Genes Moderate the Association of Trait Diurnal Cortisol and Externalizing In Boys

by

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ABSTRACT

The hypothalamus pituitary adrenal (HPA) axis and the human genome are important components of the biological etiology of externalizing disorders. By studying the associations between specific genetic variants, diurnal cortisol, and externalizing symptoms we can begin to unpack this complex etiology. It was hypothesized that genetic variants from the corticotropine releasing hormone receptor 1 (CRHR1), FK506 binding protein 51 (FKBP5), catechol-O-methyl transferase (COMT), and dopamine transporter (DAT1) genes and diurnal cortisol intercepts and slopes would separately predict externalizing symptoms. It was also hypothesized that genetic variants would moderate the association between cortisol and externalizing. Participants were 800 twins (51% boys), 88.5% Caucasian, M=7.93 years (SD=0.87) participating in the Wisconsin Twin Project. Hierarchical Linear Modeling (HLM) was used to separate the variance associated with state and trait cortisol measured across three consecutive days and trait cortisol measures were used. There were no main effects of genes on externalizing symptoms. The evening cortisol intercept, the morning cortisol slope and the evening cortisol slope predicted externalizing, but only in boys, such that boys with higher cortisol and flatter slopes across the day also had more externalizing symptoms. The morning cortisol intercept and CRHR1 rs242924 interacted to predict externalizing in both boys and girls, with GG carriers significantly higher compared to TT carriers at one standard deviation below the mean of morning cortisol. For boys only there was a significant interaction between the DAT1 variable number tandem repeat (VNTR) and the afternoon

slope and a significant slope for 9/9 carriers and 9/10 carriers such that when the slope was more steep, boys carrying a nine had fewer externalizing symptoms but when the slope was less steep, they had more. Results confirm a link between diurnal trait cortisol and externalizing in boys, as well as moderation of that association by genetic polymorphisms. This is the first study to empirically examine this association and should encourage further research on the biological etiology of externalizing disorder symptoms.

DEDICATION

To My Mother, Deborah Swann, and My Father, Dennis Swann, Without You,

None of This Would Have Been Possible

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TABLE OF CONTENTS

Page

LIST OF TABLES vi

LIST OF FIGURES vii

CHAPTER

INTRODUCTION 1

The HPA-axis and Diurnal Cortisol as Biological Underpinnings of Externalizing Symptoms 3

Genetic Influences on the HPA-axis and Externalizing Symptoms Association 5

Reactive Aggression vs. Abnormal Aggression 6

Stress-reactive Genes 7

CRHR1 7

FKBP5 9

Catecholamine Genes 11

COMT 11

DAT1 13

The Proposed Study 14

Method 15

Participants 15

Measures 16

Procedures 18

Results 19

Page

CHAPTER

Preliminary Analyses 19

Hypothesis 1 21

Hypothesis 2 22

DISCUSSION 24

Placing the Findings in the Context of the Literature 25

Implications 29

Limitations 33

Future Directions 35

Conclusions 37

REFERENCES 38

LIST OF TABLES

Table		Page
1.	Genotype Frequencies for Candidate Genes	47
2.	Descriptive Statistics	48
3.	Zero-Order Correlations	49
4.	Main Effect of Genes on Externalizing Symptoms	50
5.	Diurnal Cortisol Measures on Externalizing Symptoms	51
6.	Interactions with CRHR1 and Cortisol Measures on Externalizing Symptoms	52
7.	Interactions with FKBP5 and Cortisol Measures on Externalizing Symptoms	53
8.	Interactions with COMT and Cortisol Measures on Externalizing Symptoms	54
9.	Interaction with DAT1 and the Morning Cortisol Intercept on Externalizing Symptoms	55
10.	Interaction with DAT1 and the Afternoon Cortisol Slope on Externalizing Symptoms	56
11.	Interactions with DAT1 and Cortisol Measures on Externalizing Symptoms	57

LIST OF FIGURES

Figure		Page
1.	Evening Cortisol Intercept and Sex on Externalizing Symptoms	58
2.	Morning Cortisol Slope and Sex on Externalizing Symptoms	59
3.	Afternoon Cortisol Slope and Sex on Externalizing Symptoms	60
4.	CRHR1 and Morning Cortisol Intercept on Externalizing Symptoms	61
5.	DAT1-VNTR and Morning Cortisol Intercept on Externalizing Symptoms in Boys	62
6.	DAT1-VNTR and Afternoon Cortisol Slope on Externalizing Symptoms in Boys	63

Genes Moderate the Association of Trait Diurnal Cortisol and Externalizing Symptoms in Boys

Conduct disorder (CD) and oppositional defiance disorder (ODD) are relatively common childhood externalizing disorders characterized by aggressive and delinquent behaviors (American Psychiatric Association, 2000). Early externalizing symptoms are associated with later development of externalizing disorders and a lifetime of delinquent behavior (Moffitt, 1993). Behavioral differences for children who go on to develop externalizing disorders have been observed as early as the infancy period (Olson et al., 1999), but middle childhood is a critical time when the negative consequences of these disorders appear to be both concurrent and far-reaching. For elementary school-aged children, externalizing symptoms predict poorer self-esteem (Zhou et al., 2010), and poorer relationships with parents, siblings, and peers compared to their healthy counterparts (Hymel, Rubin, Rowden, & Lemare, 1990; Richmond & Stocker, 2006). Externalizing symptoms in children at age eight predict delinquency and substance abuse in adolescence and adulthood (Fergusson, Horwood, & Ridder, 2005; Fergusson & Lynskey, 1998).

Approximately two to three percent of children in middle childhood have a diagnosis of CD or ODD across a three month window (Costello et al., 2003). Between the ages of nine and 16, cumulative prevalence rates are nine percent for CD (3.8% for girls and 14.1% for boys) and 11 percent for ODD (9.1% for girls and 13.4% for boys), making externalizing disorders a common health concern. In order to prevent the manifestation of symptoms that may lead to externalizing

disorders and address the risk of negative outcomes associated with externalizing disorders, it is crucial to understand biological and psychological mechanisms that underlie their development.

The hypothalamic-pituitary-adrenal (HPA) axis, a key component of the human neuroendocrine system that regulates the biological reaction to stress, has shown potential as a mechanism underlying externalizing behaviors via its regulation of the stress hormone cortisol (Alink et al., 2008; Tuttle et al., 2011). The physiological pathways by which cortisol acts to influence externalizing behaviors are not fully understood, but evidence is accumulating in support of a neurobiological model in which the HPA axis plays a crucial role in the development of externalizing symptoms (van Goozen, Fairchild, Snoek, & Harold, 2007). Animal research supports two different biological pathways from HPA axis functioning to externalizing symptoms. The first is a reactive aggression pathway. Stress activates the hypothalamus which activates the HPA axis, and the resulting cortisol leads to an increase in feelings of anxiety (Kruk, Halasz, Meelis, & Haller, 2004). This increase in anxious feelings in children leads to acting out in ways that would be classified as externalizing symptoms. The second pathway is what van Goozen et al (2007) call the abnormal aggression pathway. When corticosterone, an animal hormone equivalent to cortisol in humans, was controlled in rodents, such that corticosterone exposure was abnormally low, the rodents were unable to correctly interpret social cues and behaved with abnormally high levels of aggression (Haller et al., 2001). Translating these findings to the application of human research, it may be useful

to differentiate between externalizing symptoms as the result of anxiety brought on by biological reactions to high or chronic stress, and externalizing symptoms born from low levels of cortisol leading to an inability to correctly interpret environmental cues. The current study attempted to differentiate between these two etiological pathways by distinguishing between comorbid externalizers (children with symptoms of both externalizing and anxiety) and "pure" externalizers (children with externalizing without anxiety symptoms). Both higher cortisol reactivity in response to stress, and dysregulated cortisol patterns measured across a typical day (diurnal cortisol) have been associated with externalizing symptoms in children (Alink et al., 2008).

The HPA-axis and Diurnal Cortisol as Biological Underpinnings of Externalizing Symptoms

Attempts to understand the contributions of the HPA-axis to externalizing behaviors through the measurement of diurnal cortisol have been met with conflicting results. Studies have linked externalizing to both heightened (hyper) and muted (hypo) patterns of cortisol activity throughout the day (Alink et al., 2008). From a theoretical perspective, understanding these discrepant patterns is crucial to differentiating between reactive and abnormal aggression pathways.

