout the body (Kirschbaum and Hellhammer, 1989). Glucocorticoid release regulates glucose synthesis in response to circadian rhythms, suppresses inflammatory and immune functioning, and activates receptors that regulate transcription of hundreds of glucocorticoid-responsive genes (de Kloet, 1991). These alterations in gene transcription may occur minutes or hours following the initial activation of this system, and downstream alterations in gene-protein-tissue production may occur for far longer periods of time – functionality that does not map neatly onto popular conceptualizations of the "flight-or-fight" SAM response (Sapolsky, Romero, and Munck, 2000).

Glucocorticoids bind to two types of receptors – Mineralcorticoid receptors, located primarily in the limbic system (MRs; deKloet, Joels, and Holsboer, 2005), and glucocorticoid receptors (GRs), located throughout the brain and in many organs systems – that act in contrast to one another. MRs mediate basal physiological processes (e.g., blood pressure, circadian rhythm), while GRs activate neuronal feedback pathways to the PVN, inhibiting CRH production and terminating the HPA response via inhibitory μ-opiod receptor OPRM1 and endogenous cannabinoid receptor CB1 (e.g., Barna, Zelena, Arszovszki, and Ledent, 2004). Thus, delayed activity of cortisol in humans ultimately shuts down the acute stress response system, facilitating a return to cellular homeostasis following stress (Sapolsky et al., 2000).

The Unique Role of CRH in the Stress Response

Limbic cortical regions (anterior cingulated cortex, orbital and medial prefrontal cortices) and subcortical structures (amygdala, hippocampus, thalamus, etc.) are utilized in the appraisal of potential environmental stressors. The activity of these regions is ultimately regulated by CRH neurons (Bale and Vale, 2004). In rodent models, infusion of CRH into analogous brain regions intensifies anxious behaviors (e.g., Reul and Holsboer, 2002) and CRH neurons in these same regions sensitize to CRH given repeated exposure to psychosocial stressors (Butler, Weiss, Stout, and Nemeroff, 1990). Two types of CRH receptor neurons act in contrast, but not in opposition to one another (much like MR and GR; Zhao et al., 2007). Activation of CRHR1 is associated with HPA regulation and fearful or anxious behavior, as these receptors are found in limbic regions involved with processing threatening stimuli. Activation of CRHR2 is associated with depressed or

anxiolytic states, as these receptors are found in subcortical regions associated with basic bodily functions (e.g., Muller and Wurst, 2004).

Serotonergic and Dopaminergic Alterations of the Stress Response

Recent studies have demonstrated that serotonin is involved in both activation and feedback control of the HPA axis (e.g., Lesch et al., 1996, Lowry, 2002). Exposure to acute stress increases serotonin release in the amygdala, regions of prefrontal cortex, the nucleus accumbens (NAc), and the lateral hypothalamus. In animal models, serotonin activates the HPA system by stimulating release of CRH (Murphy and Lesch, 2008). Serotonin promotes downregulation of HPA activity by binding to postsynaptic 5-HT_{1A} receptors, which are found in the hippocampus and amygdala. Stimulation of these receptors produces anxiolytic effects in rodents, and lower receptor density is associated with long term stress exposure (Charney, 2004). Reduced availability of L-tryptophan (a precursor of serotonin) impairs this negative feedback control mechanism that terminates cortisol release (Porter, Gallagher, Watson, and Young, 2004).

Exposure to uncontrollable stress also provokes dopamine release from medial prefrontal regions and inhibits release in NAc (Charney, 2004), suggesting amygdalar functioning mediates the activation of dopaminergic "reward" pathways in response to stress. Increases in synaptic dopamine effect changes in multiple neurotransmitters involved in the stress response (e.g., serotonin), which later inhibit dopamine release.

Psychobiological Variations in Acute Stress Exposure

Decades of stress research have demonstrated that subjective interpretations of environmental stimuli have direct consequences on physiological responses. Henry (1992) proposed that HPA axis activity is associated with situational uncontrollability

and perceived helplessness, while SAM activity occurs when one faces a challenge to be overcome. In a meta-analysis of studies linking chronic stress exposure to HPA functioning, Miller, Chen, and Zhou (2007) report that uncontrollable stressors, threats to physical integrity, or traumatic experiences lead to diurnal hypoactivity, but greater total cortisol release throughout the day. Controllable stressors are associated with more adaptive cortisol responses – higher morning rise, and truncated reactivity to the acute stressor. Studies of HPA axis responses to psychological laboratory stressors demonstrate similar patterns (Dickerson and Kemeny, 2004). The largest, most prolonged cortisol responses are elicited by tasks that are perceived as uncontrollable, force failure, provide no escape from negative consequences, or feature social-evaluative threat.

Allostasis and Allostatic Load

According to McEwen (1998), ***allostasis*** is the process by which an organism continues to adapt to ever-changing internal and external environmental cues through activation of neurobiological stress response systems. In the short-term, this activity is beneficial and necessary for survival, returning an organism to homeostasis. In the long-run, this same activity can be deleterious to the organism's overall functioning. Although these systems are designed to adapt continuously, this continuous adaptation damages the response system itself and associated organs and tissues. This "wear-and-tear" experienced by an organism over time as it continuously adapts has been termed ***allostatic load***. Earlier and more serious exposure to environmental threat is associated with stronger and more persistent effects on biological processes.

Cortisol responses altered by allostatic load sharply contrast with typical stress responses – short-term elevation of physiological activity followed by a recovery period

(See Figure 1; see the Appendix for all figures). Some individuals may demonstrate physiological responses to stress, but the recovery period does not readily occur, such that the individual is under a constant state of ***alert*** (i.e, hyperreactivity). Individuals who are acutely depressed or anxious are likely to display this elevated response, and are susceptible to cardiovascular disease (McEwen and Seeman, 1999). Others may not display reactivity at all (i.e., hyporeactivity), and are rendered susceptible to inflammatory and autoimmune diseases that are byproducts of hypocortisolism (Sternberg, 1997). They are likely to have experienced some form of early life trauma and their dysregulation may represent psychophysiological adaptation to the stressors themselves, and/or accumulation of neuroendocrine damage (Heim and Nemeroff, 2001).

Stress and Disease

Exposure to psychological or physiological stressors leads to changes in immune functioning, blood coagulation, and gene expression (Glaser, 2005; Weaver, 2009). Glucocorticoid elevation during stress suppresses inflammation by altering leukocyte functioning and redistributing cytokines. Dhabhar (2002) notes that chronic stressors are immunosuppressive, while acute stressors may have generally immunoenhancing effects. Also, acute stress tends to activate molecules that coagulate blood, contributing to increased risk for cardiovascular disease (e.g., von Kanel, Kudielka, Hanebuth, Preckel, and Fischer, 2005). Exposure to chronic childhood stress is associated with multiple epigenetic effects, including DNA methylation, decreased GR receptor transcription, altered activation of GR-responsive genes that regulate inflammation and other processes, and pathological consequences of these variations (McGowan et al., 2009).

As a consequence of what endocrinologist Hans Selye had termed General Adaptation Syndrome (GAS; Selye, 1936), humans are likely to experience diseases that arise from the breakdown of weakened or predisposed organ systems in the context of chronic or major stressors. In addition to cardiovascular, autoimmune, and endocrine disorders, stress exposure has been tied to a variety of psychopathological disorders, including MDD (Mazure, 1998), SUDs (e.g., Sinha, 2008) and PTSD. Chronic activation of the glucocorticoid stress response is also associated with premature systemic aging, through the shortening of telomeres (repetitive nucleotide sequences at the ends of genes that prevent their degradation; Epel et al., 2010) and oxidative damage to RNA (Aschbacher et al., 2013).

Sex Differences in the Stress Response

Sex differences observed in the prevalence of many diseases – e.g., cardiovascular and infectious diseases are more common in males, but autoimmune disorders are more common in females – may be attributable to variations in cortisol functioning (Kudielka and Kirschbaum, 2005). Both subjective interpretations of stressful events and individual variations in baseline physiological state will affect cortisol release, and both tend to vary in males and females.

Human males display greater levels of basal plasma and salivary cortisol than females in many studies (e.g., Kirschbaum, Wüst, and Hellhammer, 1992; Tersman, Collins, and Eneroth, 1991). When measured during relaxation, basal cortisol levels of males and females do not differ significantly, but differences emerge once individuals are exposed to psychosocial stressors (Kudielka and Kirschbaum, 2005). Differences in

reactivity are only observed consistently when female participants are between puberty and menopause, and few significant differences are observed outside of this time period (e.g., Fisher, Kim, Bruce, and Pears, 2012; Nicolson, Storms, Ponds, and Sulon, 1997). In some studies, basal cortisol levels are higher in males only when compared to menstruating females during their follicular phases, and were comparable to that of males in the luteal phase (Kirschbaum et al., 1992).

Sex differences are attributable to variations in neurohormonal functioning (e.g., Carey, Deterd, de Koning, Helmerhorst, and de Kloet, 1995). The adrenal cortices of females are more responsive to ACTH than those of males, as levels measured at the pituitary gland often do not differ between sexes (Kirschbaum et al., 1992). Females have demonstrated greater sensitivity to the release of AVP in neurohormonal administration tests (Born, Ditschuneit, Schreiber, Dodt, and Fehm, 1995).

Estrogen release also exhibits significant effects on HPA functioning. Estrogen regulates GR and MR functioning in rodent models, and mediates CRH synthesis in PVN. In humans, higher estradiol concentrations (naturally in women or administered to men) are associated with attenuated cardiac output (Kajantie and Phillips, 2006). Estrogen alters the functioning of multiple physiological systems by increasing parasympathetic nervous system activity and reducing sympathetic activity. Estrogen also stimulates the production of corticosteroid-binding globulin (CBG), which binds with free cortisol in plasma and reduces cortisol levels (Kirschbaum et al., 1992).

Taylor and colleagues (2000) propose that females may exhibit wholly different response pattern to stress – wherein they may "tend-and-befriend" as opposed to males who would fight or flee – attenuating SAM and HPA activation via oxytocin release,

which is stimulated by estrogen. Oxytocin – a hypothalamic neurohormone that modulates intimacy, maternal bonding, and attachment – produces anxiolytic effects in rodents and primates, and promotes affiliative behavior in response to stressors.

The developers of the Trier Social Stress Test – a laboratory psychosocial stress task (TSST; Kirschbaum, Pirke, and Hellhammer, 1993) – note that some sex differences are likely due to gender-mediated perceptions of the socially-ostracizing task, since other forms of stress induction (CRH injection, bicycle ergometry) do not demonstrate such differences. Males also demonstrate elevated cortisol responses during anticipation of this stressor, while females showed reductions in cortisol release after starting the task. In one study of young adults, Stroud and colleagues (2002) demonstrated that females exhibited greater cortisol responses to social rejection, while males responded more intensely to tasks that involved achievement, such as the TSST.

How Early Adversity Shapes the Stress Response

The damaging effects of long-term HPA activation are noteworthy throughout the lifespan, but exposure to stress during childhood is particularly impactful on subsequent long-term HPA dysregulation. Characteristics of cortisol dysregulation vary depending on age or developmental timing of exposure; and type, severity, or duration of exposure to environmental adversity (Fisher and Gunnar, 2010).

After birth, maternal nurturing behaviors regulate how many GRs will develop in the hippocampus (via methylation, which prevents transcription of genes; see Weaver et al., 2004). Increased hippocampal GR density is associated with maternal attention and, therefore, more efficient termination of stress responses. Having fewer GRs is associated

with maternal neglect, slower mobilization of the HPA response, poorer regulation of the response (i.e., slower return to baseline), and ultimately an increased vulnerability to the effects of allostatic load (Meaney and Szyf, 2005; Weaver et al., 2001).

Childhood Attachment

In general, children who have secure attachment relationships do not show elevations in HPA activity in response to distressing laboratory and naturalistic situations; toddlers with insecure attachment relationships generally exhibit elevated cortisol levels in response to such stressors (e.g., Gunnar and Donzella, 2002). Children in disorganized or disordered attachment relationships with their caregivers experience high variability in their stress responses, often characterized by elevated and poorly regulated HPA activity in preschool years, and equally dysregulated mood and behavior in the presence of the caregiver (e.g., Halligan, Herbert, Goodyer, and Murray, 2004). Further, dysfunctional parenting is associated with cortisol increase in children during parent-child discussions of conflict (Granger, Weisz, and McCracken, 1996).

Early Life Trauma and Neglect

Maltreatment during development leads to alterations in the stress response systems, which in turn produce maladaptive outcomes (see Bremner and Vermetten, 2001; De Bellis, 2001; or Heim and Nemeroff, 2001). Traumatic family events involving conflict (punishment, quarrelling, fighting) tend to be associated with brief periods of elevated cortisol activity in all children, but these return to more normal levels of functioning after a short period of time. Children repeatedly exposed to more severe traumatic events or neglect display patterns altered by exposure.

Children exposed to long-term early abuse display attenuated cortisol release later in life diurnally (e.g., Lovallo, Farag, Sorocco, Cohoon, and Vincent, 2012) and in response to stressors (e.g., Cicchetti, Rogosch, and Oshri, 2011). Maltreated preschool-aged children (subjected to either physical abuse or neglect) exhibit less cortisol reactivity in general, and in one study demonstrated especially low levels on days when the classroom environment was filled with conflict or fighting (Hart, Gunnar, and Cicchetti, 1995). If these children were also exposed to prenatal substance abuse, they are more likely to demonstrate hyporesponsiveness to the TSST-C before adolescence (Fisher, Kim, Bruce, and Pears, 2012). Sexually abused girls show blunted ACTH responses when CRH injections are administered (De Bellis et al., 1994). Also, both infants reared in orphanages and domestically-neglected children placed in foster care demonstrate significant cortisol dysregulation, characterized by low morning levels and altered diurnal rhythms (Carlson and Earls, 1997; Gunnar and Fisher, 2006).

Adolescents and adults exposed to severe stressors display exaggerated episodic cortisol responses, experience long-term upregulation of HPA activity given repeated exposure (e.g., Kirschbaum et al., 1995). However, individuals who were abused as children may be more likely to display hyposecretion as adults when exposed to acute stressors (symptomatic of PTSD). These variations likely represent cross-sections of the developmental degradation of HPA functioning. De Bellis (2001) argues that cortisol dysregulation may change over the life course – a byproduct of chronic allostatic load – though he notes that many structural effects of maltreatment persist indefinitely. Indeed, many structural changes in the hippocampus and corpus callosum may not manifest until later in life, but are activated by early life trauma (Andersen and Teicher, 2004).

Genetics of the Stress Response

Variations in genes that encode proteins regulating neuroendocrine functioning are associated with phenotypic variations in cortisol release. Many studies of reactivity have considered Single Nucleotide Polymorphisms (SNPs) – single alleles (or "letters" that correspond to the names of nucleotides) that vary in a molecular DNA sequence. Other studies have considered the phenotypic effects of longer sections of DNA that vary in length or nucleotide sequence – Variable Number Tandem Repeats (VNTRs).

Genetic Variations in HPA

When measured via urinary free cortisol, HPA activity appears to be significantly heritable, with estimates averaging 50% (Bartels, de Geus, Kirschbaum, and Sluyter, 2003); genetic variations in GR feedback mechanisms show similar heritability. The heritability of responses to laboratory stressors – behavioral or chemical – have not been conclusively studied, but at least one twin study suggests very high heritability (>97%) for sustained stress response. GRs mediate the effects of glucocorticoids on gene transcription by binding directly to regions of the genome or by interacting with other neurochemicals that regulate transcription (e.g., AP-1, NF-κB; see Wust et al., 2004, for a review). Cortisol and other glucocorticoids bind to GR receptors, which translocate to the nucleus of the cell, bind to specific regions of the genome, and modulate mRNA transcription. Several polymorphisms in GRs and MRs moderate cell-specific GR/MR expression and ultimately behavioral responses to stress, but are very rare (e.g., a variant in Asn363Ser exon 2 is associated with elevated reactivity; a variant in BclI is associated with attenuated reactivity; DeRijk and de Kloet, 2005, 2008). A more common minor allelic variation in Mineralcorticoid receptor gene SNP (MR I180V; rs5522) is associated

with increased depressive symptoms, and increased cortisol response following the TSST (DeRijk, van Leeuwen, Klok, and Zitman, 2008).

Genetic Variations in SAM

To date, several functional polymorphisms in genes associated with norepinephrine release (such as the norepinephrine transporter, NET) have been studied in the context of multiple clinical conditions, but their relationships with the stress response system have yet to be established. A notable exception is found in the catechol-O-methyltransferase gene (COMT), where a common polymorphism found in humans (Val158Met, causing a substitution of the amino acid Methionine for Valine at codon 158; rs4680) contributes to a four-fold decrease in enzyme activity for minor allele carriers (e.g., Chen et al., 2004). Since the COMT gene produces a protein that inactivates norepinephrine and dopamine, carriers of the minor "Met" variant are more susceptible to phenotypic consequences of dysfunction. Multiple studies have associated variations in this polymorphism with anxiety-related personality phenotypes (i.e., neuroticism; Stein, Fallin, Schork, and Gelernter, 2005) and risk for unipolar or bipolar depression (Mandelli et al., 2006). In conjunction with 5-HTTLPR, COMT moderates the relationship between life stress and MDD, wherein carriers of the minor allele are more likely to become depressed, and risk increases significantly given exposure to life stress.

COMT alters HPA activity, as homozygous carriers of "risk" Met alleles have demonstrated elevated diurnal cortisol release relative to carriers of "protective" Val alleles (Walder et al., 2010), and greater cortisol release following the TSST (Armbruster et al., 2012). The likely mechanism of this alteration is through reduced breakdown of norepinephrine and promotion of ACTH release released during exposure to

environmental stressors, leading to prolonged activation of the HPA system (Jabbi et al., 2007). Further, these minor allele carriers demonstrate reduced μ-opioid binding potential, greater HPA activation when a μ-opioid chemical blockade is introduced, and generally reduced pain tolerance (regulated by μ-opioid activity).