Many factors have been explored to explain these discrepancies, including the age of participants, gender, reactive (cortisol measured after exposure to a specific stressor) vs. diurnal (cortisol measured at multiple time points across a typical day) measures of cortisol, and clinical vs. normal population samples. Meta-analysis of the literature yielded associations between diurnal cortisol, but

not reactive cortisol, and externalizing symptoms (Alink et al., 2008). Only participant age consistently moderated the association between cortisol and externalizing. Specifically, higher levels of diurnal cortisol (hypercortisolism) was associated with externalizing in preschoolers, lower levels (hypocortisolism) was associated with externalizing in middle childhood, and cortisol was not associated with externalizing in adolescents.

A more recent factor that has been explored in relation to the cortisol-externalizing association is the variance in cortisol related to "trait-like" sources and the variance related to "situation specific" sources of variance (Kirschbaum et al., 1990; Shirtcliff, Granger, Booth, & Johnson, 2005). The logic is that variance in cortisol must arise from three sources: person factors (traits), person-situation interactions (states), and measurement error, and that separating trait and state-like variance allows a more in-depth study of cortisol. State cortisol variance ranged from 52-75 percent of the total variance, and trait cortisol variance ranged from 19-46 percent, with lower trait cortisol found only in boys high in externalizing symptoms, but no reported association with state cortisol (Booth, Granger, & Shirtcliff, 2008; Shirtcliff et al., 2005).

The "trait-like" component of variance of particular interest in this study is the genetic influence on the HPA-axis. Previous work has shown heritability estimates of morning cortisol in children to be as high as 60 percent (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003; Wust, Federenko, Hellhammer, & Kirschbaum, 2000), suggesting an important genetic component to HPA axis functioning. The influence of genetics on diurnal cortisol is not the same

throughout the day. Morning cortisol has a significant additive genetic component that extends to the slope from morning to afternoon cortisol, but the slope from afternoon to evening cortisol does not have a significant genetic component (Van Hulle, Shirtcliff, Lemery-Chalfant, & Goldsmith, 2012). Morning, afternoon, and evening measures have a significant influence of the shared environment in common. Concentrating on the variance associated with trait cortisol throughout the day focuses the current study on aspects of diurnal cortisol most likely to have strong genetic influences, by testing morning cortisol, and the aspects that are more environmentally driven, by testing afternoon and evening measures, all while eliminating the variance that fluctuates from day to day.

Genetic Influences on the HPA-axis and Externalizing Symptoms Association

Genetic differences, through their impact on biological systems both integral and peripheral to HPA-axis functioning, have the potential to act as moderators of the association between diurnal trait cortisol and externalizing symptoms. Previous research has separately found both externalizing disorders (Dick, Viken, Kaprio, Pulkkinen, & Rose, 2005) and diurnal cortisol (Van Hulle et al., 2012) to be moderately heritable, but it is not yet clear from twin and molecular genetic research if the association between externalizing and cortisol may vary by genetic background. Catechol-O-methyl transferase (COMT), and dopamine transporter (DAT1) genes have been associated with externalizing behaviors (Albaugh et al., 2010; Schmidt, Fox, & Hamer, 2007; Young et al., 2002). In addition, FK506 binding protein 51 (FKBP5), corticotropin releasing hormone receptor 1 (CRHR1), COMT, and DAT1 genes have all been associated

with the HPA-axis (Alexander et al., 2011; Chen, Joormann, Hallmayer, & Gotlib, 2008; Gillespie, Phifer, Bradley, & Ressler, 2009; Ising et al., 2008).

Understanding the moderating effects of genes in the cortisol-externalizing association is an integral step in understanding the mechanisms at work in the development of externalizing symptoms. The goal of this project is to understand how polymorphisms of the FKBP5, CRHR1, COMT, and DAT1 genes are associated with externalizing and moderate the relation between trait diurnal cortisol and externalizing symptoms in middle childhood.

Reactive Aggression vs. Abnormal Aggression

It is first crucial to understand whether the cortisol-externalizing pathway is better classified as reactive or abnormal. The reactive aggression pathway can be tested with a diathesis-stress model. According to the diathesis-stress model there are two components necessary for developing a disorder: a diathesis (in this case a genetic susceptibility) and stress (in this case exposure to dysregulated cortisol) (Monroe & Simmons, 1991). Even though diurnal cortisol activity is somewhat heritable in the morning, there is evidence in the literature that cortisol can act as an epigenetic factor to activate or deactivate gene expression (Lee et al., 2010). The abnormal pathway, in contrast, is brought on by a biological deficit, in this case a lack of cortisol affecting the child's ability to accurately interpret and respond to social cues.

One method for distinguishing between these two pathways is to consider the impact of anxiety symptoms as a covariate. In line with two independent pathways, children with CD or ODD have been found to be fundamentally

different based on whether they were comorbid for an anxiety disorder (Walker et al., 1991). Children with an anxiety comorbidity had fewer symptoms and were less severely impaired by their externalizing disorder compared to children with externalizing without the comorbidity. Children displaying externalizing symptoms because of the abnormal pathway can be considered "pure" externalizers, displaying symptoms of externalizing without also exhibiting symptoms of anxiety. Symptoms of anxiety, in this case, would be associated with the reactive pathway. In other words, children who display symptoms of externalizing for reactive reasons are responding to chronic stress that may also result in anxiety symptoms, and can be considered "comorbid" externalizers. By controlling for the impact of comorbid anxiety symptoms, one goal of the current study was to understand the predominate pathway through which externalizing symptoms arise for children during middle childhood. If results in the current study are only significant when anxiety is controlled, it would suggest support for the abnormal aggression pathway. On the other hand, if findings are only significant when anxiety symptoms are not controlled for, it would suggest support for the reactive aggression pathway.

Stress-reactive Genes

CRHR1. CRHR1, or corticotropin releasing hormone receptor 1, is a gene with strong links to the HPA-axis and cortisol. Neurons in the paraventricular nucleus of the hypothalamus send out signals that cause the hypothalamus to release corticotropin releasing hormone (CRH) into the blood where it travels to the anterior pituitary gland and activates CRH1 receptors (Gillespie et al., 2009;

Swanson et al., 1983). Activation of the CRH1 receptors causes an increase in the secretion of adrenocorticotrophic hormone (ACTH) which stimulates the release of cortisol. CRHR1, as the gene that dictates the number of CRH1 receptors, is of particular interest to researchers studying the HPA-axis stress response. CRHR1 has been implicated in the development of mood disorders through its influence on the activity of the CRH system (Bradley et al., 2008; Reul & Holsboer, 2002).

Very few studies so far have drawn a link between this gene and externalizing behavior. In rhesus monkeys, genetic differences in a CRHR1 haplotype, an allelic combination of adjacent chromosome locations that transmit together, were associated with CRH in the blood, and bold behavior and alcohol consumption (Barr et al., 2008). In humans, alcohol consumption, which is related to externalizing behaviors, is associated with stress and the CRHR1 gene such that greater stress and homozygosity for the C allele of CRHR1 SNP rs1876831 predicted earlier onset of drinking and greater amounts of alcohol consumption (Schmid et al., 2010).

The influence of this gene on the upregulation of CRH receptors links it to the production of cortisol and makes it a strong candidate for contributing to the dysregulated daily cortisol patterns associated with externalizing behaviors in middle childhood. If the G allele of the rs242924 SNP is associated with higher levels of cortisol, then it may play a role in the link between hypocortisolism and externalizing behaviors.

FKBP5. The FKBP5, also known as FK506 binding protein 51 gene, like CRHR1 is known to influence HPA-axis functioning and cortisol. Cortisol is

integral to the negative feedback process that downregulates the HPA axis' stress response (Binder, 2009). Cortisol binds with glucocorticoid receptors (GR) to initiate the negative feedback loop. FKBP5 binds to GRs and puts them into a low binding affinity state. When GRs are in this low binding affinity state, they are less sensitive to cortisol and it requires higher levels of cortisol to initiate the negative feedback loop. GRs are nuclear receptors that, after binding with cortisol, translocate to the cell nucleus where they mediate mRNA production for genes associated with neuronal activation and plasticity by binding to transcription factors (Maccari et al., 1992; Pavlides et al., 1995). Tatro et al (2009) inhibited FKBP5 in cells, which led to increased nuclear localization (travel to the nucleus) of GRs. Based on these studies, changes in FKBP5 mRNA expression might affect behavior through differences in neuronal activation and plasticity. This process could be particularly important when considering the epigenetic influence of cortisol on this gene.

Epigenetics is the study of changes in the expression of DNA that do not affect the underlying DNA sequence (Meaney, 2010). Methylation is an epigenetic process by which gene expression is either prevented or reduced. In research with mice, increases in corticosterone have been shown to decrease FKBP5 methylation and increase mRNA expression over two-fold (Lee et al., 2010). Similarly, in rat brains an increase in FKBP5 mRNA expression was also shown when increases in corticosterone were brought on by stress (Scharf, Liebl, Binder, Schmidt & Muller, 2011). The association between corticosterone and FKBP5 methylation and expression found in these studies suggests a utility in

testing cortisol as an epigenetic factor moderating the relationship between FKBP5 and phenotypic outcomes in humans, such as externalizing behaviors.

FKBP5 expression in humans has been found to be influenced by rs1360780; a T for C base substitution SNP in the FKBP5 gene (Binder, 2009). Homozygosity for the T allele of this SNP is associated with protein expression twice as high as C allele carriers. Previous research has associated this SNP with depression and anxiety (Binder et al., 2004; Binder et al., 2008; Zimmerman et al., 2011). It has also been found to interact with neglect during childhood to predict increased reactivity in the amygdala during magnetic resonance imaging (MRI) (White et al., 2012).

Researchers have yet to tackle, however, the possible involvement of this gene in the etiology of externalizing disorders. If school-aged children with externalizing problems have hypocortisolism, as previous research suggests, this pattern may indicate two possible pathways by which FKBP5 may interact with diurnal cortisol to predict externalizing. First, children with the C allele and hypocortisolism may be the most likely to demonstrate symptoms of externalizing because hypocortisolism associated with less FKBP5 mRNA expression coupled with a genotype already associated with less FKBP5 protein expression means these children can more quickly turn off their stress response and feel less stress in response to their aggressive actions. Second, if we consider the findings of Velder et al (2011) that the T allele is associated with a lower cortisol AUC, perhaps children with the T allele and hypocortisolism have higher externalizing because their cortisol response is not increasing to fulfill the higher requirement to

activate the negative feedback loop, putting them in a prolonged period of physiological stress resulting in externalizing symptoms.

Catecholamine Genes

COMT. Research has identified genes influencing catecholamines, the most common of which in the human brain are dopamine, epinephrine, and norepinephrine, as potential indirect influences on the HPA-axis (Alexander et al., 2011). The catechol-*O*-methyl transferase (COMT) gene regulates catecholamines by encoding an enzyme that breaks them down in the synapses (Grossman, Emanuel, & Budarf, 1992). Catecholamines have an impact on the HPA-axis through their influence on brain structures that contribute to the regulation of the HPA-axis, such as the limbic system and the prefrontal cortex (Jankord & Herman, 2008). Using a rat model, Ginsberg et al (2011) found up regulation of COMT in the cerebellum, frontal cortex, hippocampus, midbrain, and striatum of submissive rats using a resident-intruder paradigm. In the aggressive rats they discovered down regulation of COMT in the hippocampus. In humans, the rs4680 SNP is a valine (val) to methionine (met) substitution (Lachman et al., 1996). The Met allele of this SNP is associated with increased dopamine in the prefrontal areas of the brain compared to the Val (Sesack et al., 1998).

One important consideration with the functioning of this gene is the body of literature suggesting that it has a sexually dimorphic effect. In mice, males without the COMT gene were found to have a 2-3 fold increase in dopamine in the frontal cortex whereas female mice without the COMT gene showed no difference in dopamine levels (Gogos et al., 1998). In humans, COMT activity has

been shown to be greater in male brains even when COMT expression in male and female brains is equivalent (Chen et al., 2004). Differences in estrogen and dopamine functioning between men and women have been proposed as possible explanations for this sex difference; however, the exact cause of the sex disparity in COMT activity is not fully understood (Harrison & Tunbridge, 2008). The implications of this difference appear to be different depending on the phenotype of interest but what is clear is that sex should be treated as a moderator when considering this gene (Harrison & Tunbridge, 2008).

There is a sizable literature relating rs4680 with externalizing behavior. The Val allele has been associated with CD symptoms in a sample of adolescents (DeYoung et al., 2010). In children with ADHD, the Met allele has been most commonly associated with antisocial behaviors (Caspi et al., 2008; Thapar et al., 2005). Mothers have also been found to rate their children higher on the aggressive behavior scale of the Child Behavior Checklist when their children had a Met allele compared to Val homozygotes (Albaugh et al., 2010).

It remains to be seen how the rs4680 SNP might moderate the association between diurnal cortisol and externalizing symptoms in the present study. With previous findings associating the Met allele with both higher externalizing related outcomes and higher levels of both reactive and diurnal cortisol, it remains unclear how this relates to Alink et al.'s (2008) finding that externalizing is associated with lower levels of cortisol during middle childhood.

DAT1. The dopamine transporter (DAT) is a protein that binds to dopamine molecules in the synapses and reuptakes them back into nerve terminals

(Torres, Gainetdinov, & Caron, 2003). The dopamine transporter gene (DAT1) possesses a VNTR of which the 10-repeat allele and the 9-repeat allele are the most common (Heinz et al., 2000). The 9-repeat allele has been associated with reward-related activity in the ventral striatum (the piece of the striatum associated with the limbic system) (Forbes et al., 2009). The 10-repeat allele has been associated with increased post-mortem levels of DAT in the mid-brain (Brookes et al., 2007).

Reports on the functionality of the DAT1-VNTR have been mixed. Mill, Asherson, Craig, and D'Souza (2005) found no difference in gene expression between the 9 and 10-repeat alleles. VanNess et al. (2005), on the other hand, found that participants with the 10-repeat allele had 50 percent more DAT binding site density compared to participants with the 9-repeat. More recently, van de Giessen and colleagues (2009) found the 9-repeat allele to be associated with higher striatal DAT levels compared to the 10-repeat.

DAT1 has been associated with aggression. In adolescents, having the 10-repeat and fewer friends involved in criminal activity predicted increased violent aggression and criminal recidivism (Vaughn et al., 2009). Guo et al (2007) found a main effect of the DAT1-VNTR such that boys with a copy of the 10-repeat were nearly twice as high on self-reported violent delinquency scores compared to 9-repeat homozygotes and this difference was stable from the age of 12 to 23 years. Evidence in young and middle childhood, however, suggests that the 9-repeat may be the risk factor for younger children (Young et al., 2003). At ages four and seven, carriers of the 9-repeat were significantly more likely to show

symptoms of mother reported externalizing compared to children with the 10-repeat.

The Proposed Study

The general purpose was to understand main effect associations between genetic polymorphisms and symptoms of externalizing disorders and to understand the potential moderating influence of polymorphisms on the association between diurnal cortisol and symptoms of externalizing disorders, while controlling for symptoms of anxiety. Researching potential interactions between genes and an integral physiological system, such as the HPA-axis through the measurement of diurnal cortisol, may help uncover the etiology of externalizing symptoms. I tested two sets of hypotheses:

Hypothesis 1: Main Effects of Genetic Polymorphisms and Diurnal Cortisol on Externalizing Symptoms

1a) I hypothesized that children would have significant differences in externalizing symptoms because of allelic differences in CRHR1 SNP rs242924, FKBP5 SNP rs1360780, COMT SNP rs4680, and the DAT1-VNTR.

1b) I hypothesized that children lower (intercepts) or flatter (slopes) on each trait diurnal cortisol measure would have more externalizing symptoms.

Hypothesis 2: Genetic Polymorphisms Moderate Diurnal Cortisol and Externalizing Symptom Associations

2) I hypothesized that genetic polymorphisms in CRHR1 SNP rs242924, FKBP5 SNP rs1360780, COMT SNP rs4680, and the DAT1-VNTR would interact with diurnal cortisol to predict externalizing symptoms in children.