Genetics of Limbic CRH Functioning

Polymorphisms in CRHR1 have been associated with variations in response to psychosocial stress. Mahon and colleagues (2013) reported that minor alleles of several SNPs – rs110402, rs242924, and rs7209436 – were associated with reduced levels of cortisol in response to the TSST in "one of the largest TSST cohorts yet examined" (n = 368). Also, Sheikh and colleagues (2013) identified several SNPs within the CRH system (including rs1776310 in CRHR1) that predicted reduced reactivity in children who carried minor alleles of these genes. CRHR1 has demonstrated inconsistent interactive effects in the prediction of cortisol reactivity, which are discussed in the next section.

Variations in CRHR1 are associated with the occurrence of psychopathology in the presence of life stress, including alcohol abuse (SNP rs1876831; Blomeyer et al., 2008) and MDD (SNP rs110402; Papiol et al., 2007). SNP rs110402 also moderated the effects of child abuse on adult depressive symptoms (Bradley et al., 2008), such that adults who are homozygous for the risk allele (GG) who have experienced child abuse are most likely to be depressed, while homozygous AA carriers are protected from the depressogenic effects of child abuse later in life.

Genetic Variations in the Serotonergic System

The serotonin transporter (SERT) gene-linked polymorphic promoter region (5-HTTLPR) is a widely-studied VNTR polymorphism, wherein individuals generally carry

14- or 16- repeats of a three-letter sequence, resulting in a 44-base-pair deletion/insertion variation and "short" or "long" 5-HTTLPR alleles. In general, carrying at least one copy of the short allele is associated with reduced transcription, reduced SERT expression, and reduced serotonin reuptake. An associated SNP (rs25531) interacts with 5-HTTLPR to alter further serotonergic functioning in some individuals, rendering "long" alleles "short" functionally (Wendland et al., 2006).

SERT contains a glucocorticoid response element, contributing to elevated emotional responses in the presence of stress-induced HPA activity. Dexamethasone administration increases SERT gene activity and serotonin uptake capacity (Glatz, Mossner, Heils, and Lesch, 2003). Both rodents who carry short alleles of the equivalent gene to SERT in humans (SLC6A4) and those who have had the serotonin transporter gene knocked out exhibit increased HPA activity in response to acute stressors (Murphy and Lesch, 2008). Variations in human cortisol reactivity in response to laboratory stress are also moderated by the 5-HTTLPR polymorphism, such that carriers of the short allele exhibited higher and prolonged cortisol responses to laboratory stressors (e.g., in adolescent girls, Gotlib, Joorman, Minor, and Hallmayer, 2008; and in young adults, Way and Taylor, 2010). A recent meta-analysis by Miller and colleagues (2013) reports a small effect for the short allele of 5-HTTLPR contributing to elevated cortisol reactivity to psychosocial stress across studies. Interactive effects on reactivity associated with 5-HTTLPR are inconsistent (See next section).

The short allele of 5-HTTLPR is associated with increased anxiety and neuroticism in humans (e.g., Lesch et al., 1996); as well as multiple psychopathological outcomes, such as MDD (Caspi et al., 2003), SUDs (Covault et al., 2007), and anxiety

disorders (Hariri and Holmes, 2006) given exposure to life stressors. Another polymorphism in SERT (rs25532) is associated with liability for obsessive compulsive disorder (Wendland et al., 2008), but has not yet been associated with variations in cortisol reactivity or other phenotypic manifestations of dysfunction.

Genetic Variations in the Dopaminergic System

Although dopamine binds to at least five different receptors, the DRD2 receptor has received considerable attention in psychopathology literature. Specifically, a polymorphism referred to as Taq1a – located within the ankyrin repeat and kinase domain containing 1 gene (ANKK1) 9.5 Kilobase-pairs downstream from the coding region of the DRD2 gene – has been identified as highly discriminatory among patients and control individuals. The A1 allele of ANKK1 is generally associated with a reduced number of dopamine binding sites in the brain (e.g., Thompson et al., 1997) and several pathological outcomes, including alcohol abuse (Munafo, Matheson, and Flint, 2007), nicotine dependence (Swan et al., 2005), and amphetamine abuse (Ujike, 2009). The A2 polymorphism has been found to moderate the association between life stress and MDD, such that homozygous A2 carriers are at increased risk for depression following stressful life events (Elovainio et al., 2007).

Polymorphisms in genes related to dopaminergic functioning have not been extensively researched relative to cortisol reactivity. A variation in a dopamine receptor D4 gene (the 7R VNTR) is associated with attenuated reactivity, and interacts with variations in 5-HTTLPR to produce greater drops in cortisol release (Armbruster et al., 2009). The integration of this system with other components of the human stress response suggests it may be involved.

Genetic Variations in the Opioid System

Opiates (e.g., heroin, morphine, hydrocodone, etc.) interact with μ-opioid receptors that drive reward and pain responses, as well as the human stress response. This system operates with the dopaminergic reward pathway, such that motivation for opiates in addicted mammals originates from mesolimbic dopaminergic structures. In rodents exposed to laboratory social stressors, μ-opioid mRNA expression is elevated (e.g., Nikulina, Miczek, and Hammer, 2005).

A functional polymorphism in the μ-opioid receptor gene (OPRM1 N40D; SNP: rs1799971) has been shown to modify expression of the OPRM1 transcript and to modify β-endorphin binding to the μ-opiod receptor (e.g., Kreek et al., 2005). This nonsynonymous SNP substitutes an Aspartic Acid (D) for an Asparagine (N) in the extracellular domain of the receptor. The D/Asp40 variant of this allele is associated with a three-fold increase in β-endorphin affinity, increasing inhibition of CRH neurons and reducing HPA stress reactivity (Bond et al., 1998). The minor N allele is associated with increased levels of baseline cortisol and with increased cortisol response to both naltrexone administration and to laboratory stressors (e.g., Chong et al., 2006; Hernandez-Avila et al., 2007).

Gene-Environment Interactions in Cortisol Reactivity

Studies of gene-environment (GxE) interactions in psychopathologies (such as MDD or SUDs) demonstrate associations between genes related to HPA functioning and environmental threat exposure, suggesting cortisol dysregulation may be a mechanism driving pathogenesis of these disorders.

CRHR1 Interactions

SNPs in the CRHR1 gene have differentiated participants' cortisol reactivity and diurnal regulation given exposure to adversity. Carriers of the GG "risk" genotype of SNP rs242924 who were maltreated as children display elevated cortisol release following the Dexamethasone/CRH test (Tyrka et al., 2009). Also, in another study of SNP rs110402, male carriers of the A "protective" allele demonstrated attenuated reactivity to this test relative to female carriers and male homozygous carriers of the "risk" allele; when childhood abuse history was considered, abused female A-allele carriers displayed elevated cortisol reactivity relative to abused male carriers (Heim et al., 2009). Carriers of a "risk" haplotype including SNP rs110402 have demonstrated attenuated diurnal cortisol regulation if they were abused or maltreated as children; however, non-abused carriers of the risk haplotype resemble those individuals who carry protective copies of the gene, demonstrating expected diurnal cortisol variations (Cicchetti et al., 2011).

5-HTTLPR Interactions

Numerous studies have reported that carriers of two copies of the short (S) allele of 5-HTTLPR showed increased cortisol release following a laboratory stressor when compared to carriers of at least one long (L) allele (Miller et al., 2013). Yet, findings regarding how this gene interacts with stressful life experiences remain mixed. Adult male homozygous S carriers who experienced recent stressful life events displayed elevated cortisol reactivity (Alexander et al., 2009), but another study of young adults found the opposite – carriers of at least one S allele showed generally reduced cortisol reactivity relative to homozygous L allele carriers, who also showed attenuated cortisol

release if they had experienced stressful life events during the first five years of childhood (Mueller et al., 2011).

5-HTTLPR has also been studied in concert with other gene implicated in cortisol dysregulation. Carriers of a vulnerability in a dopaminergic receptor D4 gene who also carry long allele subtype L_A of 5-HTTLPR demonstrated attenuated reactivity compared to those who carry long L_S or short alleles (Armbruster et al., 2009). Also, children exposed to laboratory stress demonstrated divergent effects of short alleles in 5-HTTLPR given variants of another SNP in a BDNF gene (Dougherty et al., 2010) – carriers of Met-alleles in BDNF displayed low baseline levels of cortisol, but significant reactivity; Val-allele carriers displayed low levels of cortisol throughout the stress tasks. Overall, the diversity of these findings suggests that stable interactions involving 5-HTTLPR may be difficult to detect.

The Present Study

This study will consider how an individual's sex, genotype, and exposure to early and recent adversity shape cortisol responses to a laboratory psychosocial stress task (the TSST). Specifically, it will report on a simultaneous assessment of multiple GxE interactions implicated in previous research on cortisol functioning, including those related to HPA activation and termination, and systems that function reciprocally with HPA activity.

This study will test the following hypotheses concerning cortisol reactivity:

1. Independent effect of sex on reactivity – Females will display attenuated responses to psychosocial stress, relative to males.

2. Independent environmental effects on reactivity – Individuals who were exposed to childhood adversity (physical abuse, sexual abuse, or neglect) will display attenuated responses to psychosocial stress, compared to individuals exposed to less or no early adversity. Also, individuals exposed to recent stressful life events or difficulties will display attenuated reactivity, compared to individuals not exposed to recent adversity.
3. Independent genetic effect on reactivity – Polymorphic variations at sites that encode functional components of the HPA system will alter cortisol reactivity, including those associated with the following:
    a. Response Activation:
        i. COMT SNP **rs4680**. Homozygous carriers of risk alleles will demonstrate elevated response to psychosocial stressors.
        ii. CRHR1 SNPs **rs110402** and **rs1876831**. These genes will demonstrate interactive effects with adversity and sex.
        iii. MR SNP **rs5522** in gene NR3C2. Homozygous carriers of risk alleles will demonstrate elevated response to psychosocial stressors.
    b. Termination:
        i. SERT. Carriers of the short allele of **5-HTTLPR** will display elevated reactivity with or without exposure to adversity. Also, carriers of the risk allele of SNP **rs25532** will display elevated reactivity.

ii. OPRM1 SNP **rs1799971.** Carriers of minor alleles will demonstrate a prolonged response to the TSST.

c. Associated Activation:

i. DRD2. The effects of SNP **rs1800497** in ANKK1 on cortisol reactivity will be explored.

4. Interactive effects on reactivity – Individuals who carry multiple vulnerabilities to altered cortisol functioning (e.g. exposure to early life stress, genetic polymorphisms) will display more extreme forms of reactivity than individuals who do not carry such vulnerabilities. This study will assess all potential interactions, with particular consideration given to the following:

a. CRHR1 x Early Adversity x Sex – reported by Heim et al. (2009); Tyrka et al. (2009) report a significant GxE effect for another SNP.

b. 5-HTTLPR x Early Adversity – reported by Mueller et al. (2011) in young adults, but not supported in a meta-analysis by Miller et al. (2013).

c. 5-HTTLPR x Recent Adversity – reported by Alexander et al. (2009), but not supported in recent meta-analysis.

CHAPTER II

METHODS

Overview of Study and Design

Data for this project were obtained from an ongoing longitudinal cohort-sequential study assessing risk factors associated with substance use in children, adolescents, and emerging adults, the Oregon Youth Substance Use Project (OYSUP; Andrews et al., 2003). One-thousand seventy-five students from five grade cohorts (1st through 5th grade at the first assessment, during the 1998-1999 academic year) were assessed annually until one year after completing high school. At age 20-22, students primarily from two cohorts (in 3rd and 4th grade during the first assessment) completed interviews about recent stressful experiences (the Life Events and Difficulties Schedule, LEDS; Brown and Harris, 1978) and their childhood care experiences (the Childhood Experiences of Care and Abuse interview, CECA; Bifulco et al., 2003), and participated in a laboratory-based task designed to induce stress (the TSST; Kirschbaum, Pirke, and Hellhammer, 1993). Saliva samples were obtained to assay cortisol preceding and following the TSST, and also to assay specific polymorphisms of candidate genes that may be related to the human neuroendocrine stress response.

Participants

The 1075 students originally recruited for OYSUP were randomly selected from 15 elementary schools in one school district in Western Oregon (see Andrews et al., 2003, for additional information about the representativeness of the original sample). Students (N = 403) in the current study were contacted within one year of their 21st

birthdays (ages 20-22; Mean age = 21.14) to participate in a day-long assessment. The current sample was primarily middle- and lower-middle class European-Americans (84.6%) and Latinos (6.5%), with a nearly-equal proportion of males (n = 189, 50.7%) and females. Many of these students had been or were currently enrolled in college at the time of assessment (64.6% of students reported at least some coursework after high school; only 7.0% of students did not complete high school). Most students were employed during the last six months (83.7%), and many were employed currently (70.3%). A small number (4.0%) were active-duty or reserve military personnel.

Thirty individuals (19 females, 11 males) did not participate in the TSST. Five of these individuals were pregnant at the time of the assessment, and the remaining 25 individuals declined participation. Other than being disproportionately female, this subset of individuals did not differ in terms of demographic or adversity variables from the rest of the sample. Additionally, five individuals had incomplete or missing genetic or cortisol data (two individuals declined to provide a saliva sample for DNA analysis, and the cortisol samples provided by three individuals could not be assayed).

Attrition

Cohorts Three and Four consisted of 450 students who participated in the first OYSUP assessment. Forty-seven of these individuals did not participate in the age 20-22 assessment. Demographic differences were not observed between individuals who participated in the first assessment and those who participated in the current assessment. These individuals did not significantly differ from study participants in terms of demographics, parental education, childhood SES (measured by free/reduced lunch in grade school and maternal education level) or racial composition.

Assessment Procedures and Interviews

At age 20-22, participants were assessed at Oregon Research Institute (ORI; 95% completed within one day; Mean participation time = 5.55 hours). Most participants were interviewed in the morning, and participated in a laboratory-induced stress task in the afternoon. Before the stress task, participants provided saliva samples to assay DNA and baseline cortisol concentrations. Additional saliva samples were collected to assess cortisol reactivity in response to the stress task.

LEDS

Considered the gold standard for assessing, defining, and rating life stressors and long-term difficulties, the LEDS employs an interview-based, contextual threat methodology. The LEDS is a semi-structured interview that identifies discrete events or chronic difficulties in all life domains (school, work, living arrangements, child-rearing, finances, criminal activity, health, romantic and platonic relationships, and other events), placing these occurrences in the context of the interviewee's life, and allowing for assessment of immediate and long-term threat, event focus and independence. Events were defined as singular occurrences lasting a couple weeks at most. Difficulties were ongoing problems associated with a life domain that lasted for at least a month. Participants were interviewed about events and difficulties that occurred during 12 months preceding date of interview.

Using manuals providing thousands of case examples, raters who were blind to the interviewee's subjective responses assigned standardized ratings to events and difficulties from cases presented by the interviewer. For this study, events that were rated 1 or 2 in terms of long-term threat (on a five-point scale, where lower numbers indicate

greater threat) were counted as stressful life events (SLEs). A high rating of long-term threat suggests that the negative effects of a SLE will be felt for weeks or months following the event, despite the level of immediate impact. Similarly, chronic difficulties were rated in terms of threat on a seven-point scale, where ratings of 1 or 2 applied to high threat difficulties. High threat difficulties have no immediate sign of resolution, and may contribute to significant ongoing life problems for the foreseeable future. Event or difficulty domain was not evaluated in this study. Instead, individuals were dichotomized according to whether they experienced a SLE during the last year or not; also, they were dichotomized according to exposure to a high threat difficulty during the last year or not.

CECA

Developed using the same contextual-threat methodology as the LEDS, the CECA was used to assess early childhood adverse experiences. The CECA is a semi-structured interview that collects data on five domains – antipathy, hostility, or criticism directed at a participant by a childhood caregiver; emotional or material neglect from a caregiver; psychological abuse perpetrated by household members; physical abuse, including punishment; and sexual abuse by any perpetrator. In previous research, these domains have been associated with lifetime occurrence of mood and anxiety disorders, and are also associated with substance use disorders. Although both instruments are retrospective interviews, and many participants cannot vividly recall some aspects of childhood functioning, an interview-based method is more likely to capture accurate information when compared to a questionnaire covering the same information (McQuaid, Monroe, Roberts, Kupfer, and Frank, 2000).

A team blind to the subjective reports of the interviewees rated the severity of abuse by each caregiver or perpetrator within each domain (Antipathy; Neglect; Physical, Psychological, and Sexual Abuse) using a 5-point ordinal scale. A score of one indicates the highest level of severity, and four indicates a low level of severity; if no abuse was reported within a domain, a summary score of five was assigned to the participant. Case examples and standardized rules were used to guide ratings, and scores were dated to the nearest year of age of the participant. Summary variables for a given participant were generated based on the highest severity score reported before age 12 within a domain.

To reduce the number of variables examined in final regression equations, only three domains of abuse were utilized – physical and sexual abuse, and neglect. Neglect was chosen over Antipathy and Psychological Abuse for several reasons. Neglect demonstrated more consistent (yet comparable) relationships with genetic and cortisol variables in preliminary analyses, compared to Antipathy, Psychological Abuse, or an average of all three psychosocial abuse variables. The variable was strongly correlated with Antipathy (r=0.54), and to a lesser degree with Psychological Abuse (r=0.42). Also, it demonstrated the most favorable psychometric properties of the three variables (range, skew, etc.). This resulted in three independent measures of early adversity, with lower values representing ***more*** severe occurrences of abuse in childhood.