Method

Participants

Monozygotic and dizygotic twin pairs were recruited from Wisconsin birth records as part of the Wisconsin Twin Project (WTP). The WTP is a large multi-phase longitudinal twin study that seeks to understand the development of temperament, emotions, psychopathology and other behavioral and biological phenotypes from birth to adolescents (Schmidt et al., 2013). A subset of the full WTP sample was selected at age seven in order to achieve a balance of children with externalizing, internalizing, and comorbid disorders, as well as a control sample of healthy children, and all cotwins. Eight-hundred twins (400 pairs) and their families recruited during infancy between 1989 and 2004 participated at this phase and provided genetic data.

The sample is predominantly Caucasian (88.5%). Other ethnicities include African American (4.1%), mixed ethnicity (5.8%), Native American (0.3%), and Hmong (0.1%). The mean age is 7.93 (SD = .087 years) and 51% are boys. Monozygotic twins made up 32.2 percent of the sample, 33.4% were same sex dizygotic twins, and 34.4% were opposite sex dizygotic twins. Total family income ranged from unemployment to over $200,000 a year. The median income was $60,000 to $70,000 a year. Parent's level of education ranged from no formal

education to a graduate degree, with most parents having completed some or all of a college degree (M mothers = 14.88 years of school, M fathers = 14.43).

Measures

Socio-economic Status (SES). Total family income, mother education, and father education were found to be moderately correlated (r's = .45-.55, p<.0001). A mean composite was formed from the three measures after standardization of scales. SES was tested as a covariate.

Externalizing Symptoms. Scores on CD and ODD were obtained from the primary caregiver for each twin separately at age seven using the Diagnostic Interview Schedule for Children IV (DISC-P) (Shaffer, Fisher, Lucas, Dulcan, & Schwab-Stone, 2000). The DISC-P is a 40-70 minute interview designed to assess child psychopathology within the previous year. The DISC-P is highly structured, with a branching tree question structure. Responses to questions in the DISC-P are almost always limited to "yes", "no", and "maybe". Validity for the DISC-IV has not been formally tested, but for previous versions of the DISC-P validity ranged from .40 to .74 when comparing DISC diagnoses to clinician evaluations (Hersen, 2004). CD and ODD symptom count scales were moderately correlated (r = .50). For the purposes of this study, CD and ODD scales were used to form a sum externalizing symptom composite. Reliability for the externalizing composite was serviceable (α = .61).

Anxiety. The general anxiety symptom count scale was also taken from the DISC-P. Reliability for the general anxiety scale has previously been found to be decent (α = .65) (Shaffer et al., 2000).

Diurnal Cortisol. Hierarchical linear modeling was used to separate the variance associated with state and trait cortisol measured across three consecutive days for all diurnal cortisol measures (Van Hulle, Shirtcliff, Lemery-Chalfant, & Goldsmith, 2012). This was done by taking the samples from each of the three days cortisol was collected and separating the variance that is consistent across the three days (trait cortisol) from the variance that changed across the days (state cortisol). This allowed for the formation of intercept and slope variables using trait cortisol. The diurnal cortisol intercepts are the individual cortisol measures taken at three time points throughout the day. They include the morning intercept measured at waking, the afternoon intercept measured seven hours after waking, and the evening intercept measured 14 hours after waking. Diurnal cortisol slopes were calculated using the intercepts to form the morning slope (the slope from the morning intercept to the afternoon intercept) and the afternoon slope (the slope from the afternoon intercept to the evening intercept). The current study used the morning and evening cortisol intercepts, as well as the morning and afternoon cortisol slopes.

Genes. CRHR1 was measured using SNP rs242924. FKBP5 was measured using SNP rs1360780. COMT was measured using SNP rs4680. DAT1 was measured using the DAT1-VNTR located at the 3' end of the DAT1 gene. SNPs and VNTRs were coded additively. Because there is little known about how these SNPs and VNTRs may operate, and even less known about how they might function together, each gene was analyzed separately. All SNPs and VNTRs were found to be in Hardy-Weinberg Equilibrium (see Table 1), indicating that allelic

frequencies for the sample were in line with expected frequencies from the general population.

Procedures

SES. Phone interviews were conducted with the twins' primary caregiver when the twins were age seven. During these phone interviews, research staff administered demographics questionnaires that asked for family income and years of education of both parents.

Externalizing & Anxiety. Participating families took part in a four-five hour home visit during which research staff administered the DISC-P to the primary caregiver, separately for each twin.

Salivary cortisol. Diurnal cortisol was collected three times a day for three consecutive days from each twin. Cortisol was collected through passive drool. Each twin was instructed not to eat or drink an hour before sampling. Parents collected saliva from their twins using salivettes, tagged the salivettes with the date and time, and stored the salivettes in their freezer until salivettes were retrieved by research staff at the home visit. Samples were collected at waking, seven hours after waking (afternoon), and 14 hours after waking (evening).

In order to assay the cortisol, samples were centrifuged for 10 minutes at 5000 rpm to remove impurities. Salivary enzymeimmunoassay kits (Salimetrics, State College, PA), used in duplicate, were used to assess the cortisol samples. Internal controls were included in each assay. For the low control, the average value was 0.082 μg/dL with inter- and intra-assay Coefficent of Variations (CVs) of 7.2% and 6.1%, respectively. For the high control, the average value was 0.84

μg/dL with inter- and intra-assay CVs of 8.1% and 5.3% respectively. The CV of the duplicates had to be < 20% for the results to be considered suitable.

Genotyping. Cheek swabs were used to collect buccal cells for DNA testing at the home visits. MasterPure DNA kits obtained from Epicentre Biotechnologies at the Translational Genomics Research Institute (for SNP data) and the University of Wisconsin-Madison Biotechnology Center (for VNTR data) were used to extract DNA. Individual genotyping for SNP data was done using Sequenom technology. Sequenom technology directly reads the base sequences that make up the DNA strands. Eleven plates Direct Lysis Plasmid96 DNA Purification Kits that included four randomly chosen intraplate replicates and two positive CEPH (Centre d'etude du polymorphisme humain) controls from the Utah reference sample (CEU) of the International HapMap Consortium (2005) were used. HAPMAP concordance and the identification of Mendelian errors were accomplished by the addition of a plate of CEPH trios into each plex. VNTR genotyping was performed on agarose gels or via capillary electrophoresis. Agarose gels are used in a process called gel electrophoresis. Gel electrophoresis and capillary electrophoresis are methods used to separate fragments of DNA by length.

Results

Preliminary Analyses

All variables were within West, Curran, and Finch's (1995) guidelines for skewness and kurtosis. Multivariate outliers were tested using Mahalanobis D^2 (Cohen, Cohen, Aiken, & West, 2002). and no significant outliers were found.

Age, sex, and SES were examined as covariates in all models, both as main effects and in interactions with the predictors. Age and SES were nonsignificant covariates across all models, and were thus dropped. Main and interactive effects of sex were often significant, and interactions were probed using procedures outlined by Aiken and West (1991). In the case of significant three-way interactions, follow up analysis were conducted to elucidate the nature of the interactions. Specifically, when three-way interactions with sex were significant, they were followed up by testing the model again separately for boys and girls.

DISC general anxiety symptoms were controlled for in every model. This was done in order to inform whether the reactive aggression or abnormal aggression pathway was most influential on the current findings. Models in which results changed based on the inclusion of anxiety symptoms are noted below.

Means and standard deviations for the morning and evening cortisol intercepts and slopes, as well as DISC general anxiety symptoms and externalizing symptoms and the covariates are reported in Table 2. As expected, the highest levels of cortisol came at the beginning of the day. The means of the morning and afternoon slopes indicate on average that cortisol is decreasing throughout the day which is consistent with the evening intercept having a lower mean than the morning intercept, and is in line with previous research with this age group.

Zero-order correlations are presented in Table 3. The morning cortisol intercept was positively, but moderately correlated with the evening cortisol

intercept and negatively correlated with the morning and afternoon cortisol slopes, suggesting that higher morning cortisol is associated with steeper cortisol slopes and vice versa. The evening cortisol intercept, morning cortisol slope, and afternoon cortisol slope were all highly positively correlated with each other. DISC general anxiety symptoms were moderately positively correlated with DISC externalizing symptoms. Age was positively correlated with the evening cortisol intercept and both cortisol slopes. Genetic polymorphisms were not significantly correlated with other measures.