Psychosocial Stress Task and Cortisol Data Collection

TSST

The Trier Social Stress Test (Kirschbaum et al., 1993) is a standardized laboratory procedure used to assess cortisol reactivity to a moderate psychosocial stressor. This

procedure has previously been established to raise salivary cortisol levels two- to three-fold in at least 70% to 80% of participants, in addition to evoking similar cardiovascular and affective responses (Kudielka, Hellhammer, and Wüst, 2009). These effects are attributed to the uncontrollable and evaluative nature of two tasks – a speech delivered in front of an audience, and a mental arithmetic assignment – which are known to provoke HPA reactivity. After meeting two unknown confederate "scientists" who rate behaviors and direct the tasks, participants prepared for a speech about their qualifications for a hypothetical job. Upon returning to the evaluation room, they delivered the speech for five minutes, and were then instructed to perform mental arithmetic for five minutes (either subtraction or addition in intervals of 13). The confederates were instructed to show no emotional or supportive reactions to task performance, instead informing the participant of additional time remaining for the speech or errors during the math task.

It was anticipated that 20% to 30% of participants in the TSST might not demonstrate cortisol reactivity (Kudielka et al., 2009), so two additional measures of stress reactivity were utilized to validate the salivary data – changes in heart rate (HR) and emotional state. Self-reported ratings of positive and negative affect (Subjective State Scale; al'Absi, Hatsukami, Davis, and Wittmers, 2004) completed before and after the TSST task were compared. On average, most participants showed changes in affect following the TSST [decreases in positive affect ($t(372)=-11.77$, $p<.001$) and increases in distress ($t(372)=26.65$, $p<.001$)].

To record HR variability, most participants ($n = 347$) wore a commercially-available digital heart rate monitor (CMS 50-F; ChoiceMed Electronic Tech Co., Beijing, China) on the wrists of their non-dominant arms, and heart rate data were recorded to the

device using a rubber finger probe[1]. Twenty percent (n = 70) of these participants also wore a second heart rate monitor that utilized a probe strapped to the chest, considered the gold-standard in psychometric assessment (Polar RSX-800; Polar Electro, Kempele, Finland). Data from the chest and finger probes were nearly identical over the course of the TSST for these participants (r = 0.94, p < .001), validating the finger probe data. Changes in HR during the preparation period were compared to changes following termination of the stress task. As a group, all participants showed significant changes in heart rate following the task – a quadratic trend, peaking during the ten-minute period following termination of the TSST [$F(1, 345) = 33.45$, $p<.001$].

As expected, nearly 25% of participants displayed either unexpected (e.g., >1 SD than mean change) or no changes in HR, affect, or cortisol release during the TSST, but no participants displayed unusual changes in all three constructs. Thus, no cases were dropped from analyses, and these individuals were considered hyporeactive.

Cortisol

Salivary free cortisol was collected using salivettes (Salimetrics, State College, PA) before, during, and after the TSST at six time points – once after a 20 minute relaxation period preceding the TSST (***t1***), once after meeting the panel of TSST "scientist" evaluators (***t2*** – approximately 10 minutes after t1), once immediately after completing the speech and arithmetic tasks (***t3*** –approximately 20 minutes after t2), and three more times following the tasks [10 minutes (***t4***), 20 minutes (***t5***), and 50 minutes (***t6***) after t3 collection]. Peak cortisol release was captured by the t4 collection, 30

[1] HR data collection was initiated after 30 participants had been evaluated. The self-reported affect and cortisol data obtained from these individuals did not significant differ from the rest of the sample. Further, their affect data showed no anomalies, so their cortisol data were included without HR validation.

minutes after initial stress exposure (t2). Cortisol returns to pre-task levels after one hour for most individuals, and this was reflected in the t6 collection.

Saliva collection occurred during the late afternoon and evening, when participants return from a lunch break (15:00-17:00h), allowing for control of diurnal variation. Since salivary cortisol can be affected by tobacco, food or drink, the baseline saliva sample was collected at least two hours after lunch, following a 20 minute observed rest period in the laboratory. All participants were encouraged to drink water throughout the day to promote salivation. Further, women were scheduled during the luteal phase of their menstrual cycles (days 15-26), controlling for estrogen levels.

At each collection time, participants placed cotton swabs in their mouths until these were saturated with saliva. The swabs were placed the salivettes, which were stored at -20°C and later shipped on dry ice to Salimetrics. Salivary cortisol was assessed by immunoassay with mean intra- and inter-assay coefficients of variation of 3.5% and 5.1%, respectively. Two assays per sample were averaged to assess cortisol levels at each collection time, yielding highly consistent results ($r = 0.99$, $p<0.001$).

These averages of salivary free cortisol over time were used to obtain measures of area under the curve (Pruessner, Kirschbaum, Meinlschmid, and Hellhammer, 2003) – area with respect to ground or zero (AUCg), measuring the total cortisol release across time; and area under the curve with respect to increase (AUCi), measuring reactivity to the stress task by accounting for change relative to baseline (t1 assay). AUCg is calculated by summing the area of the five trapezoidal shapes created between the x-axis and each cortisol sampling point over a given time interval (t1 to t2, etc.). AUCi is calculated by multiplying the t1 value by the sum of all time differences {mean 1 * [(t2 -

t1) + (t3 - t2) + (t4 - t3), etc.]} and subtracting this value from AUCg. Subcomponents of AUCi were also calculated for each individual – Rise, defined as the area of AUCi from t2 to peak (t4), representing activation of the HPA system; and Decline, defined as the area from peak to t6 collection, representing the termination of HPA functioning. An attenuated or elevated Rise in response to the stressor, extreme AUCi/AUCg values (high or low), and reduced or elevated Decline were all considered forms of cortisol dysregulation. A visual depiction of these constructs is shown in Figure 2.

Genetic Polymorphisms

Candidate SNPs and VNTR sequences were selected based on a priori association with neurobiological variations in the human stress response. These polymorphisms showed the greatest effects across the most studies, and include the following:

1. ANKK1 SNP rs1800497
2. COMT SNP rs4680
3. CRHR1 SNP rs110402
4. CRHR1 SNP rs1876831
5. MR SNP rs5522
6. OPRM1 SNP rs1799971
7. SERT SNP rs25532
8. SERT VNTR 5-HTTLPR

Saliva collection for DNA was performed using commercially available kits (Oragene®•DNA 10 Self-Collection Kit, OG-500; DNA Genotek, Ontario, Canada). Salivary collection carries several benefits – higher quality of DNA, and preservation and

stabilization of samples relative to buccal swabs; non-invasive, safe, and painless collection relative to blood draw. Participants provided 2 mL of saliva into each collection tube before participating in the TSST and cortisol collection. When capped, the contents were stabilized by a reagent released by the container. DNA samples were stored and shipped to a laboratory at SRI, International (SRI, Menlo Park, CA) at room temperature. DNA was extracted from saliva samples using Oragene® purifier and ethanol precipitation (Nishita et al., 2009) using the manufacturer's protocol for manual purification of DNA from 4.0 mL, PD-PR-015 Issue 3.1 instead of 2.0 mL. DNA was quantified by OD, Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen™, Carlsbad, CA), and qPCR (Nishita et al, 2009).

Genotyping of candidate gene polymorphisms was performed at SRI using standard TaqMan® assays on a ViiA7™ Real-Time PCR system from Life Technologies with the exception of the following: SNP rs25532 was genotyped using primers and custom Taqman® probes (Wendland, et al, 2008). The genotyping of the 5-HTTLPR VNTR was performed using both a restriction fragment length polymorphism method (RFLP; Wendland, et al., 2006) and a Taqman® assay (see Hu et al., 2006, for a description) as stage one. Two samples (0.5%) of the total subjects genotyped were discordant between the two methods for the 5-HTTLPR genotyping and were changed to undetermined. SNP rs25531 was also genotyped using the Wendland et al. (2006) RFLP method. All genotyping was performed with positive and negative controls. The minimum genotype completion rate was 99.0%, for SNP rs25532.

Hardy-Weinberg Equilibrium

Hardy-Weinberg (H-W) equilibrium refers to the distribution of alleles in a population, where homozygous and heterozygous carriers of alleles will remain in balanced ratios across generations. Violations of H-W equilibrium suggest an environmental influence on the distribution of genes. The distribution of alleles in the sample data was examined using Haploview 4.2 (Broad Institute, Cambridge, MA), and most genes were found to be in H-W equilibrium, evidenced by non-significant χ^2 deviation tests (*p*s range 0.27-0.69). Exceptions include CRHR1 SNPs (rs110402, p=0.02; rs1876831, p=0.11) and COMT SNP rs4680 (p=0.06). For these genes, participants displayed minor allele frequencies higher than what is expected by chance. Since analyses will only compare homozygous minor allele carriers to all others, these violations of equilibrium were ignored.

Linkage Disequilibrium

Linkage Disequilibrium (LD) refers to alleles at two or more loci that are associated with each other. LD is most often calculated for alleles located on the same chromosome, since linked alleles tend to be close together in the genome. Only SNPs within CRHR1 and SERT were correlated with one another, so LD was calculated for these. Both pairs of SNPs were found to be in strong LD (CRHR1: rs110402 and rs1876831, D'=0.98, 95% CI: 0.91-1.00; SERT: rs25531 and rs25532, D' = 1.00, 95% CI: 0.19-1.00), suggesting that their effects are interpretable independently.

Triallelic versus Biallelic Variations in 5-HTTLPR

Recent studies of SERT have identified another SNP (rs25531) that alters functional expression of SERT (Wendland et al., 2006). Thus, carriers of the "risk" allele

of rs25531 who do not carry short alleles of 5-HTTLPR may still display ineffective serotonergic reuptake and transport. This has been described in literature as a "triallelic" variation in SERT. Separately, this study incorporated three iterations of this vulnerability into models predicting reactivity – 1) the 5-HTTLPR variants alone; 2) 5-HTTLPR and rs25531 variants, and their interaction; and 3) the triallelic variations recommended by Wendland, et al. (2006). Neither the triallelic variations, nor interactions between 5-HTTLPR and rs25531 were significant in predicting reactivity in single-gene models. Further, collinearity tolerances were exceeded when rs25531 was included as a unique term. Thus, this study only considered biallelic 5-HTTLPR.

Model Variables

Genotype data were reduced to single dichotomous variables, defined as homozygous "risk" allele carriers (i.e., an allele that is associated with atypical cortisol reactivity or risk for development of disease) compared to all "protective" allele carriers (heterozygous or homozygous). In preliminary analyses, heterozygous and homozygous carriers of "protective" alleles were indistinguishable in terms of cortisol reactivity. Thus, for purposes of parsimony, analyses were restricted to these dichotomous variables.

Statistical Analyses

Preliminary analyses were conducted to examine associations among predictor variables and cortisol AUC components (See Tables 1 to 3; see the Appendix for all tables). To address multicollinearity in regression models among these variables, continuous predictors were standardized. All analyses were conducted using SPSS 21.0 (IBM Corp., Armonk, NY).

A series of multiple regression models were used to predict each measure of cortisol reactivity from genes, early and recent adversity, sex, and interactions among these variables. Except where noted, effects of current psychopathology were not considered. Significant interactions were decomposed using techniques proposed by Aiken and West (1991). The effect of each polymorphism was first assessed separately, in a regression model which included all five adversity measures, sex, and their interactions. Predictors that exceeded collinearity tolerances (tolerance statistics <0.20) were removed automatically. Non-significant main effects and interactions (with *p*-values greater than 0.25) were removed from each model using backward elimination, starting with three-way interactions. If a higher order interaction was significant, all lower order interactions and main effects were retained in the single-gene model.

All retained main effects and interactions from each of these single-gene models were then entered simultaneously into four regression equations, to examine the independent effects of each predictor and its interactions on each construct of reactivity. If multiple genes were predictive of a given variable in these models, gene-by-gene-by-environment-by-sex interactions and their lower-order subcomponents were entered into predictive models. Again, non-significant main effects and interactions (with *p*-values greater than 0.10) were removed from final models using backward elimination, except when they were subcomponents of a higher-order significant interaction. All missing data were resolved by listwise deletion, resulting in reduced numbers of cases given availability of genomic data. Even though all analyses were conducted with continuous measures of early adversity, visual depictions are limited to dichotomous comparisons of "severe" and "non-severe" levels of stress for the sake of parsimony.

CHAPTER III

RESULTS

Preliminary Analyses

Preliminary analyses revealed significant associations among predictor and outcome variables. Peak levels of early childhood abuse were all positively correlated with one another (*r*s range 0.54 to 0.20, $p<0.001$; See Table 1); Antipathy and neglect showed the strongest correlation, while physical and sexual abuse were weakly associated. Recent exposure to a severe stressful life event or high threat difficulty were also associated ($\phi=0.32$, $p<0.001$). Notably, greater childhood exposure to neglect, psychological abuse, and sexual abuse were significantly associated with experiencing a stressful life event during the last year (*r*s range -0.13 to -0.20,*p*s range <0.01 to <0.001); and, greater levels of all forms of early adversity were significantly associated with exposure to a high threat difficulty during the last year (*r*s range -0.24 to -0.29, $p<0.001$).

Correlations among measures of cortisol reactivity are reported in Table 1. Greater childhood neglect was associated with reduced cortisol Rise ($r=0.12$, $p=0.02$), and sexual abuse exposure was associated with greater AUCi and Rise in cortisol ($r=0.12$, $p<0.05$; and $r=0.14$, $p<0.01$, respectively). Exposure to a stressful life event during the last year was associated with decreased cortisol release across measures (AUCg: $r=-0.13$, $p<0.05$; AUCi: $r=-0.16$, $p<0.01$; Rise: $r=-0.16$, $p<0.01$). Exposure to a high threat difficulty was also associated with attenuated reactivity in response to the stress task (AUCi: $r=-0.14$, $p<0.01$; Rise: $r=-0.14$, $p<0.01$).

Sex Differences

Participants significantly differed in terms of cortisol reactivity with regard to sex. Females displayed significantly lower cortisol responses than males [AUCg: $t(211.44) = 3.20$, $p = .002$; AUCi: $t(356.60) = 2.85$, $p = .005$; Rise: $t(309.99) = 4.99$, $p<.001$; Decline: $t(302.92) = 4.05$, $p<.001$; see Table 2]. All comparisons were corrected for inequality of variances, reflecting high variability in samples obtained from males. Notably, participants did not differ when t1 or t2 assays were compared, suggesting differences appear only during reactivity. Participants did not differ in terms of cortisol reactivity as a function of childhood SES or age at time of data collection.

Only one association between sex and homozygous genetic "risk" was significant – more females than males carried two copies of the C allele of SNP rs25532, located in the serotonin transporter gene [$\chi^2(1, N=363) = 5.53$, $p = .019$; see Table 3]. Groups did not significantly differ in terms of exposure to early adversity, with the exception of sexual abuse, which was more severe in females (M = 4.47, SD = 1.15) than in males (M = 4.82, SD = 0.68; $t(371)=3.613$, $p<.001$; see Table 2).

Prediction of Cortisol Reactivity

Sex, adversity, several candidate polymorphisms, and their interactions were independently predictive of variations in cortisol reactivity. Each multi-gene model accounted for a significant percentage of variance in reactivity – AUCg $F(15,352) = 49.53$, $p<0.001$, Adjusted $R^2 = 0.67$; AUCi $F(18,346) = 4.22$, $p<0.001$, Adjusted $R^2 = 0.14$; Rise $F(10,357) = 6.12$, $p<0.001$, Adjusted $R^2 = 0.12$; Decline $F(10,360) = 5.99$, $p<0.001$, Adjusted $R^2 =0 .12$ – even after correcting for the effects of conducting multiple

tests. Main effects and interactions that were significantly predictive of each cortisol AUC component are reported in Table 4.

Sex

As shown in Table 4, a significant main effect for sex was observed in all models predicting cortisol reactivity, controlling for all other significant genetic or environmental effects and interactions (*t*s range 3.18 – 5.57, $p<0.01$ or lower). A visual depiction of this effect, demonstrating greater cortisol release (AUCg), greater reactivity (AUCi), and greater variability in males, is shown in Figure 3.

Early Adversity

Peak measures of neglect and sexual abuse encountered before age 12 were significantly predictive of cortisol reactivity at age 21 in single predictor models, and were marginally significant in multi-gene models. As shown in Figure 4, childhood exposure to either form of abuse was associated with reduced cortisol reactivity to psychosocial stress in young adulthood. Specifically, participants displayed less of a rise in cortisol if exposed to higher levels of neglect from caregivers (B = 0.39, SE = 0.22, p = .07; see Figure 4a). Also, participants displayed more attenuated reactivity given greater exposure to sexual abuse (B = 0.93, SE = 0.50, p = .07; see Figure 4b).

Recent Adversity

Exposure to one or more severe stressful life events or one or more high threat difficulties during the year preceding the interview was associated with attenuated cortisol reactivity. As shown in Figure 5a, exposure to a severe stressful life event was associated with significantly attenuated Rise in participants' cortisol compared to individuals not exposed to a severe event (B = -1.54, SE = 0.47, p = .001), and was

marginally associated with decreased overall cortisol release (B = -2.50, SE = 1.52, p = .10). Similarly, participants exposed to an ongoing severe difficulty displayed attenuated reactivity to the TSST (B = -2.74, SE = 1.31, p = 0.04).

Genetic Polymorphisms of SERT, OPRM1, and NR3C2

Homozygous carriers of "risk" alleles demonstrated elevated cortisol reactivity to the psychosocial stress task, compared to carriers of at least one copy of the protective allele. As shown in Figure 6, both risk polymorphisms examined in SERT (5-HTTLPR: B = 3.48, SE = 1.30, p = 0.06; SNP rs25532: B = 3.52, SE = 1.27, p = 0.006) predicted greater cortisol reactivity. Homozygous carriers of a risk allele in OPRM1 demonstrated greater Rise in cortisol, compared to carriers of one or more protective alleles (B = 3.83, SE = 1.92, p = 0.05; See Figure 7). Notably, the relationship between 5-HTTLPR polymorphisms and elevated reactivity was not mediated by exposure to early life stress or recent adversity. Also, variations in MR gene NR3C2 were not associated with cortisol reactivity in single-gene tests, so this variable was excluded from further analyses.