Hypothesis 1: Genetic Polymorphisms and Diurnal Cortisol Predict Externalizing Symptoms

Multilevel regression in SPSS Statistics 20 (IBM, Inc., 2011) was used to regress externalizing symptoms on cortisol and candidate genetic polymorphisms. This method accounts for the independence violation of using twins as participants by controlling for the within dyad correlation (in this case the correlation between cotwins on externalizing symptoms). Each SNP and VNTR was tested in a separate model. Likewise each measure of diurnal cortisol was considered in a separate model. There were no main effects of CRHR1, FKBP5, COMT, or DAT1 on children's externalizing symptoms (See Table 4). None of the genetic polymorphisms were found to interact with sex, SES, or age to predict externalizing symptoms.

Models predicting externalizing symptoms from cortisol measures are reported in Table 4. Whereas the morning cortisol intercept did not predict

externalizing symptoms, the evening cortisol intercept interacted with sex to predict externalizing symptoms. Further probing of the interaction found the slope for boys (β = 1.1665, s.e. = 0.438, z = 2.6635, p = .0077) was significant, such that boys with higher evening cortisol had greater externalizing symptoms (See Figure 1). Similarly, the morning and afternoon cortisol slopes interacted with sex to predict externalizing symptoms. Probing of the interaction found the slopes for boys (morning: β = 24.3084, s.e. = 9.9345, z = 2.4469, p = .0144; afternoon: β = 9.5291, s.e. = 3.5038, z = 2.7197, p = .0065) were significant such that boys with a less steep decline in cortisol had greater externalizing symptoms (See Figures 2 and 3).

Hypothesis 2: Genetic Polymorphisms Moderate Diurnal Cortisol and Externalizing Symptom Associations

Multilevel regression in SPSS Statistics 20 (IBM, Inc., 2011) was used to test moderating associations. Product terms were formed by first centering the four cortisol measures. CRHR1 SNP rs242924 was found to interact with the morning cortisol intercept to predict externalizing symptoms (See Table 5 and Figure 4). The slope for GG carriers was marginal (β = -1.4822, s.e. = 0.8748, z = -1.6944, p = .0902). Regions of significance testing revealed that GG carriers were significantly different from TT carriers at one standard deviation below the mean for morning cortisol (β = -1.2731, s.e. = 0.3206, z = -3.9704, p = .0001). There was no significant difference at one standard deviation above the mean (β = -0.4044, s.e. = 0.3206, z = -1.2612, p = .2073). No significant interactions were

found with CRHR1 SNP rs242924 and any of the other three cortisol measures (See Table 6).

No significant interactions were found between FKBP5 SNP rs1360780 and any of the four cortisol measures when predicting externalizing symptoms (See Table 7).

No interactions were significant between the COMT Val/Met and any of the four cortisol measures when predicting externalizing symptoms (See Table 8).

The DAT1-VNTR, the morning intercept, and sex interacted to significantly predict externalizing symptoms (See Table 9). Follow up analysis with boys revealed a marginal interaction and a marginal slope for 9/9 carriers (β = -1.4822, s.e. = 0.8748, z = -1.6944, p = .0902) such that at lower levels of morning cortisol, boys with the 9/9 genotype had more externalizing symptoms (See Figure 5). Region of significance testing found no significant differences at either one standard deviation below the mean (β = -1.0006, s.e. = 0.8872, z = -1.1279, p = .2594) or one standard deviation above the mean (β = 1.4219, s.e. = 0.8872, z = 1.6027, p = .109) on morning cortisol. No interaction was found between the DAT1-VNTR and the morning intercept for girls. The DAT1-VNTR and the afternoon slope were also found to interact with sex to predict externalizing symptoms (See Table 10). Follow up analysis with boys resulted in a significant interaction between the DAT1-VNTR and the afternoon slope and a significant slope for 9/9 carriers (β = 37.4428, s.e. = 10.098, z = 3.7079, p = .0002) and 9/10 carriers (β = 19.9677, s.e. = 5.9536, z = 3.3539, p = .0008) such that when the slope was more steep, boys carrying a nine had fewer externalizing

symptoms but when the slope was less steep, they had more (See Figure 6). This association became marginal when symptoms of general anxiety were not controlled for. Region of significance testing found no significant differences at either one standard deviation below the mean ($\beta = 1.2345$, s.e. = 0.7766, $z = 1.5896$, $p = .112$) or one standard deviation above the mean ($\beta = -0.9713$, s.e. = 0.7766, $z = -1.2506$, $p = .2111$) on the afternoon cortisol slope. No interaction was found between the DAT1-VNTR and the afternoon slope for girls. DAT1 did not significantly interact with either the evening intercept or the morning slope to predict externalizing symptoms (See Table 11).

Genetic analyses were rerun with Caucasian only subsample in order to address population stratification, the differences in ancestry that can cause differences in allelic frequencies and confound genetic findings when populations of different origins are studied together, and results were the same.

Discussion

Integral to the treatment of complex psychological disorders such as externalizing disorders, is a greater understanding of the genetic and biological pathways that significantly impact them. The primary goal of the current study was to explore the association between diurnal cortisol, a hormone key to the HPA-axis stress response, and externalizing symptoms, as well as the moderating role of specific genetic variants on this association during middle childhood. Results suggest not only a link between diurnal cortisol and externalizing symptoms in boys, but confirmation that genetic polymorphisms moderate this association. This is the first study to empirically examine the moderating impact

of measured genes on the cortisol-externalizing dynamic and findings should encourage a continued exploration of the physiological etiology of symptoms of externalizing disorders.

Placing the Findings in the Context of the Literature

The first part of the first hypothesis, that genetic polymorphisms on CRHR1, FKBP5, COMT, and DAT1 would be associated with externalizing symptoms was not supported. For the two HPA-axis genes, CRHR1 and FKBP5, this is not entirely unexpected in the absence of current literature directly relating either gene to externalizing outcomes. For the catecholamine genes, COMT and DAT1, this result is a little more surprising. The COMT Val allele in adolescents (DeYoung et al., 2010) and the Met allele in children (Albaugh et al., 2010; Caspi et al., 2008; Thapar et al., 2005) have been associated with symptoms of conduct disorder and antisocial behavior. Similarly, the 10-repeat of the DAT1-VNTR (Guo et al., 2007) and the 9-repeat (Young et al., 2003) have been associated with violent behavior and externalizing symptoms in children. However, lack of consistency across studies associating measured genes with complex traits are not uncommon (Colhoun, McKeigue, & Smith, 2003). Inconsistencies in this literature can result from issues of power, the bias in publication toward significant findings, or more theoretical issues such as population structure. Measured gene studies are performed all over the world with samples that are not necessarily consistent from one study to another in terms of ancestral background. Lack of replication may indicate that an association between gene and phenotype only exists for some populations and not others.

The second half of the first hypothesis, that children lower on trait cortisol would demonstrate higher externalizing symptoms, was also not supported. In fact, my findings demonstrated the opposite pattern in boys and showed no significant association with externalizing symptoms in girls. Three of the four diurnal cortisol measures, the evening intercept, morning slope, and afternoon slope, were significantly associated with number of externalizing symptoms, but unlike the meta-analysis finding from Alink et al. (2008) for the middle childhood age group, higher levels of cortisol on these three measures were associated with higher externalizing symptoms. Differences in the methodology of the current study and the Alink meta-analysis may help explain the discrepancy in findings. Alink et al considered a broader range of "school-aged" children (5-12 years) and reported a different direction of effects for children under five. I examined a more narrow age range (7-8 years) and that may have contributed to the difference.

Perhaps more importantly, I used trait cortisol measures, which have only been used in a handful of studies, as opposed to raw measures of cortisol. The difference in results may be because patterns of trait cortisol with externalizing symptoms are uniquely different from patterns of raw cortisol with externalizing symptoms. It may be that the pattern of hypocortisolism most often found in association with externalizing is largely due to state cortisol and that by separating out the two I have uncovered a different pattern associated specifically with trait cortisol. The single previous study to examine the association between trait cortisol and externalizing symptoms found lower cortisol to be associated with externalizing in boys, but their measurement of cortisol was limited to one sample

each day at waking (Shirtcliff et al., 2005). The one cortisol measure I did not find a significant main effect for was the morning intercept. Unlike the trait morning intercept, the trait afternoon slope and evening intercept have previously been found to have no significant additive genetic component in twin research (Van Hulle, Shirtcliff, Lemery-Chalfant, & Goldsmith, 2012). The differences between the Alink et al (2008) meta-analysis and Shirtcliff et al. (2005) may be because trait measures of afternoon and evening cortisol are uniquely able to capture differences in the HPA-axis stress response associated with the shared family environment.