Gene-Environment Interactions in Cortisol Reactivity

Several genes were associated with altered HPA functioning only in individuals exposed to early or recent adversity. These GxE interactions varied as a function of participant sex in many cases.

ANKK1 x Physical Abuse x Sex

As shown in Table 4, the three-way interaction of the ANKK1 SNP with physical abuse and sex was significant in the prediction of total cortisol release (AUCg). Decomposition of this three way interaction showed that the interaction between ANKK1

and physical abuse was significant for males, but it was not significant for females (B = 1.20, SE = 9.39, p = .45). The three-way interaction in the prediction of cortisol reactivity (AUCi) showed a similar pattern – when decomposed by sex, the two-way interaction between ANKK1 and physical abuse was significant for males, but not for females (B = 1.67, SE = 4.29, p = .70). In this sample, no female carriers of risk alleles for this gene were exposed to very severe physical abuse.

Male homozygous carriers of the "risk" allele displayed greater cortisol release and reactivity depending on physical abuse exposure. Exposure to more severe abuse was associated with greater total release and elevated basal cortisol levels (AUCg physical abuse B = 23.64, SE = 5.66, p<.001), but also less reactivity (negative AUCi values, indicating a drop in cortisol over the task period); individuals who were exposed to less severe forms of abuse demonstrated exaggerated reactivity (AUCi physical abuse B = -14.61, SE = 3.30, p < .001), but also greater total cortisol release compared to male carriers of protective alleles.

Female homozygous carriers of the risk allele did not display a consistent pattern in AUCg or AUCi when physical abuse history was considered – exposure was not associated with variations in release or reactivity (AUCg, physical abuse B = 1.60, SE = 9.41, p = .87; AUCi, physical abuse B = 1.24, SE = 4.27, p = .77). Similarly, male and female carriers of a "protective" allele did not demonstrate variations in cortisol functioning given abuse exposure (Males: AUCg, physical abuse B = -1.26, SE = 1.76, p = .48; AUCi, physical abuse B = -0.71, SE = .84, p = .40; Females: AUCg, physical abuse B = 0.40, SE = 1.66, p = .81; AUCi, physical abuse B = -0.42, SE = 0.81, p = .61). Variations in HPA responses to the TSST are displayed in Figure 8.

ANKK1 x Neglect x Sex

A significant interaction between ANKK1 genotype, level of exposure to childhood neglect, and sex predicted variations in termination of the HPA response (Decline; See Table 4). Decomposition of this three way interaction showed that the interaction between ANKK1 and neglect was significant for females (B = 6.54, SE = 2.00, $p = 0.001$), but it was not significant for males (B = -1.98, SE = 2.74, $p = 0.47$). Female "protective" allele carriers of ANKK1 demonstrated a strong relationship between exposure to childhood neglect and cortisol Decline – greater exposure to neglect was associated with attenuated cortisol release after peak (neglect B = 7.06, SE = 1.98, $p < 0.001$). Female homozygous carriers of the ANKK1 "risk" allele showed a weaker relationship, as reduced Decline values were marginally associated with more severe exposure (neglect B = 0.53, SE = 0.34, p = .12). Male "protective" allele carriers did not differ in terms of cortisol Decline due to neglect history (Males: neglect B = -1.80, SE = 2.73, p=0.51). Further, males carrying two copies of the "risk" allele appeared to diverge in terms of Decline in a pattern opposite to that of females; however, these males showed no consistent relationship between severity of neglect and Decline (neglect B = 0.18, SE = 0.32, p = 0.58). These variations are portrayed in Figure 9.

COMT x Physical Abuse x Sex

The three-way interaction of the COMT SNP with physical abuse and sex was also significant in predicting total cortisol release (AUCg). When decomposed by sex, as shown in Table 4, the two-way interaction between physical abuse and COMT was significant for males, but not for females (B = -2.60, SE = 3.40, $p = .45$). In male homozygous carriers of the COMT "risk" allele, extent of physical abuse exposure

predicted cortisol release. Attenuated cortisol release was associated with more severe physical abuse exposure, and elevated cortisol release was associated with less severe exposure (physical abuse B = 6.30, SE = 2.82, p = .026). Male and female "protective" allele carriers did not differ in terms of cortisol release due to physical abuse history (Males: physical abuse B = -1.26, SE = 1.76, p=.48; Females: physical abuse B = 0.40, SE = 1.66, p = .81). Females carrying two copies of the "risk" allele appeared to diverge in terms of AUCg in a pattern opposite to that of males; however, these females showed no consistent relationship between severity of physical abuse and AUCg (physical abuse B = -2.19, SE = 2.99, p = .46). These Variations are displayed in Figure 10.

COMT x Recent Difficulty

A significant interaction between COMT and exposure to high threat difficulty was observed (See Table 4). Specifically, carriers of at least one protective allele demonstrated variations in reactivity depending on exposure to a recent difficulty, where attenuated HPA activation (lower Decline values) is marginally associated with exposure to a high threat difficulty during the last year (B = -1.59, SE = 1.03, p = 0.12). Carriers of two copies of the "risk" allele did not show a relationship between stress exposure and termination of HPA functioning (B = 0.65, SE = 0.61, p = 0.29). These variations are displayed in Figure 11. Notably, variations in COMT significantly interacted with exposure to recent stressful events in the prediction of total cortisol release and the initiation of reactivity (Rise), but these tests were not significant in multi-gene analyses.

CRHR1 x Physical Abuse x Sex

Three-way interactions of the CRHR1 SNP rs110402 with physical abuse and sex were significant in the prediction of cortisol reactivity and activation of the cortisol

response (Rise). When decomposed by sex, the interaction between CRHR1 and physical abuse was significant for males, and approached significance for females in the prediction of reactivity (AUCi B = -2.32, SE = 1.43, p = .10). Also, the interaction between CRHR1 and physical abuse predicting Rise was not significant for males, but was for females (B = -1.18, SE = 0.61, p = .05). As shown in Table 4, male homozygous carriers of the "risk" allele demonstrated attenuated reactivity given greater exposure to abuse. Female homozygous carriers of the CRHR risk allele displayed the opposite pattern to males – more severe abuse was associated with exaggerated cortisol reactivity (AUCi, physical abuse B = -2.74, SE = 1.21, p = .02) and cortisol Rise (B = -1.19, SE = 0.52, p = .02). Male and female carriers of a "protective" allele did not demonstrate variations in overall reactivity or activation of HPA given abuse exposure (Males: AUCi, physical abuse B = -0.71, SE = 0.84, p = .40; Rise, physical abuse B = 0.05, SE = 0.36, p = .89; Females: AUCi, physical abuse B = -0.42, SE = 0.81, p = .61; Rise, physical abuse B = -0.01, SE = 0.36, p = .98). These variations are portrayed in Figure 12.

ANKK1 x COMT x Physical Abuse x Sex

The four-way interaction among these risk alleles, physical abuse, and sex was considered in the prediction of total cortisol release (AUCg); however, extreme multicollineraity between the interaction term and other lower-order predictors prevented analysis. Nevertheless, the two way interaction between ANKK1 x COMT, and the three way interactions between these genes and both sex and physical abuse were significant (See Table 4). In general, the effects of physical abuse on cortisol reactivity depended strongly on haplotype and gender. Both male and female carriers of protective alleles in both genes demonstrated significantly attenuated cortisol release given greater abuse

exposure (Males: B = 45.72, SE = 6.79, $p < 0.001$; Females: B = 131.37, SE = 7.49, $p < 0.001$). Male and female carriers of "risk" alleles for both genes did not show consistent relationships between total release and physical abuse exposure (Males: B = 1.31, SE = 1.06, $p = 0.22$; Females: B = 0.13, SE = 1.13, $p = 0.91$). Carriers of one risk allele or the other showed relationships comparable to Sex-by-GxE interactions previously interpreted.

ANKK1 x CRHR1 x Physical Abuse x Sex

The four-way interaction among these risk alleles, physical abuse, and sex was also considered in the prediction of AUCi. Extreme multicollineraity between the interaction term and other lower-order predictors prevented analysis. Lower-order interaction terms were not significant, except those already reported.

Effects of CRHR1 SNP rs1876831

A significant three-way interaction was observed among CRHR1 SNP rs1876831, childhood neglect, and sex predicting termination of HPA functioning (B = 1.35, SE = 0.61, $p = 0.03$). However, this interaction was not significant in multi-gene analyses.

CHAPTER IV

DISCUSSION

The present study was developed to examine the interactive effects of sex, early and recent adversity, and polymorphisms associated with variations in the human stress response on cortisol reactivity. In most cases, the primary hypotheses of this study were supported. Overall, young adult females displayed reduced cortisol reactivity to the TSST compared to males. Individuals exposed to greater levels of early adversity or recent life stress generally displayed attenuated reactivity, while homozygous carriers of risk alleles in SERT and OPRM1 displayed elevated reactivity. Exposure to physical abuse interacted with three polymorphisms – SNPs in COMT and ANKK1, and SNP rs110402 in CRHR1 – and the nature of these interactions depended on sex. In general, physically abused males who carried risk alleles displayed attenuated reactivity, while females displayed elevated reactivity under the same circumstances. Individuals who carry alleles identified by previous studies or implicated by the corpus of research to be at-risk for cortisol dysregulation have shown that they are indeed demonstrating the varied effects of allostatic load when exposed to stressors (i.e., hypo- or hyperreactivity).

To date, this is the first study to measure the multiple independent effects of GxE interactions simultaneously. The pattern of significance observed in these interactions informs a complex developmental model of cortisol dysregulation. Partial replications and non-replications of previous studies may be due to random variations in study samples, or may occur because GxE interactions were previously examined in a statistical (and, by proxy, psychobiological) vacuum. Gene-by-GxE interactions were also evaluated, and explain more variability in reactivity than single-gene models.

Effect of Participant Sex

Differences attributable to participant sex have been demonstrated in the current study, consistent with the body of literature suggesting human females release less cortisol in response to psychosocial stress than males (e.g., Kudielka and Kirschbaum, 2005). It is conceivable that the effect of sex observed in these studies could have been due to increased exposure to early adversity, which is also associated with attenuated reactivity. In the present study, females reported significantly more exposure to sexual abuse than males, and this exposure might explain their attenuated reactivity as adults. However, this study evaluated the effect of sexual abuse exposure, sex, and their interaction, and found that both sexual abuse exposure and participant sex were independently predictive of attenuated reactivity. Thus, divergent biological responses and coping mechanisms (but not differential exposure) likely explain sex differences.

Effect of Exposure to Adversity

Regardless of sex, individuals who were exposed to both distal and proximal forms of adversity exhibited attenuated reactivity compared to individuals exposed to lesser or no adversity. This finding is consistent with research associating childhood exposure to neglect or sexual abuse with reduced cortisol reactivity in adolescence or adulthood (e.g., Lovallo et al., 2012). The association between recent life stress and reactivity is consistent with some research (e.g., Armbruster et al., 2012), but other studies often demonstrate effects of recent life stress given genetic vulnerabilities (e.g., Alexander et al., 2009). Taken together, these findings reinforce the idea that repeated or ongoing exposure to environmental stressors can independently alter cortisol functioning

during any period in life, as opposed to effects being limited to exposure during early childhood or to individuals with "risk" alleles. In all cases, participants were exposed to highly-threatening experiences, and responded to subsequent stressors with reduced reactivity (i.e., demonstrated effects of allostatic load).

Effects of Genetic Vulnerabilities

Several genetic vulnerabilities identified in previous research demonstrated effects on cortisol reactivity. As hypothesized, homozygous carriers of "risk" polymorphisms in SERT (5-HTTLPR, and SNP rs25532) and OPRM1 (rs1799971) demonstrated elevated cortisol reactivity compared to "protective" allele carriers.

The finding regarding 5-HTTLPR is consistent with a recent meta-analysis of studies on cortisol reactivity and this polymorphism, which found a small effect for homozygous carriers of the short allele on increased reactivity (Miller et al., 2013). The current study also verifies the independence of this effect, as opposed to inconsistent interactive effects reported by Mueller et al. (2011), Alexander et al. (2009), and others. Both "risk" alleles and environmental stressors may be risk factors for the onset of these disorders, but the effects of stressors and genotype may not depend on one another. The current study also tested both the biallelic and triallelic variations in 5-HTTLPR, and found no significant results associated with the latter.

The reported association between the "risk" allele in SERT SNP rs25532 and reactivity is new, and may reflect the overall importance of SERT in the regulation of the HPA system. Any genetic variation that alters the expression or functioning of this gene should be considered in future research on cortisol reactivity.

The observed effect of OPRM1 vulnerability as a promoter of elevated HPA activation (i.e., Rise) is generally consistent with a study by Chong et al. (2006), who found differences only in peak cortisol release following the TSST. One would expect genes that alter termination of HPA activity to predict Decline, but both studies show effects for activation of HPA. The current data include only five individuals with this vulnerability; thus, high variability in Decline may have prevented observation of a significant effect. Further study is needed to examine the effects of this polymorphism.

No Effect of NR3C2

In contrast to previous studies of participants in the TSST, the current study did not find global or interactive effects associated with MR polymorphism SNP rs5522 in gene NR3C2. The vulnerability, previously associated with exaggerated cortisol reactivity, did not discriminate among typical or dysregulated responses, and was the only predictor selected before initiation of the study that did not produce a statistically significant result in single-gene models. Given the associations of MRs with basal activity rather than reactivity, the finding is not surprising. deKloet (2008) notes that variations in this gene are rare, but not to the degree observed in the current data; only five individuals carried two copies of the risk allele. Secondary tests using heterozygous risk allele carriers and multiple genotype groups were also not successful. A larger sample of at-risk individuals will be required to measure the effects of this gene.

Gene-Environment Interactions

Several genetic and environmental risk factors were found to interact in predicting dysregulation, and these effects were moderated by sex. Specifically, males who carry

two copies of "risk" alleles in three genes – SNP rs110402 in CRHR1, and SNPs in COMT and ANKK1 – displayed attenuated reactivity given greater exposure to childhood physical abuse, while females display elevated reactivity given the same circumstances. Males and females were exposed nearly equally to physical abuse before age 12, but their responses to psychosocial stress as adults diverged significantly. These GxE-by-sex effects could be caused by divergence in adaptation to physical abuse in childhood, by differences in cognitive or emotional processing of the stressor (i.e., males may be more ego-threatened by both physical violence and the TSST, compared to females), or they could manifest as extensions of the basal sex differences in stress reactivity found in all participants. Differential coping, rather than differential exposure, explains observed sex differences in GxE interactions.

CRHR1 Interactions

Results of the current study suggest that CRHR1 SNP rs110402 is the strongest indicator of phenotypic risk, as its associations with reactivity explained more variability than those of SNP rs1876831 in multi-gene analyses. Current findings bear some resemblance to those of previous research on CRHR1, but are very different upon close inspection. Gene-by-abuse-by-sex interactions reported by Heim et al. (2009) and in this study differ by genotype, such that phenotypic expressions of reactivity are opposites. Heim et al. note that long term changes in CRH functioning are only associated with physical and not sexual abuse (supported by the current study), but also suggest and do not test the idea that differential exposure explains the sex difference (not supported by the current study). On the other hand, the at-risk sample in the Tyrka et al. study was over 70% female, and the pattern of response reported more closely resembles that of females

in the present study (where maltreated homozygous GG allele carriers displayed elevated reactivity), albeit involving a different SNP. Although the present findings are interesting, they are also difficult to reconcile with extant literature on GxE involving CRHR1.

COMT Interactions

The divergent effects of sex on GxE in reactivity are not limited to a single gene, but also independently involve other genes. The effects of COMT on reactivity have previously been reported as main effects, where carriers of risk alleles demonstrate elevated reactivity, and this finding was not supported in the current study. Instead, the current study reports novel GxE interactions that are moderated by sex – male carriers of risk alleles demonstrate attenuated reactivity given exposure to greater physical abuse, and females display elevated reactivity given greater exposure and genetic risk.

COMT also interacts with recent stressors in the prediction of cortisol reactivity. Homozygous carriers of risk alleles who were not exposed to recent high threat difficulties demonstrate an effect similar to that reported by previous studies – an elevated response. In comparison, those carriers exposed to a recent difficulty demonstrate reduced responses (already associated with attenuated reactivity). Carriers of protective alleles demonstrate comparable (i.e., normal) reactivity, even if they were exposed to a recent difficulty. The parallel results in the current study suggest that the genetic vulnerability within COMT affects HPA functioning over the entire life course, but these effects may depend on exposure to certain forms of abuse (physical abuse in males, ongoing difficulties in all individuals). These effects also depend on sex, as males and females show different responses to physical abuse and acute psychosocial stress.

ANKK1 Interactions

The finding that variations in ANKK1 are associated with cortisol reactivity has not been previously reported. Homozygous males carriers of the genetic vulnerability displayed dramatically elevated reactivity – on the order of 2-3x greater than most participants. Males with this predisposition and a history of severe physical abuse displayed low resting cortisol, but were the most "activated" by the TSST (i.e., AUCi values were greatest); males exposed to less severe physical abuse displayed elevated reactivity throughout the TSST. Females did not display a consistent pattern, but this was due to limited exposure to severe physical abuse in female risk allele carriers.

Given that male risk allele carriers displayed such unusual reactivity, in the absence of firm evidence for functional variation in existing literature, one must postulate reasons for these findings. These individuals may have high basal cortisol levels due to a disorder associated with dopaminergic variation (such as substance abuse), or ANKK1 has some effect on HPA functioning outside of the expected alterations of dopaminergic functioning. Exposure to uncontrollable stress promotes dopamine release, but the indirect actions of dopamine ***on*** cortisol reactivity are less extensively tested.

Within the scope of this study, some of these issues can be addressed. ANKK1 genotype was not significantly associated with any current SUD diagnosis (alcohol, cannabis, nicotine, opiates, or stimulants), any mood disorder diagnosis, or any anxiety disorder diagnosis. Further, inclusion of variables representing current MDD or SUD diagnoses did not alter observed relationships among sex, early abuse exposure, and ANKK1 genotype in single-gene analyses. Future research will be required not only to replicate this effect, but also to elucidate the mechanisms behind it.

Consistent Systemic Effects on HPA Functioning

Most of the proposed hypotheses were supported by the results of the current study, patterned in ways that inform models of cortisol dysregulation over the developmental course. Global effects on reactivity were found involving genes that regulate the ***termination*** of HPA activity, while genes that regulate ***activation*** of the HPA system demonstrated interactive effects with stress exposure and sex. Greater stress exposure is also associated with attenuated reactivity over the life course.

Both CRHR1 and COMT genetic vulnerabilities likely alter the HPA system through prolonged activation. COMT degrades catecholamines such as norepinephrine, which activates the HPA axis. The genetic vulnerability in COMT is associated with a four-fold decrease in production of the enzyme, which would lead to the presence of norepinephrine for longer periods of time. CRH acts as a relay in the HPA axis, stimulating the release of ACTH; yet, the genetic vulnerability in CRHR1 is inconsistently related to reactivity. In both cases, neuroendocrine mechanisms that initiate and maintain HPA activity interact with sex and early life stress, suggesting that these systems change in response to environmental experiences that occur during critical periods. Given the similarity of findings, it is possible that variations in ANKK1 also effect change during early critical periods, altering activation of the HPA system.

The divergent patterns of these developmental changes (with vulnerable males displaying attenuated reactivity, and vulnerable females displaying elevated reactivity) counter the typical models of functioning in this population, where males displayed greater reactivity than females. In individuals who carry "risk" alleles that alter activation, the phenotypic manifestation of that "risk" may be HPA functioning that is the

opposite of what would develop typically. These patterns of response may diverge over time, as the child is exposed to repeated instances of physical abuse.

The main effects of SERT and OPRM1 polymorphisms – resulting in elevated reactivity in carriers of "risk" alleles – likely alter HPA functioning through termination of the response. Although serotonin is also implicated in its activation, the inability to quickly "turn-off" the HPA response by serotonergic feedback mechanisms likely contributes to elevated measures of reactivity. In both cases, termination of HPA appears to be genetically determined, rather than being affected by sex or adversity. Epigenetic effects (e.g., altered SERT gene expression given repeated glucocorticoid influx) likely shape the actions of HPA termination, suggesting an indirect role for stress exposure.

Risk versus Resilience

This study has perpetuated a basic idea in studies of phenotypic variations in disease processes – *one gene is "good," and the other is "bad."* This notion of risk, and its complement resilience, is related to broadly-defined cortisol dysregulation in the current study, where risk is either elevated or attenuated cortisol release. Experientially, elevated short term cortisol release is the "ideal" response, inhibiting key physiological functions while the individual copes with the stressor. Yet, elevated reactivity to laboratory-controlled psychosocial stress is considered a maladaptive outcome in some studies, so the construct is labelled "risky" by virtue of being stress-reactive and associated with cardiovascular diseases. Attenuated reactivity is also considered a maladaptive response, since it does not allow for effective mobilization of resources, and leaves the individual vulnerable to autoimmune diseases.

Concepts like risk and resilience should be defined relative to the context of the individual and a specific disease process. This study has addressed this issue by enumerating as much of the individual's context (biologically and environmentally) as is practical in longitudinal social science research. In doing so, this study has demonstrated that genetic risk may contribute to very different functional outcomes depending on other factors (e.g., participant sex, stress exposure). In turn, these outcomes will only lead to further risk for or resilience from disease given individuals' biological and environmental predispositions. Future studies of risk and resilience for psychological or physiological disorders must consider a wide variety of contextual information. Otherwise, mathematical models of disease processes will lack face validity, and our understanding of pathology will be diminished.

Strengths

This study considers the interactive effects of genes, adversity, and sex, with regard to other independent effects on cortisol reactivity. Although this method tests the robustness of multiple regression, it also allows for consideration of how effects may be shaped or negated by other effects. Several single-factor findings were significant, only to be called non-significant in multi-gene analyses. This suggests that future examinations of stress reactivity should consider sex, life stress, and other key genes when examining the effects of a given gene or stressor. Overall, one must question the utility of single-gene studies, which are advantageous in terms of hypothesis testing, but disadvantageous in terms of the applicability of results. The present study is not powerful enough to show

very weak main effects, but it also has the predictive resolution necessary to examine GxE effects carefully and in great detail.

The study verifies many previously reported global effects on cortisol reactivity (e.g., sexual abuse is associated with attenuation of HPA activity; 5-HTTLPR is associated with slight elevation), and embellishes on some inconsistent findings (e.g., CRHR1 interacts with sex and physical abuse). Novel GxE-by-Sex interactions robustly elucidate a system-level model of cortisol reactivity, shaped over time by genetically-moderated developmental experiences. These effects are reported on a racially-homogenous cohort of young adults, controlling by design for variations in germ-line and developmental status that may alter cortisol secretion. The study utilized salivary cortisol samples from 373 individuals, making it one of the largest TSST samples collected.

Although measures of genetic polymorphisms and salivary cortisol are consistent across most studies, measures of life stress utilized in other studies are of varying quality, to the detriment of the field in general (Monroe and Reid, 2008). The present study utilized the gold-standards in life stress assessment – the LEDS and CECA – that provide more accurate information in greater detail than any self-report measure of life stress. GxE interactions reported in this study should be considered more reliable measures of effects, since they are based on these high-resolution stress measurement interviews.

Limitations

The attention-to-detail required for a study like this also contributes to a number of limitations. The sample size in the current study is large compared to other studies of cortisol reactivity, but small compared to other studies assessing the effects of genes on

pathology. Oftentimes, these studies aggregate results from a variety of samples or examine the entire genome, and measure the general contributions of genes to variations in pathology. Yet, adding sample size does not necessarily lead to more precise findings. Indeed, the myopia that affects smaller studies of GxE effects (where objects examined are clear, but decontextualized) is not corrected in large studies, which blur effects of genes by poorly aggregating outcome measures.

The method employed by this study was to choose genes that "tag" a particular neuroendocrine system associated with HPA activity. This limited examination of eight genes – chosen for their predictive power, and for the sake of efficiency – means that gene-specific knowledge gained from this study is limited. Further, genes that were examined were reduced to dichotomous variables. This prevented analysis of dose-response relationships for genes, where more of a "risk-associated" or "protective" allele is associated with greater or lesser variation. Studies that examine heterozygosity are highly inconsistent, suggesting that establishment of the absolute "risk" or "protection" conferred by a gene should be a priority in research.

Many of the GxE effects reported in the current study are novel, and must be verified on additional samples. Specifically, the significant amount of variability accounted for by GxE effects involving ANKK1 is suspect, and these effects must be replicated. Yet, other findings show a consistent pattern and fit with other known GxE observations. Further, this sample is primarily representative of young adults who are of European-American origin, so results will need to be replicated in individuals from other racial groups. While the relative homogeny of this sample allowed for easier examination of genetic effects, GxE interactions describing a subset of humans are of limited utility.

With the exception of the inclusion of diagnostic covariates in some preliminary analyses, this study ignored an individual's history of psycho- or physiopathology in regression models of cortisol reactivity. Many forms of pathology are associated with variations in reactivity – depression, anxiety, and some forms of substance abuse are associated with elevated cortisol release; PTSD and other forms of substance abuse are associated with attenuated cortisol release. This study presumed that these pathologies are manifestations of the underlying processes being assessed. Thus, it is unclear whether the development of pathology contributes to dysregulation, or vice versa. The processes underlying dysregulation and disease are likely interactive, and would need to be assessed as they develop. Future studies should examine the developmental progression of cortisol reactivity given differential exposure to stress and genetic risk, and also examine the parallel progression of associated diseases to establish causality.

Implications for Prevention and Treatment

This study has wide implications on future efforts to prevent and treat a variety of psychological and physiological diseases. Cortisol reactivity is an endophenotype of those diseases, and may represent a useful biomarker for assessing disease risk, or projecting or tracking therapeutic change. For example, multiple SNPs in CRHR1 are associated with irritable bowel syndrome (IBS) symptom subtypes (e.g., Sato et al., 2012), and treatments are often focused on those symptom subtypes. IBS is a chronic functional gastrointestinal disorder characterized by bowel inflammation; strong associations with depression and anxiety suggest that neuroendocrine dysregulation may underlie both disorders. If certain SNPs modulate HPA functioning through exposure to

psychosocial stress, and responses to those stressors vary by sex, then both prevalence of IBS subtypes and their treatments could be related to GxE-by-Sex processes reported herein. For example, abuse history may predict poor IBS treatment outcomes in female carriers of CRHR1 risk alleles.

Cortisol reactivity may, in itself, represent a risk factor for poor outcomes of treatment efforts. Variations in HPA functioning are likely associated with altered functioning of drugs used to treat a variety of disorders (e.g., selective serotonin reuptake inhibitors), but HPA activity also affects responses to psychosocial interventions. In one study, dropping out of a residential treatment program for SUDs was associated with higher salivary cortisol responses to computer-administered psychological stress tasks (Daughters, Richards, Gorka, and Sinha, 2009). In another study, attenuated cortisol release was associated with alcohol administration to heavy drinkers, but elevated reactivity in light drinkers (King, Munisamy, de Wit, and Lin, 2006), suggesting those demonstrating attenuated reactivity may be more difficult to treat. Given that cortisol reactivity to psychosocial stress is affected by sex-moderated GxE effects, future examinations of the efficacy of these and other psychosocial treatments should include assessments of patients' HPA functioning.

Future Directions

Future studies on cortisol reactivity should account for well-established effects when validating novel GxE effects. Like any study, a larger sample size could be used to identify global and interactive risk factors for hypo- or hyperreactivity more reliably, and to identify more nuanced patterns of functioning associated with genetic vulnerability. In

addition to examining biallelic variations, future studies should also examine haplotypes – common combinations of genetic polymorphisms that may be associated with negative outcomes or risk endophenotypes.

Also, the associations between genes and environmental risk factors on reactivity should be examined relative to diurnal variation. This construct of cortisol functioning may be associated with different disease processes than reactivity – it may represent an endophenotype of a subset of disorders, or may represent a useful indicator for measuring symptomatic change during an intervention. Also, the connections between diurnal variation and reactivity need to be established more clearly.

Conclusion

Variations in cortisol reactivity are associated with multiple independent and interactive factors, including sex, genetic polymorphisms, and exposure to early or recent adversity. The impact of ongoing stress exposure is clear when examining changes within a person, but obfuscated when examining changes across people. Thus, cortisol reactivity and associated diseases processes should be evaluated with regard to the developmental and neurobiological idiosyncrasies of the individual. Our understanding of the transactional processes that underlie diseases would benefit from this contextualized approach to clinical research.

APPENDIX

FIGURES AND TABLES

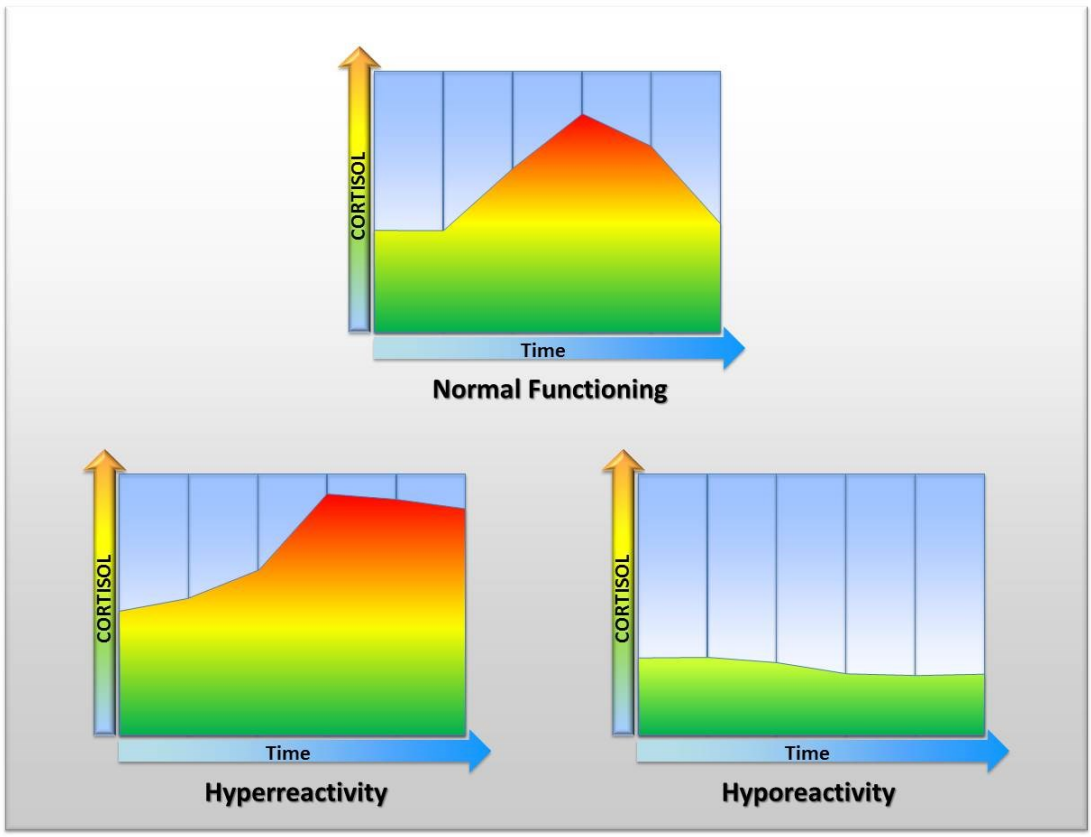


Figure 1. Typical cortisol functioning, and two forms of dysregulation caused by prolonged stress exposure (i.e., allostatic load). Hyperreactivity is characterized by elevated cortisol levels throughout exposure to stressors. Hyporeactivity is characterized by a blunted response to stress exposure.

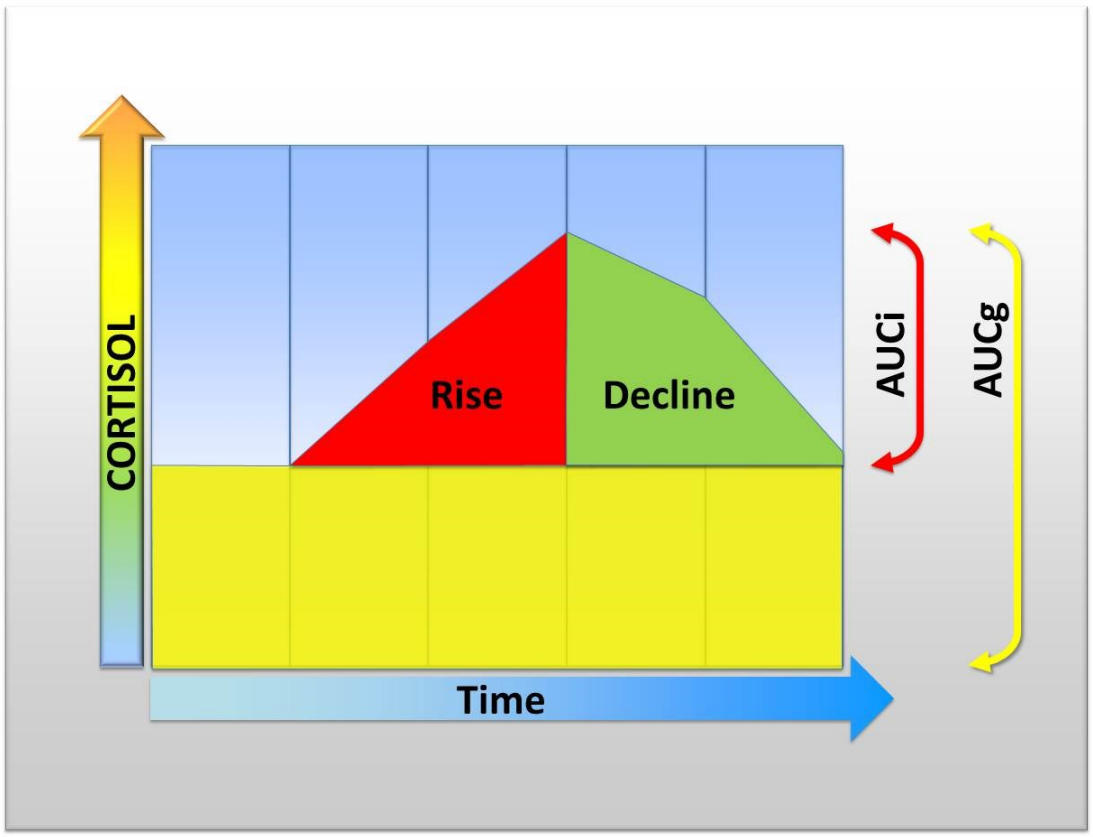


Figure 2. AUC components of cortisol reactivity. AUCg measures total cortisol release. AUCi measures cortisol released during task, relative to baseline (i.e., reactivity). Rise measures the activation of HPA activity. Decline measures the termination of HPA activity.