The second hypothesis, that genetic variants on CRHR1, FKBP5, COMT, and DAT1 would moderate the association between diurnal cortisol and externalizing symptoms, was partially supported. CRHR1 SNP rs242924 moderated the association between the morning cortisol intercept and externalizing in boys and girls. The marginal slope for GG carriers suggests that this group has higher externalizing symptoms at lower levels of morning cortisol compared to GG carriers at higher levels of morning cortisol. GG carriers and TT carriers were also found to be significantly different at lower levels of morning cortisol, with GG carriers demonstrating higher levels of externalizing and TT carriers demonstrating lower levels of externalizing when compared to each other. These results should be interpreted with caution. This is not only the first study to find this SNP as a moderator of the association between cortisol and externalizing, but is the first to associate CRHR1 with externalizing symptoms and disorders. CRHR1 has been found to moderate the association between

trauma and cortisol, such that the GG genotype and childhood maltreatment predicted higher reactive cortisol reactions for adult men, but those results are not directly comparable to these novel findings (Heim et al., 2009; Tryka et al., 2009).

There was no significant moderation by FKBP5 SNP rs1360780. This is in line with the absence of literature relating this gene with externalizing outcomes. FKBP5 has been related to both reactive (Ising et al., 2008; Luijk et al., 2010), and diurnal cortisol (Velders et al., 2011), but never as a moderator of cortisol and psychopathological outcomes. Due to the role FKBP5 plays in shutting down the HPA-axis stress response, it is possible that the impact it may have on the cortisol-externalizing association is more proximal and better modeled in a mediation framework or in a design relating it to a more immediate byproduct of the HPA-axis such as CRH or ACTH.

Similar to FKBP5, there was no significant moderation by COMT SNP rs4680. Unlike FKBP5, COMT has been associated with externalizing outcomes in children (Albaugh et al., 2010; Caspi et al., 2008; Thapar et al., 2005). It has also been associated with diurnal cortisol (Walder et al., 2010), although it has more frequently been associated with reactive HPA-axis functioning (Alexander et al., 2011; Jabbi et al., 2007). Perhaps the reason no association was found was because COMT has a greater impact on the HPA-axis response to a specific stressor than on daily cortisol functioning.

Finally, the DAT1-VNTR significantly moderated the cortisol-externalizing association but only for boys. Specifically, the VNTR interacted with the morning intercept such that boys with the 9/9 genotype and lower

morning cortisol had marginally higher externalizing symptoms compared to those with the 9/10 and 10/10 genotypes. Similarly, boys with at least one 9-repeat were found to have higher externalizing symptoms when they had less steep afternoon cortisol slopes and had fewer externalizing symptoms when they had steeper afternoon slopes. The relation between DAT1 and externalizing symptoms has previously been documented in the psychopathology literature (Guo et al., 2007; Vaughn et al., 2009; Young et al., 2003). The current study is the first to suggest that HPA-axis functioning assessed with diurnal cortisol may be an important component of the system. As with the findings for CRHR1, the results for DAT1 should be interpreted with caution. Only one previous study has associated this gene with reactive cortisol (Alexander et al., 2011) and none have associated it with diurnal cortisol.

Implications

Findings contribute to the literature in three ways: 1) they begin to uncover a physiological pathway through which genetics and cortisol may contribute to the development of externalizing symptoms, 2) they suggest that this pathway may function uniquely for boys compared to girls, and 3) they suggest that this pathway functions independently of the association between anxiety symptoms and externalizing symptoms. One of the primary goals was to help unveil one of the biological mechanisms involved in the etiology of externalizing symptoms. The impact of genetic polymorphisms and diurnal cortisol furthers our understanding of those mechanisms.

The finding that genes involved directly with the HPA-axis and that indirectly impact the HPA-axis through the dopaminergic system in the brain are associated with the cortisol-externalizing dynamic provide insight into how human physiology may contribute to psychopathology. CRHR1, as a gene that influences the number of CRH1 receptors, contributes to ACTH and cortisol levels in the brain. The specific function of SNP rs242924 is not currently known (Heim et al., 2009). However, this SNP has been found to be in high linkage disequilibrium with CRHR1 SNPs rs110402 and rs7209436 (Bradley et al., 2008). Together these three SNPs have been used to form haplotypes, of which the TAT variation has been found to be a protective factor from depression. Perhaps the finding in the current study of lower externalizing symptoms for TT carriers on rs242924 in the presence of lower morning cortisol is an indication of a broader protective effect for the previously studied haplotype beyond just depression. It is not yet clear how the interaction between low morning cortisol and CRHR1 genotype may result in increased externalizing, but the dysregulation of multiple components of the HPA-axis, in this case, change in the number of CRH1 receptors and lower levels of morning cortisol, may represent a form of additive risk for these children.

The path by which DAT1, by virtue of its role in dopaminergic functioning, interacts with cortisol to potentially impact externalizing is likely different than CRHR1. The finding that cortisol, via both the morning intercept and afternoon slope, and the DAT1-VNTR are associated with externalizing symptoms is in line with research in rodents suggesting an interaction between the

HPA-axis and dopaminergic systems of the brain. In rats, evidence suggests the mesocortical dopamine system is involved in modulating HPA-axis activity in response to high or chronic stress (Sullivan & Dufresne, 2006). Rats exposed to chronic stress have also been found to have lower levels of dopamine transmission in the prefrontal cortex jointly with dyregulated HPA-axis functioning (Mizoguchi et al., 2008). This joint dysregulation has been associated with depressive behaviors in rats but it is conceivable that a similar joint dysfunction may be important to understanding the development of externalizing. The findings in the current study suggest the need to examine both irregular functioning in a single biological system, and the interaction of multiple systems and how they contribute to the development of psychopathological symptoms.

For the majority of findings, a significant sex interaction was found. Follow up analyses suggested these interactions were present for boys but not for girls. Boys (M = 6.89, SD = 4.55) had more externalizing symptoms than girls (M = 5.87, SD = 4.03), $t(848) = 3.467$, p = .001. The differences between boys and girls in the current study may simply be due to the higher number and greater range in symptoms found in this sample of boys compared to girls. Alternatively, biological or environmental factors may have a differential effect on boys than girls in the development of externalizing symptoms. Aggression was higher in boys during middle childhood compared to girls, which put them at higher risk for externalizing outcomes (Card et al., 2008). The higher rates of aggression and externalizing in boys may simply make biological differences in genes and cortisol easier to detect in this group, but it seems equally as likely that biological

differences in genes and cortisol are contributing to higher rates of aggression and externalizing and represent a gender difference in physiological functioning. Twin research on the etiology of externalizing has uncovered few genetic gender differences despite the higher levels of externalizing found in boys (Burt, Krueger, McGue, & Iacono, 2003; Hudziak et al., 2003). When twin studies have found a difference in the additive genetic influence on externalizing, boys have shown a greater additive genetic component compared to girls (Saudino, Ronald, & Plomin, 2005), but findings of a gender difference appear to be the exception not the rule. These findings lend credence to the hypothesis that findings were limited to boys in this study because of the greater variation in externalizing symptoms. At the very least, they suggest a need to test cortisol as a moderator of twin ACE models to see if it changes the genetic and environmental variance in externalizing differently by gender.

Integral to the theoretical basis was previous research suggesting different pathways for reactive and abnormal aggression. Symptoms of anxiety were controlled for in an attempt to differentiate between these two pathways but findings indicate no significant difference on genetic and cortisol associations with externalizing controlling for anxiety symptoms. Although anxiety symptoms significantly predicted externalizing symptoms, the pathway from anxiety symptoms to externalizing symptoms was independent of the pathway associating diurnal cortisol with externalizing. This independence suggests that cortisol may not be one of the biological mechanisms that separate children with comorbid anxiety and externalizing symptomology from more severely impaired

counterparts who only suffer from externalizing (Walker et al., 1991). It was hypothesized in this study that controlling for anxiety would differentiate reactive from abnormal pathways from HPA-axis functioning to externalizing. The findings suggest that either controlling for anxiety symptoms was a poor method for differentiating the pathways or that these two pathways supported by the animal literature do not inform the development of psychopathology in humans. It is also possible that controlling for anxiety did not adequately capture differential etiology of externalizing problems, and it would be more effective to test a broader model of internalizing or to use a more person-centered approach.