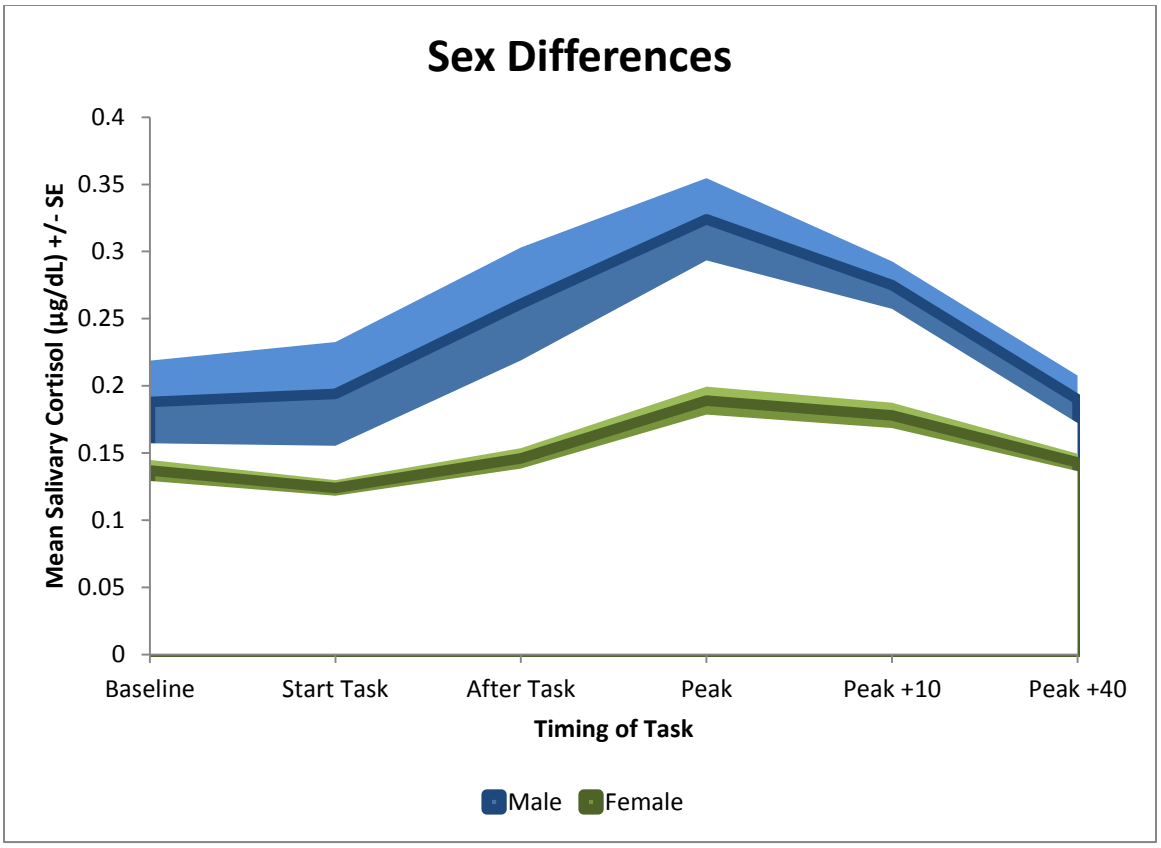
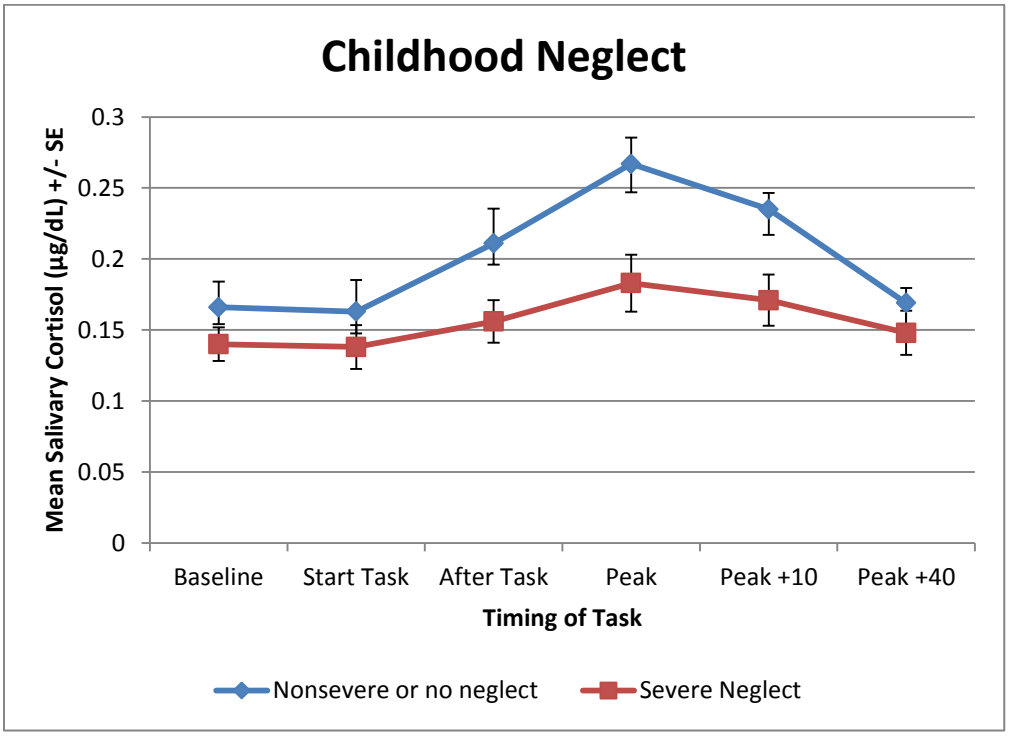


Figure 3. Mean cortisol concentrations over time by sex.

4a. Neglect



4b. Sexual Abuse

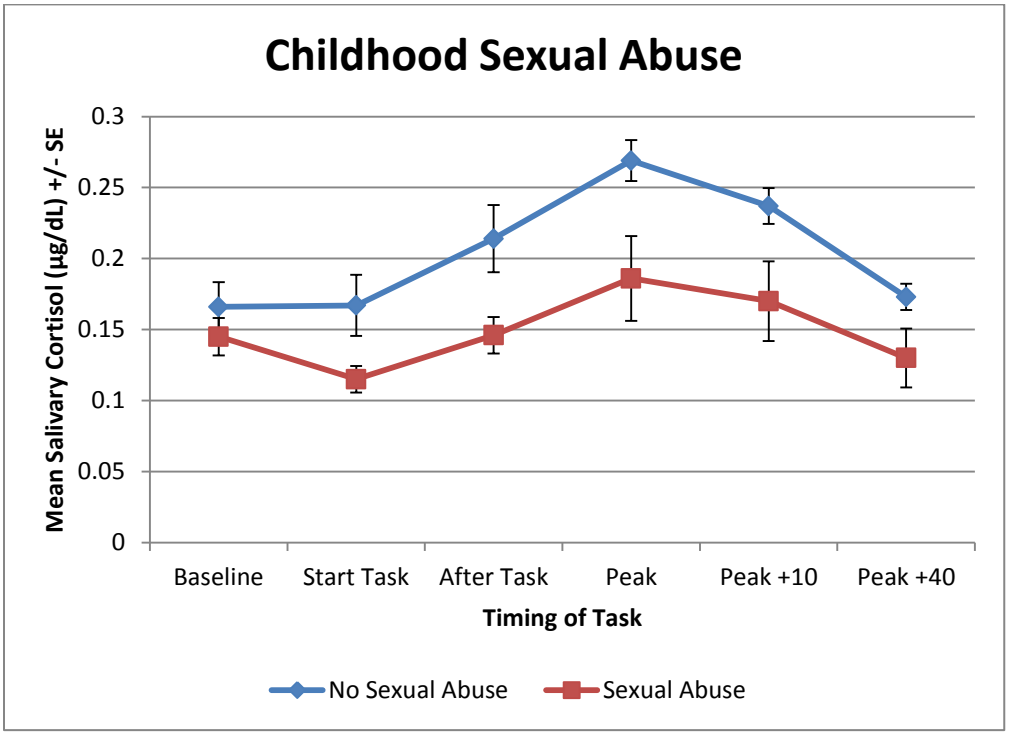
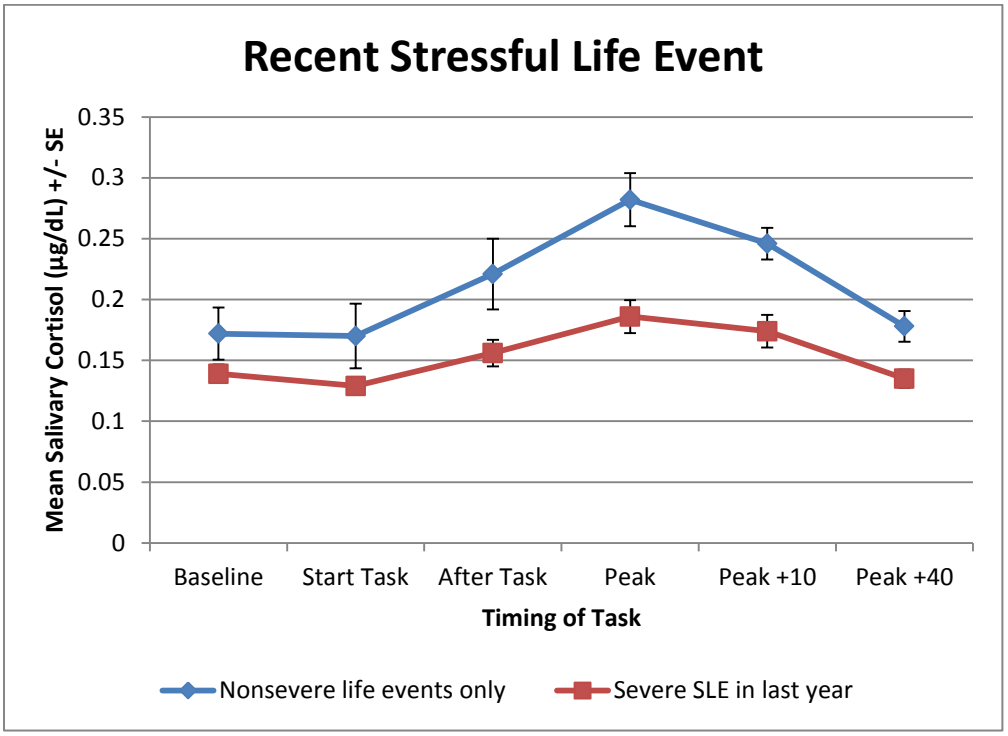


Figure 4. Mean cortisol concentrations over time by exposure to childhood abuse.

5a. SLE during last year.



5b. High threat difficulty during last year.

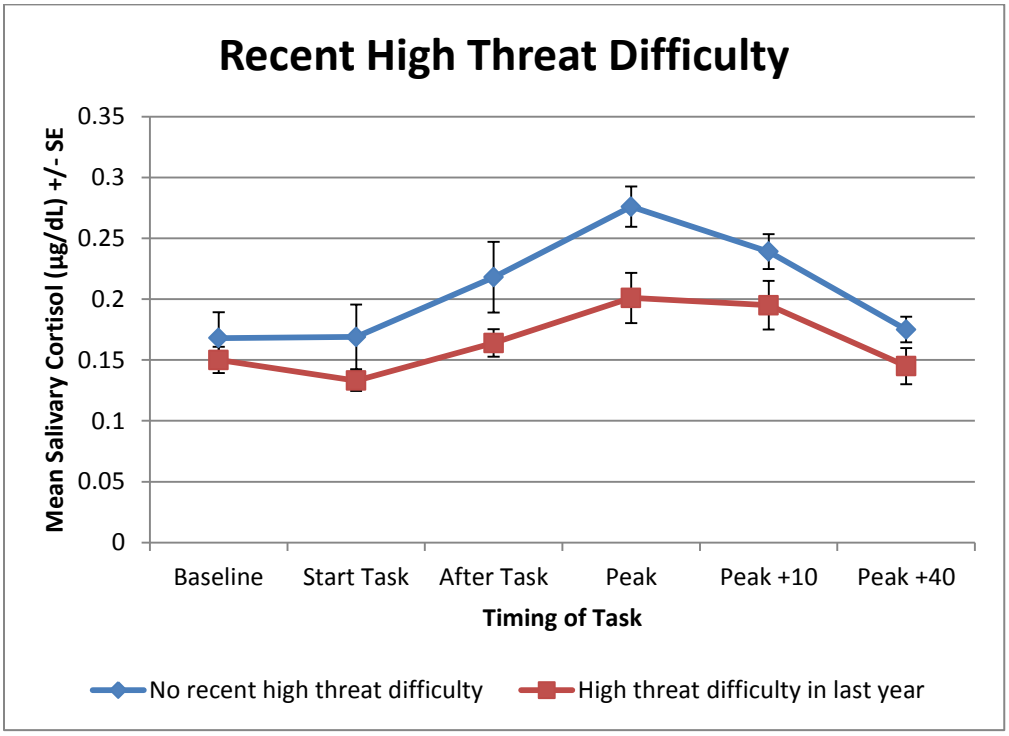
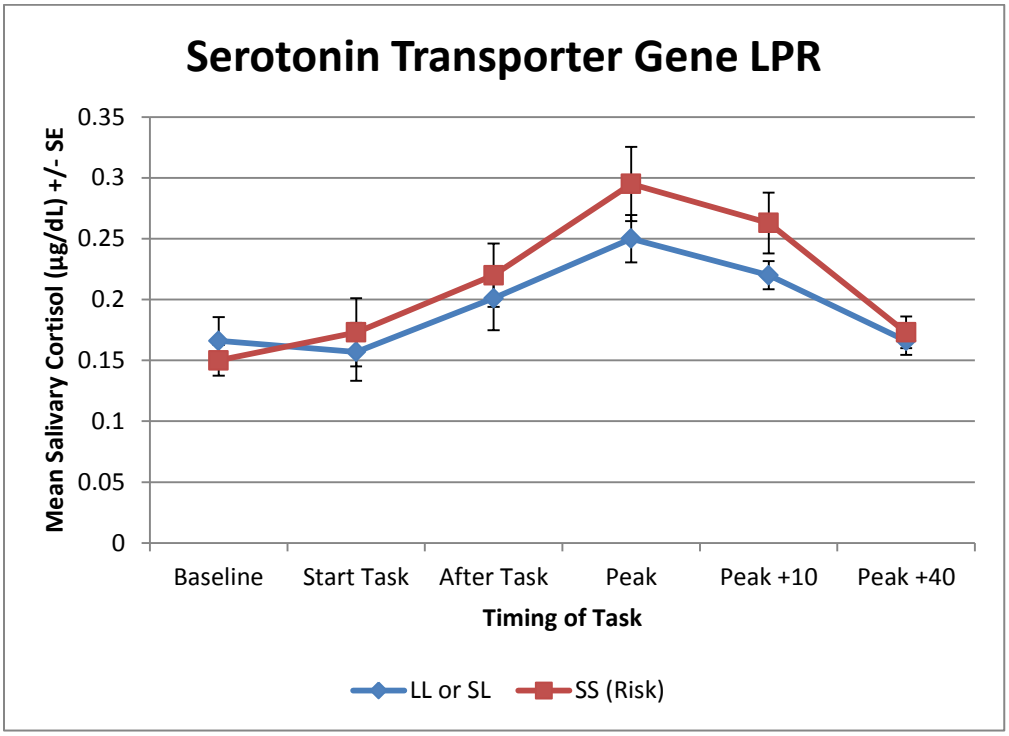


Figure 5. Mean cortisol concentrations over time by exposure to recent adversity.

6a. 5HTTLPR (VNTR).



6b. SERT rs25532.

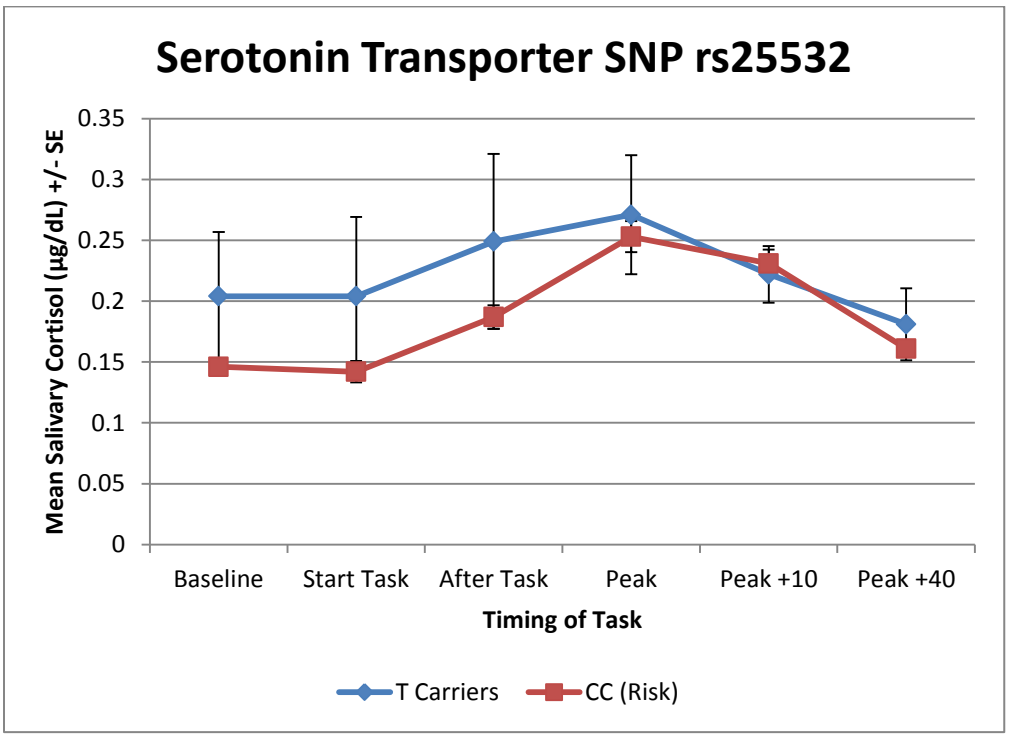


Figure 6. Mean cortisol concentrations over time by serotonin transporter genotypes.