Limitations of the Current Study

The primary limitations are the concurrent nature of the design, the limited physiological measures for attempting to understand the biological etiology of externalizing symptoms, and the use of the measured gene approach to understand the role of genetics in the development of externalizing. The application of a longitudinal design would have clarified temporal precedence in the associations of genes, cortisol, and externalizing. Knowing, not only that changes in diurnal cortisol preceded changes in externalizing symptoms, but at what period of child development that diurnal cortisol shifted and how long after that shift they impacted externalizing levels would provide a far better understanding of the dynamic. Previous research has shown differences between concurrent and longitudinal associations between diurnal cortisol and mental health (Shirtcliff & Essex, 2008). By examining both concurrent and longitudinal findings, the picture

of how these physiological processes impact externalizing would be more complete.

The present study took two complex processes in human functioning, genetics and the HPA-axis, and analyzed two small aspects of those processes, variants on four measured genes and diurnal cortisol output. This simplified method allowed for testable hypotheses but the results must be placed within the larger biological framework. The HPA-axis is a complex system and cortisol, as the byproduct of that system, can be affected by other HPA-axis processes and other systems within the human body such as the dopaminergic and serotoninergic systems within the brain (Jankord & Herman, 2008). By only testing measured levels of diurnal cortisol and selected genetic variants, the full biological system within which these findings operate is not completely understood and the context of the broader system must be acknowledged when attempting to interpret the results.

Lastly, the measured gene approach to studying the impact of genetics on psychopathological outcomes has been criticized. The primary concerns of this method are that of replication and power of effect (Taber, Risch, & Meyers, 2002). Some measured gene associations have proven difficult or impossible to replicate leading to questions of the validity of the method. The chance of findings that cannot be replicated in the current study was minimized by selecting variants from genes with known biological functions that impact HPA-axis functioning and that have previously been associated in the literature with externalizing symptoms, cortisol, or both. The issue remains, however, that the

associations tested in interaction with diurnal cortisol are novel and must be replicated in independent samples. The second issue with this method is one of meaning. The variants studied represent a miniscule fraction of the human genome. The question of whether or not the present findings represent meaningful differences within the context of a much more complicated biological system cannot be fully answered by the current study, but as with the issue of replication, the selection of biologically meaningful variants was done, in part, to help address this issue.

Future Directions

Future research should expand on these findings by incorporating a longitudinal element in order to better understand the developmental impact of genes and cortisol throughout childhood. Other genes that impact the HPA-axis and the dopamine system should also be tested and the model should be expanded to consider other systems of neurotransmitters that impact the HPA-axis, such as the serotonergic system. Trait cortisol measures should be compared with raw cortisol measures in order to better understand the similarities and differences in the pattern of results from each. In addition to expanding upon the biological measures, future studies should consider environmental measures. The biological systems that effect externalizing do not exist in a vacuum and understanding the psychosocial environment in relation to these biological systems is integral to fully understanding how externalizing symptoms and disorders develop. For instance, family conflict has been shown to impact diurnal cortisol levels in young children (Slatcher & Robles, 2012). Finally, the comorbidity between

externalizing and anxiety symptoms should be more fully explored, along with symptoms of depression in order to understand the unique and similar biological pathways between externalizing and internalizing disorders, as well as to further explore the implications of the aggression pathways found in the animal literature on human development. One method that could prove effective in this endeavor going forward, is a person-centered approach. Instead of taking the symptom-based approach used here to understand the associations between cortisol and externalizing, latent class groups would be formed based on different patterns of externalizing and internalizing within individuals. Researchers could then look at how genes and cortisol predict latent class group membership.

Finally, the current study took a moderating approach, theorizing that the interaction between genetic variants and cortisol would impact externalizing. In order to understand the full picture of how these two biological mechanisms interact to influence externalizing, future research should test cortisol as an endophenotype, a more proximal phenotype directly linked to both genotype and outcome phenotype, in a mediation model. Especially for genes such as CRHR1 and FKBP5, that have functions that may directly impact HPA-axis functioning, testing whether cortisol mediates the association between gene and externalizing is necessary to fully understanding the biological system at play in the development of externalizing disorders.

Conclusions

Findings represent a step forward in understanding the biological etiology of externalizing symptoms by demonstrating an association between trait diurnal

cortisol and symptoms of externalizing disorders, moderated by functional genetic variants. Understanding this pathway has the potential to inform pharmacological treatment and hopefully will encourage future research that can build upon the findings to better understand the biological systems at play for both prevention and intervention purposes. The current study was not able to support a distinction between a reactive aggression and an abnormal aggression pathway, but supports the role of cortisol in the etiology of externalizing and illustrates the potential of targeting the HPA axis in pharmacological interventions. By increasing our knowledge of biological pathways to psychopathology, I hope to address the significant health concern that externalizing symptoms and disorders impose, and take a step forward in a line of research with the potential to have a positive impact on children.

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Table 1: Genotype Frequencies for Candidate Genes

CRHR1 (rs242924)		FKBP5 (rs1360780)		COMT (rs4680)		DAT1-VNTR	
Total	1168		1142		1192		866
AA	249	TT	103	AA	317	nine/nine	43
AC	565	CT	430	AG	581	nine/ten	316
CC	354	CC	609	GG	294	ten/ten	489
Hardy-Weinberg	$p < .05$		$p < .05$		$p < .05$		$p < .05$

Table 2: Descriptive Statistics

	N	Mean	SD	Min	Max
Morning Cortisol Intercept	869	3.89	0.31	2.56	5.06
Evening Cortisol Intercept	869	2.34	0.51	0.76	4.51
Morning Cortisol Slope	869	-0.13	0.02	-0.21	-0.04
Evening Cortisol Slope	869	-0.10	0.06	-0.30	0.12
DISC General Anxiety Symptoms	1598	1.60	1.89	0.00	12.00
DISC Externalizing Symptoms	1597	6.58	4.54	0.00	26.00

Note. DISC stands for Diagnostic Interview Schedule for Children IV

Table 3: Zero-Order Correlations

	Morning CORT Intercept	Evening CORT Intercept	Morning CORT Slope	Afternoon CORT Slope	DISC General Anxiety Symptoms	DISC Externalizing Symptoms Sum	SES	Age	Sex	CRHR1 rs242924	FKBP5 rs1360780	COMT rs4680	DAT1 VNTR
Morning Intercept	1	.358**	-.289**	-.216**	-.033	-.035	.044	-.002	-.024	-.015	.022	-.017	.046
Evening Intercept		1	.736**	.790**	-.030	.036	-.019	.213**	-.079	-.026	.036	.020	-.020
Morning Slope			1	.804**	-.025	.037	-.070*	.209**	-.063	-.031	.032	.009	-.029
Afternoon Slope				1	.006	0.067	-.027	.215**	-.060	.001	.008	.046	-.058
DISC General Anxiety					1	.252**	-.121**	-.051	.045	.015	.003	.034	.010
DISC Externalizing						1	-.088**	-.077*	-.152**	-.025	.010	.018	.006
SES							1	.060	-.033	.067*	.005	-.024	-.067
Age								1	.002	.029	-.040	.007	-.015
Sex									1	-.008	-.032	.001	.027
CRHR1 rs242924										1	.020	.020	.013
FKBP5 rs1360780											1	.028	.021
COMT rs4680												1	.040
DAT1-VNTR													1

Note. * $p < .05$, ** $p < .01$. *DISC is Diagnostic Interview Schedule for Children IV. CORT is cortisol. SES is Socioeconomic Status. For Sex, boys were coded 0 and girls were coded 1.*

Table 4: Main Effect of Genes on Externalizing Symptoms

	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 1: CRHR1			
Intercept	6.507***	0.229	416.342
Sex	-1.265***	0.245	378.243
DISC Anxiety	0.468***	0.063	170.384
rs242924	-0.177	0.178	1079.616
Hypothesis 1: FKBP5			
Intercept	6.537***	0.323	270.334
Sex	-1.102**	0.338	249.079
DISC Anxiety	0.381***	0.077	68.851
rs1360780	-0.23552	0.251	563.910
Hypothesis 1: COMT			
Intercept	6.499***	0.223	423.864
Sex	-1.323***	0.241	381.320
DISC Anxiety	0.472***	0.062	181.322
rs4680	-0.027	0.169	1112.608
Hypothesis 1: DAT1			
Intercept	6.369***	0.311	338.320
Sex	-1.063**	0.307	274.836
DISC Anxiety	0.406***	0.074	155.023
DAT1-VNTR	0.149	0.253	782.517
Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.			