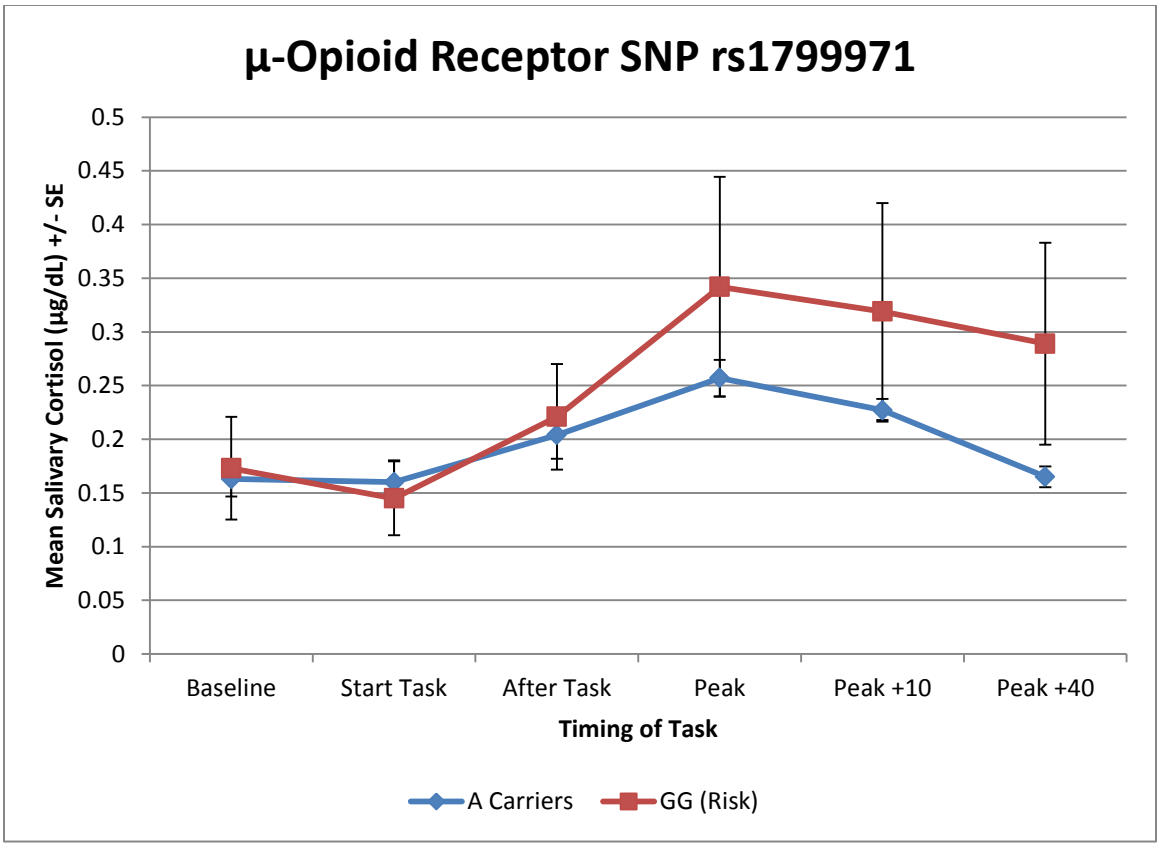
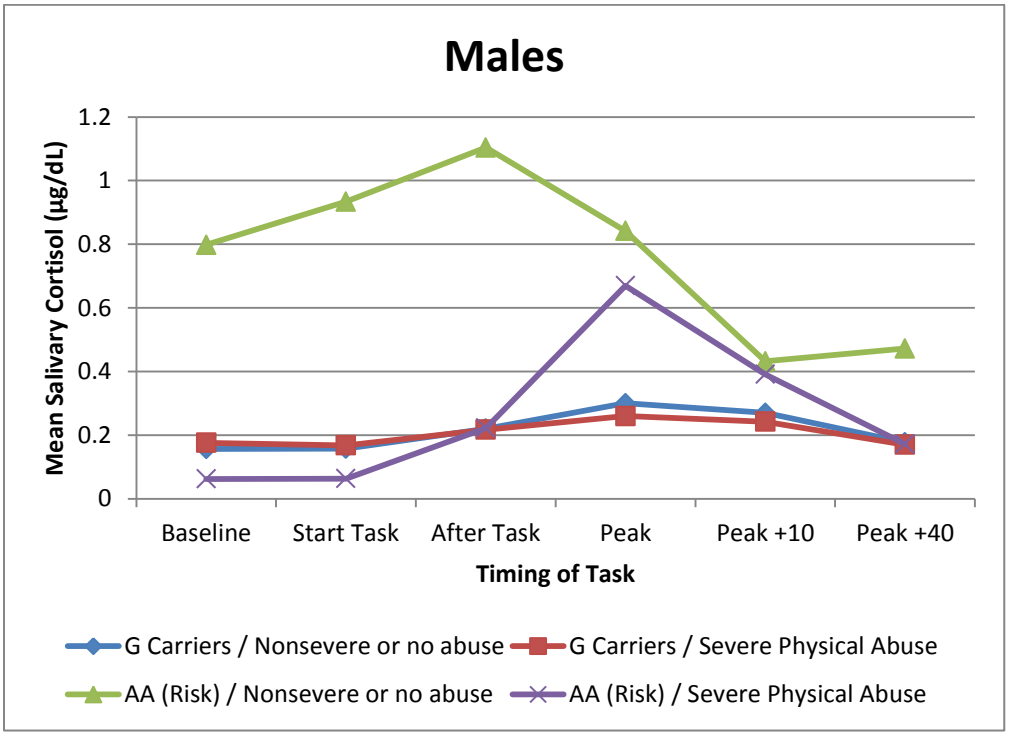


Figure 7. Mean cortisol concentrations over time by OPRM1 genotype.

8a. Males.



8b. Females.

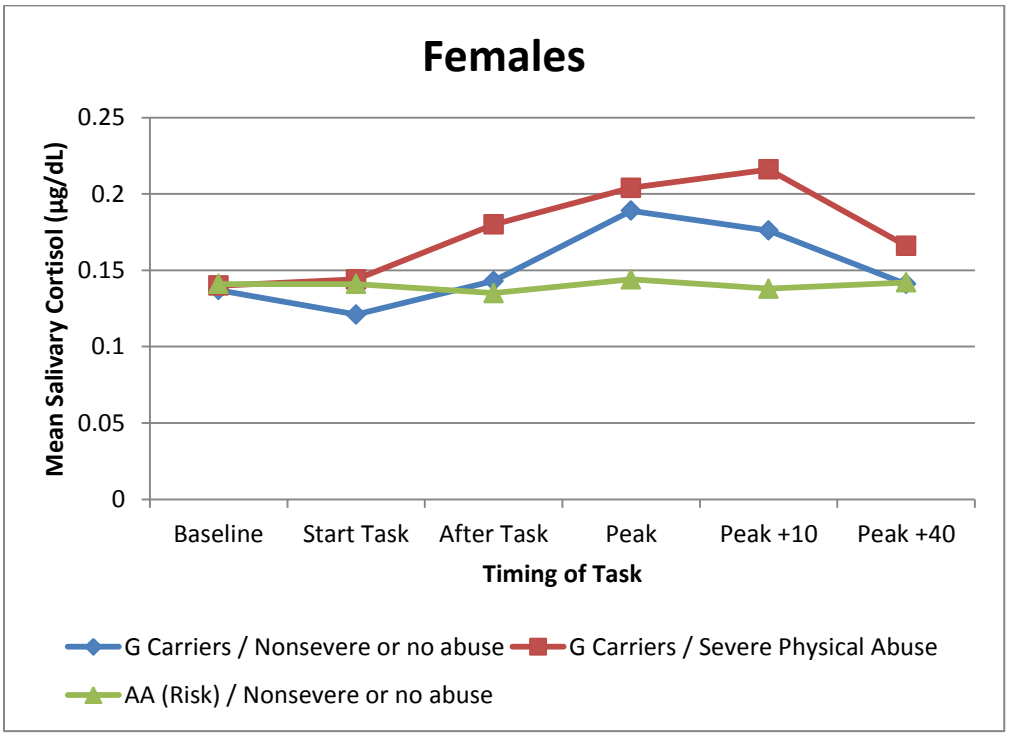
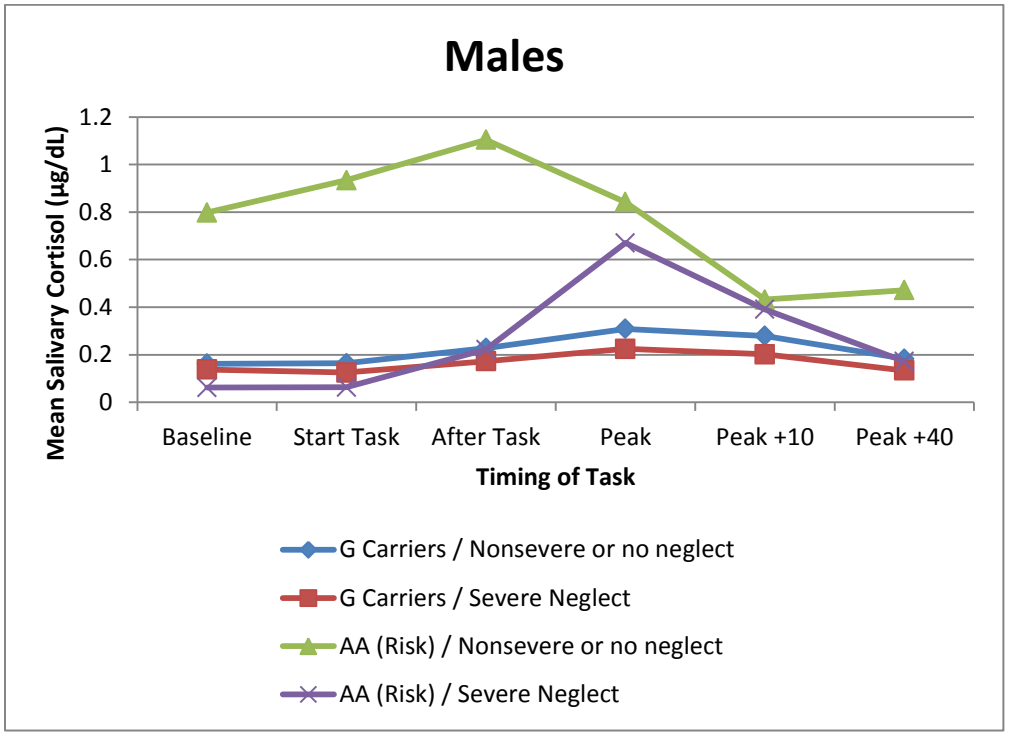


Figure 8. Mean cortisol concentrations over time by ANKK1 genotype and physical abuse history.

9a. Males.



9b. Females.

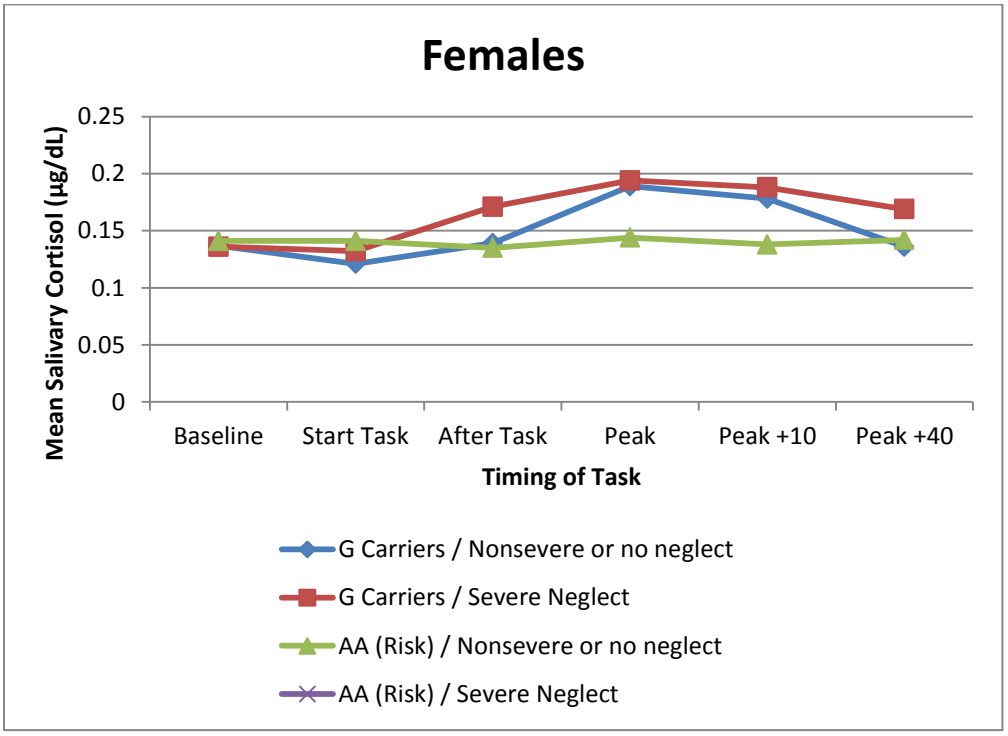
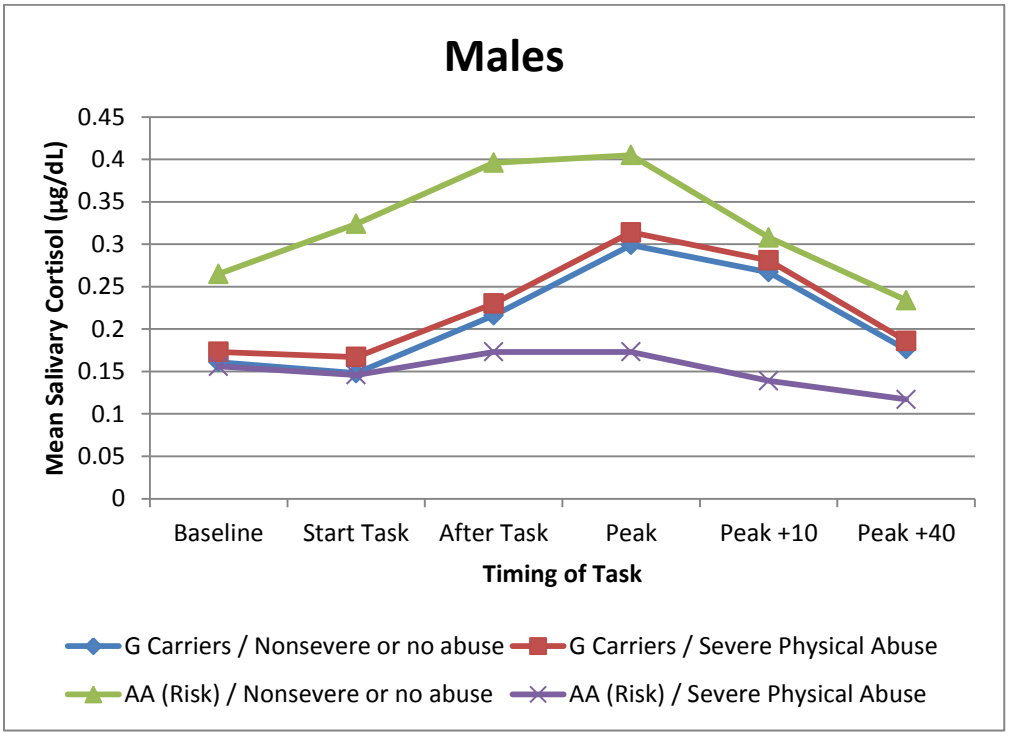


Figure 9. Mean cortisol concentrations over time by ANKK1 genotype and neglect history.

10a. Males.



10b. Females.

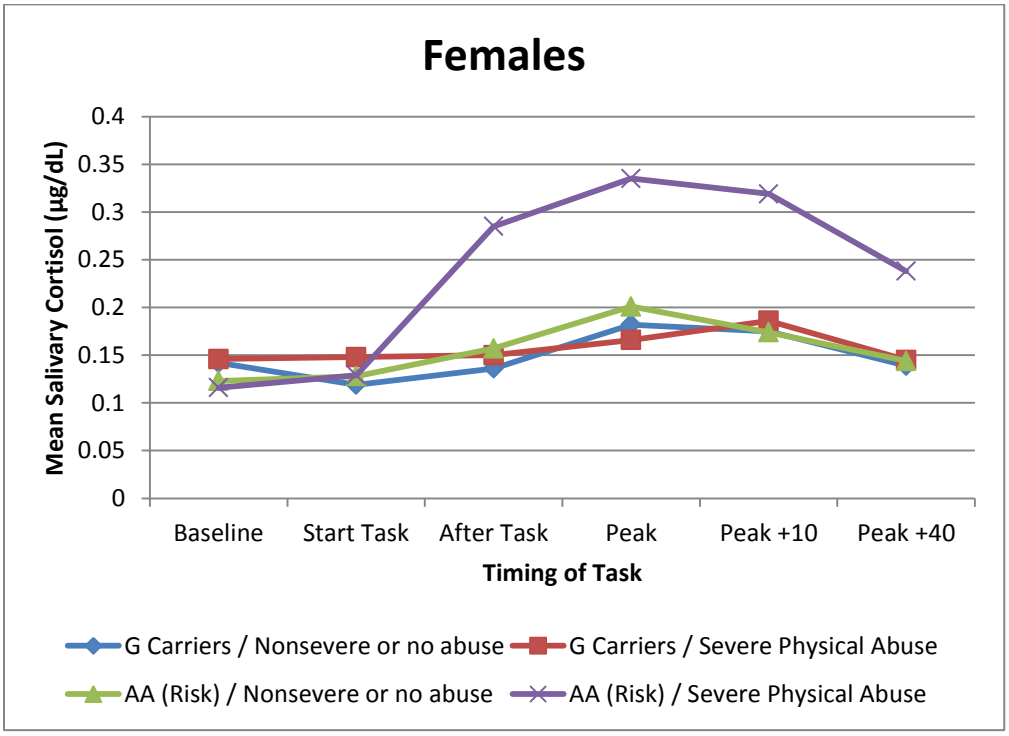


Figure 10. Mean cortisol concentrations over time by COMT genotype and physical abuse history.

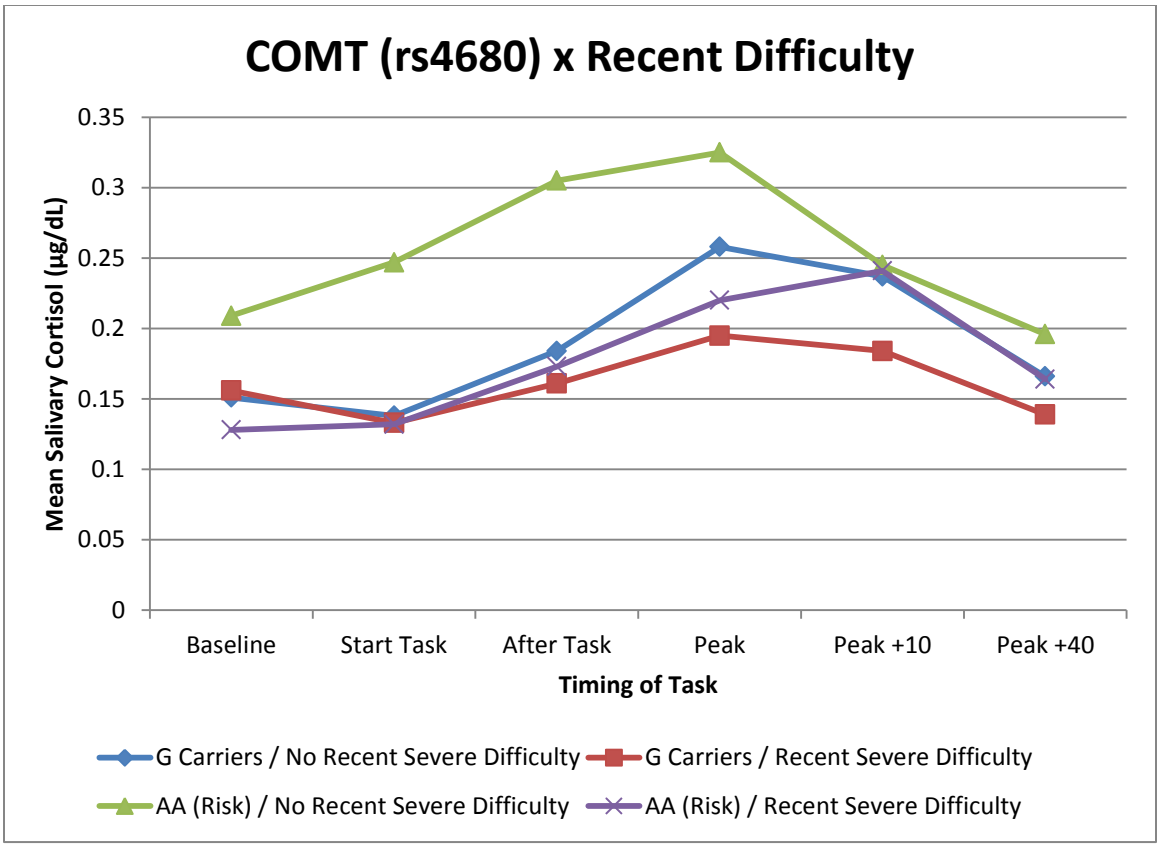
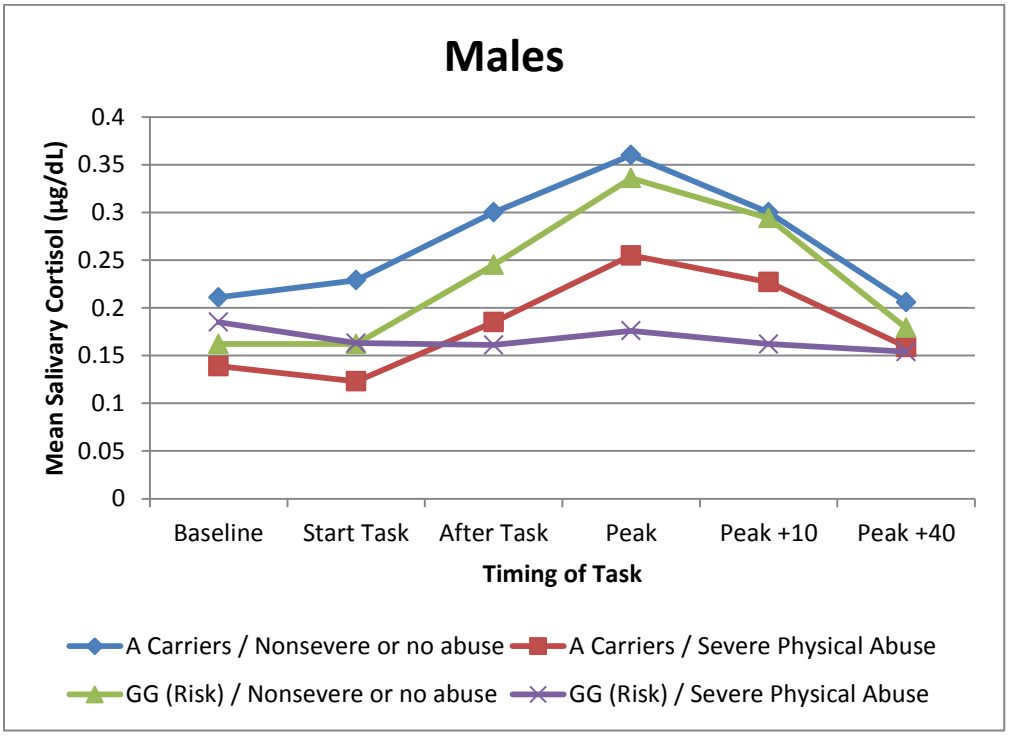


Figure 11. Mean cortisol concentrations over time by COMT genotype and recent high threat difficulty.

12a. Males.



12b. Females.

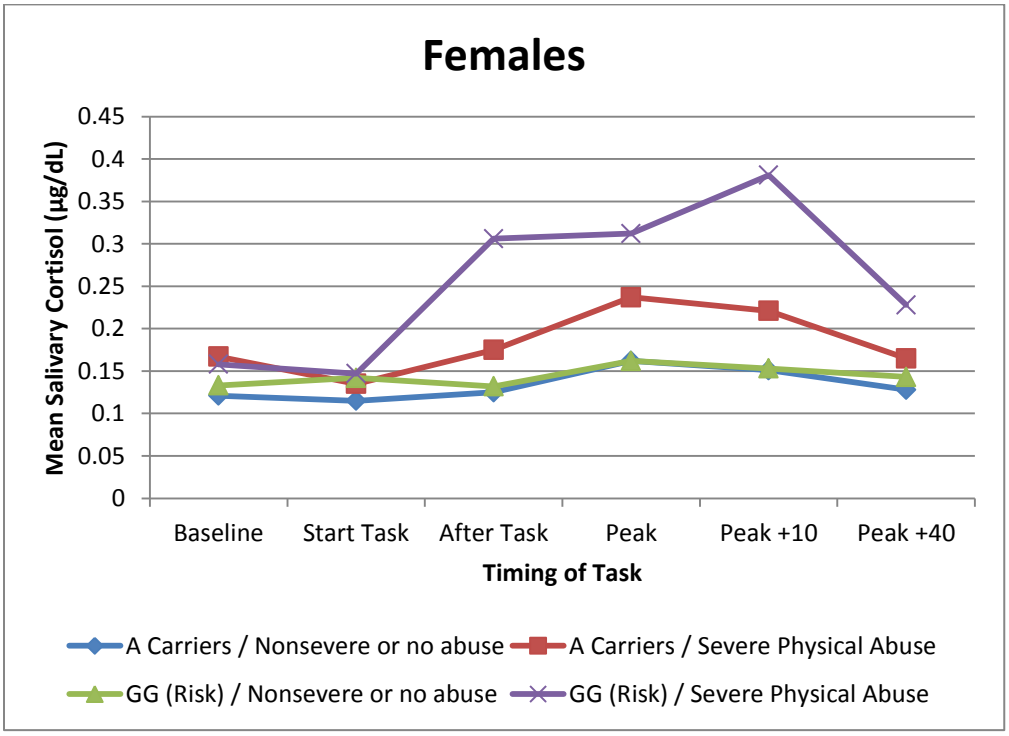


Figure 12. Mean cortisol concentrations over time by CRHR1 genotype and physical abuse history.

Table 1. Associations between Measures of Adversity and Cortisol Reactivity.

Variable	1	2	3	4	5	6	7	8	9	10
Early Adversity										
1. Antipathy	**--**									
2. Neglect	.54***	--								
3. Psychological Abuse	.42***	.45***	--							
4. Physical Abuse	.36***	.29***	.38***	--						
5. Sexual Abuse	.26***	.24***	.23***	.20***	--					
Recent Adversity										
6. Severe SLE in last year	-.50	-.20***	-.11*	-.09	-.13*	--				
7. High Threat Difficulty	-.27***	-.27***	-.29***	-.25***	-.24***	.32***	--			
Cortisol Reactivity										
8. AUCg	.03	.07	.07	.05	.08	-.09	-.07	**--**		
9. AUCi	.03	.04	.05	-.05	.12*	-.09	-.10	-.15**	**--**	
10. Rise	.07	.12*	.08	.02	.14**	-.18***	-.11*	.62***	.51***	**--**
11. Decline	.03	.10	.07	.01	.04	-.10	-.03	.81***	-.14**	.57***

Notes: Associations between continuous variables (CECA, Cortisol) are Pearson's *r*; associations between these variables and Recent Adversity measures (dichotomous) are Spearman's *r*; association between Recent Adversity variables is a phi-coefficient.
**p<.05 **p<.01 ***p<.001*

Table 2. Cortisol Reactivity Measures and Adversity Summary Scores by Sex.

Cortisol Reactivity Mean (SD)	**Males (n = 189)**	**Females (n = 184)**
AUCg	19.16 (28.78)**	12.25 (7.11)**
AUCi	4.12 (10.48)**	1.33 (8.32)**
Rise	4.06 (4.73)***	2.05 (2.85)***
Decline	3.86 (5.42)***	2.00 (3.14)***
CECA Domain Mean (SD)		
Antipathy	4.43 (1.08)	4.33 (1.20)
Neglect	4.47 (1.10)	4.34 (1.18)
Psychological Abuse	4.62 (0.88)	4.60 (0.90)
Physical Abuse	3.92 (1.00)	4.07 (1.05)
Sexual Abuse	4.82 (0.68)***	4.47 (1.15)***
LEDS Domain *n* (%) Yes during last year		
Severe Stressful Life Event	43 (22.8%)	50 (27.2%)
High Threat Difficulty	44 (23.3%)	51 (27.7%)

Notes: Lower CECA domain scores indicate more severe levels of abuse.
Groups were compared using Student's *t*-test.
***p<.01 ***p<.001*

Table 3. Distributions of Sample Participants by Genotype and Sex.

Genetic Polymorphism (SNP or VNTR) (N = 373)	**Sex:**	**Males (n = 189)**	**Females (n = 184)**
5-HTTLPR (VNTR)	**Homozygous Risk**	39 (10.5%)	29 (7.8%)
	Protective	147 (39.7%)	155 (41.9%)
ANKK1 (rs1800497)	**Homozygous Risk**	10 (2.7%)	6 (1.6%)
	Protective	178 (47.8%)	178 (47.8%)
COMT (rs4680)	**Homozygous Risk**	52 (14.0%)	50 (13.5%)
	Protective	136 (36.7%)	133 (35.8%)
CRHR1 (rs110402)	**Homozygous Risk**	56 (15.1%)	61 (16.4%)
	Protective	132 (35.6%)	122 (32.9%)
CRHR1 (rs1876831)	**Homozygous Risk**	115 (31.0%)	121 (32.6%)
	Protective	73 (19.7%)	62 (16.7%)
NR3C2 (rs5522)	**Homozygous Risk**	5 (1.3%)	3 (0.8%)
	Protective	183 (49.3%)	180 (48.5%)
OPRM1 (rs1799971)	**Homozygous Risk**	3 (0.8%)	2 (0.5%)
	Protective	185 (49.9%)	181 (48.8%)
SERT (rs25532)	**Homozygous Risk**	121 (33.0%)*	138 (37.6%)*
	Protective	65 (17.7%)*	43 (11.7%)*

Notes: "Homozygous Risk" refers to individuals who are homozygous carriers of alleles associated with extreme variations in CR. "Protective" refers to individuals who are carriers of at least one copy of an allele not associated with abnormal CR (often a major allele). Totals vary by gene due to missing data. Groups were compared using χ^2 tests.
**p<.05*

Table 4. Prediction of Cortisol Reactivity from Sex, Adversity, Polymorphisms, and Their Interactions.

Cortisol Reactivity:	**AUCg**			**AUCi**			**Rise**			**Decline**		
Predictors:	B	SE	*t*	B	SE	*t*	B	SE	*t*	B	SE	*t*
Sex	5.14	1.55	3.32***	3.76	1.19	3.18**	2.69	0.48	5.62***	1.42	0.46	3.13**
Early Adversity:												
Neglect	--	--	--	--	--	--	0.39	0.22	1.80†	0.18	0.32	0.55
Physical Abuse	1.31	1.06	1.23	-0.60	0.82	-0.73	0.14	0.34	0.41	--	--	--
Sexual Abuse	--	--	--	0.93	.50	1.85†	--	--	--	--	--	--
Recent Adversity:												
SLE during last year	-2.50	1.52	-1.64†	--	--	--	-1.49	0.47	-3.16**	--	--	--
High Threat Difficulty in last year	--	--	--	-2.74	1.31	-2.08*	--	--	--	0.65	0.61	1.06
Polymorphisms:												
5HTTLPR (VNTR)	--	--	--	3.48	1.30	3.14*	--	--	--	--	--	--
ANKK1 (rs1800497)	-2.90	8.87	-0.33	-1.42	3.84	-0.37	--	--	--	-0.77	1.92	-0.40
COMT (rs4680)	2.94	2.25	1.31	0.61	1.23	0.05	--	--	--	1.09	0.58	1.89†
CRHR1 (rs110402)	--	--	--	1.14	1.42	0.80	1.68	0.60	2.79**	--	--	--
OPRM1 (rs1799971)	--	--	--	--	--	--	3.90	1.92	2.04*	--	--	--
SERT (rs25532)	--	--	--	3.52	1.27	2.78**	--	--	--	--	--	--

Table 4. (Continued)

Cortisol Reactivity:	**AUCg**			**AUCi**			**Rise**			**Decline**		
Interactions:	B	SE	*t*	B	SE	*t*	B	SE	*t*	B	SE	*t*
Neglect x Sex	--	--	--	--	--	--	--	--	--	0.35	0.46	0.77
Physical Abuse x Sex	-1.19	1.56	-0.77	-0.42	1.17	-0.36	-0.11	0.49	-0.22	--	--	--
ANKK1 x Sex	1.59	9.97	0.16	-7.48	4.92	-1.52	--	--	--	6.07	2.40	2.53**
COMT x Sex	-2.67	3.07	-0.87	--	--	--	--	--	--	--	--	--
CRHR1 x Sex	--	--	--	-1.99	2.05	-.97	-2.41	0.87	-2.78**	--	--	--
ANKK1 x Neglect	--	--	--	--	--	--	--	--	--	-1.98	2.74	-0.72
ANKK1 x Physical Abuse	-64.64	7.52	-8.59***	1.95	4.29	0.45	--	--	--	--	--	--
COMT x Physical Abuse	-6.52	2.20	-2.97**	--	--	--	--	--	--	--	--	--
CRHR1 x Physical Abuse	--	--	--	-2.47	1.43	-1.72*	-1.32	0.61	-2.18*	--	--	--
COMT x Recent Difficulty	--	--	--	4.13	2.50	1.65†	--	--	--	-2.24	1.18	-1.90*
ANKK1 x COMT	11.17	11.14	1.00	--	--	--	--	--	--			

Table 4. (Continued)

Cortisol Reactivity:	**AUCg**			**AUCi**			**Rise**			**Decline**		
Interactions:	B	SE	*t*	B	SE	*t*	B	SE	*t*	B	SE	*t*
ANKK1 x Neglect x Sex	--	--	--	--	--	--	--	--	--	8.51	3.39	2.51**
ANKK1 x Physical Abuse x Sex	78.48	7.94	9.88***	-15.90	5.46	-2.91**	--	--	--	--	--	--
COMT x Physical Abuse x Sex	8.39	3.03	2.77**	--		--	--	--	--	--	--	--
CRHR1 x Physical Abuse x Sex	--	--	--	5.45	2.07	2.63**	1.99	0.87	2.30*	--	--	--
ANKK1 x COMT x Sex	166.38	15.03	11.07***									
ANKK1 x COMT x Physical Abuse	115.57	7.96	14.52***									

Notes: Males are coded as 0; homozygous carriers of risk alleles are coded as 0. Coefficients are missing for variables not used in final regression equation to predict a cortisol variable.
*† p<.10 *p<.05 **p<.01 ***p<.001*

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