51

Table 5: Diurnal Cortisol Measures on Externalizing Symptoms

	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 1: Morning CORT Intercept			
Intercept	6.312**	1.817058	800.3287
Sex	-1.102***	0.307349	324.1948
DISC Anxiety	0.402***	0.077661	142.9056
Morning CORT Intercept	0.002	0.461481	792.0098
Hypothesis 1: Evening CORT Intercept			
Intercept	3.651**	1.067	471.178
Sex	-1.06**	0.305	321.708
DISC Anxiety	0.401***	0.078	143.638
Evening CORT Intercept	1.124**	0.436	477.577
Evening CORT Intercept*Sex	-1.473**	0.548	527.364
Hypothesis 1: Afternoon CORT Slope			
Intercept	7.122***	0.423	477.712
Sex	-1.073***	0.303	314.278
DISC Anxiety	0.404***	0.078	145.990
Afternoon CORT Slope	8.464*	3.507	518.597
Afternoon CORT Slope*Sex	-10.755*	4.373	575.296
Hypothesis 1: Morning CORT Slope			
Intercept	9.049***	1.299	518.175
Sex	-1.068***	0.303	316.206
DISC Anxiety	0.400***	0.078	145.719
Morning CORT Slope	21.436*	9.984	514.545
Morning CORT Slope*Sex	-29.788*	12.669	594.951

Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.

Table 6: Interactions with CRHR1 and Cortisol Measures on Externalizing Symptoms

	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 2: CRHR1 x Morning CORT Intercept			
Intercept	6.526**	2.091	597.528
Sex	-1.153**	0.337	229.937
DISC Anxiety	0.465***	0.086	96.911
Morning CORT Intercept	-0.081	0.532	594.645
rs242924	-0.839***	0.238	569.226
rs242924*Morning CORT Intercept	1.401*	0.694	548.332
Hypothesis 2: CRHR1 x Evening CORT Intercept			
Intercept	5.994***	0.821	566.102
Sex	-1.150**	0.338	232.178
DISC Anxiety	0.462***	0.086	96.036
Evening CORT Intercept	0.092	0.331	532.585
rs242924	-0.857***	0.241	565.198
rs242924*Evening CORT Intercept	-0.067	0.397	558.149
Hypothesis 2: CRHR1 x Morning CORT Slope			
Intercept	6.496***	0.987	608.001
Sex	-1.176**	0.337	231.599
DISC Anxiety	0.462***	0.086	95.109
Morning CORT Slope	2.165	7.295	580.424
rs242924	-0.887***	0.240	569.710
rs242924*Morning CORT Slope	-12.464	9.473	538.770
Hypothesis 2: CRHR1 x Afternoon CORT Slope			
Intercept	6.270***	0.384	403.672
Sex	-1.164**	0.337	231.507
DISC Anxiety	0.463***	0.086	96.303
Afternoon CORT Slope	0.553	2.514	534.457
rs242924	-0.895***	0.240	566.732
rs242924*Afternoon CORT Slope	-3.899	3.234	508.010

Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.

Table 7: Interactions with FKBP5 and Cortisol Measures on Externalizing Symptoms

	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 2: FKBP5 x Morning CORT Intercept			
Intercept	3.791	4.797	224.643
Sex	-0.787	0.515	248.740
DISC Anxiety	0.379**	0.140	67.176
Morning CORT Intercept	0.562	1.242	223.205
rs1360780	0.118	0.442	241.102
rs1360780*Morning CORT Intercept	-0.454	1.613	222.908
Hypothesis 2: FKBP5 x Evening CORT Intercept			
Intercept	5.370***	1.473	242.046
Sex	-0.774	0.514	249.314
DISC Anxiety	0.370*	0.140	63.182
Evening CORT Intercept	0.286	0.683	237.479
rs1360780	0.090	0.505	245.976
rs1360780*Evening CORT Intercept	-0.168	0.805	249.465
Hypothesis 2: FKBP5 x Morning CORT Slope			
Intercept	5.559*	2.291	235.853
Sex	-0.786	0.515	249.907
DISC Anxiety	0.381**	0.141	63.722
Morning CORT Slope	-2.709	16.127	230.550
rs1360780	0.281	0.482	243.641
rs1360780*Morning CORT Slope	12.746	20.024	245.537
Hypothesis 2: FKBP5 x Afternoon CORT Slope			
Intercept	6.148***	0.836	244.681
Sex	-0.786	0.515	249.050
DISC Anxiety	0.370*	0.139	62.767
Afternoon CORT Slope	1.388	4.889	240.680
rs1360780	0.047	0.488	243.473
rs1360780*Afternoon CORT Slope	-2.873	6.215	245.231
Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.			

Table 8: Interactions with COMT and Cortisol Measures on Externalizing Symptoms

	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 2: COMT x Morning CORT Intercept			
Intercept	5.852**	2.102	586.040
Sex	-1.198**	0.343	230.800
DISC Anxiety	0.469***	0.086	106.815
Morning CORT Intercept	0.061	0.535	581.549
rs4680	-0.129	0.251	189.441
rs4680*Morning CORT Intercept	0.300	0.726	323.613
Hypothesis 2: COMT x Evening CORT Intercept			
Intercept	5.730***	0.831	531.353
Sex	-1.182**	0.344	230.887
DISC Anxiety	0.469***	0.086	106.534
Evening CORT Intercept	0.151	0.334	498.065
rs4680	-0.136	0.251	191.094
rs4680*Evening CORT Intercept	0.090	0.484	310.187
Hypothesis 2: COMT x Morning CORT Slope			
Intercept	6.392***	1.006	575.733
Sex	-1.197**	0.343	233.251
DISC Anxiety	0.465***	0.086	105.818
Morning CORT Slope	2.287	7.469	539.868
rs4680	-0.143	0.250	187.734
rs4680*Morning CORT Slope	-6.690	10.443	362.143
Hypothesis 2: COMT x Afternoon CORT Slope			
Intercept	6.097***	0.384	387.136
Sex	-1.193**	0.343	231.205
DISC Anxiety	0.471***	0.086	106.347
Afternoon CORT Slope	0.205	2.507	514.892
rs4680	-0.137	0.251	189.303
rs4680*Afternoon CORT Slope	1.348	3.302	345.247
Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.			

Table 9: Interaction with DAT1 and the Morning Cortisol Intercept on Externalizing Symptoms

	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 2: DAT1 x Morning CORT Intercept			
Intercept	15.324*	6.409	195.001
Sex	-0.805	0.581	160.723
DISC Anxiety	0.445***	0.111	106.645
Morning CORT Intercept	-2.364	1.640	193.763
DAT1-VNTR	-0.094	0.542	207.440
DAT1-VNTR*Morning CORT Intercept	3.521+	1.884	209.501
Morning CORT Intercept*Sex	3.193	1.956	224.094
DAT1-VNTR*Sex	-0.393	0.686	250.839
DAT1-VNTR*Morning CORT Intercept*Sex	-4.775*	2.313	270.893
Hypothesis 2: DAT1 x Morning CORT Intercept - Boys Only			
Intercept	17.401*	6.505	26.520
DISC Anxiety	0.589**	0.195	52.730
Morning CORT Intercept	-3.035+	1.669	24.974
DAT1-VNTR	0.211	0.623	19.632
DAT1-VNTR*Morning CORT Intercept	3.907+	2.039	26.919
Hypothesis 2: DAT1 x Morning CORT Intercept - Girls Only			
Intercept	5.042	4.499	96.334
DISC Anxiety	0.338**	0.117	137.410
Morning CORT Intercept	0.112	1.163	96.363
DAT1-VNTR	-0.486	0.444	60.798
DAT1-VNTR*Morning CORT Intercept	-0.989	1.386	99.209

Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.

Table 10: Interaction with DAT1 and the Afternoon Cortisol Slope on Externalizing Symptoms

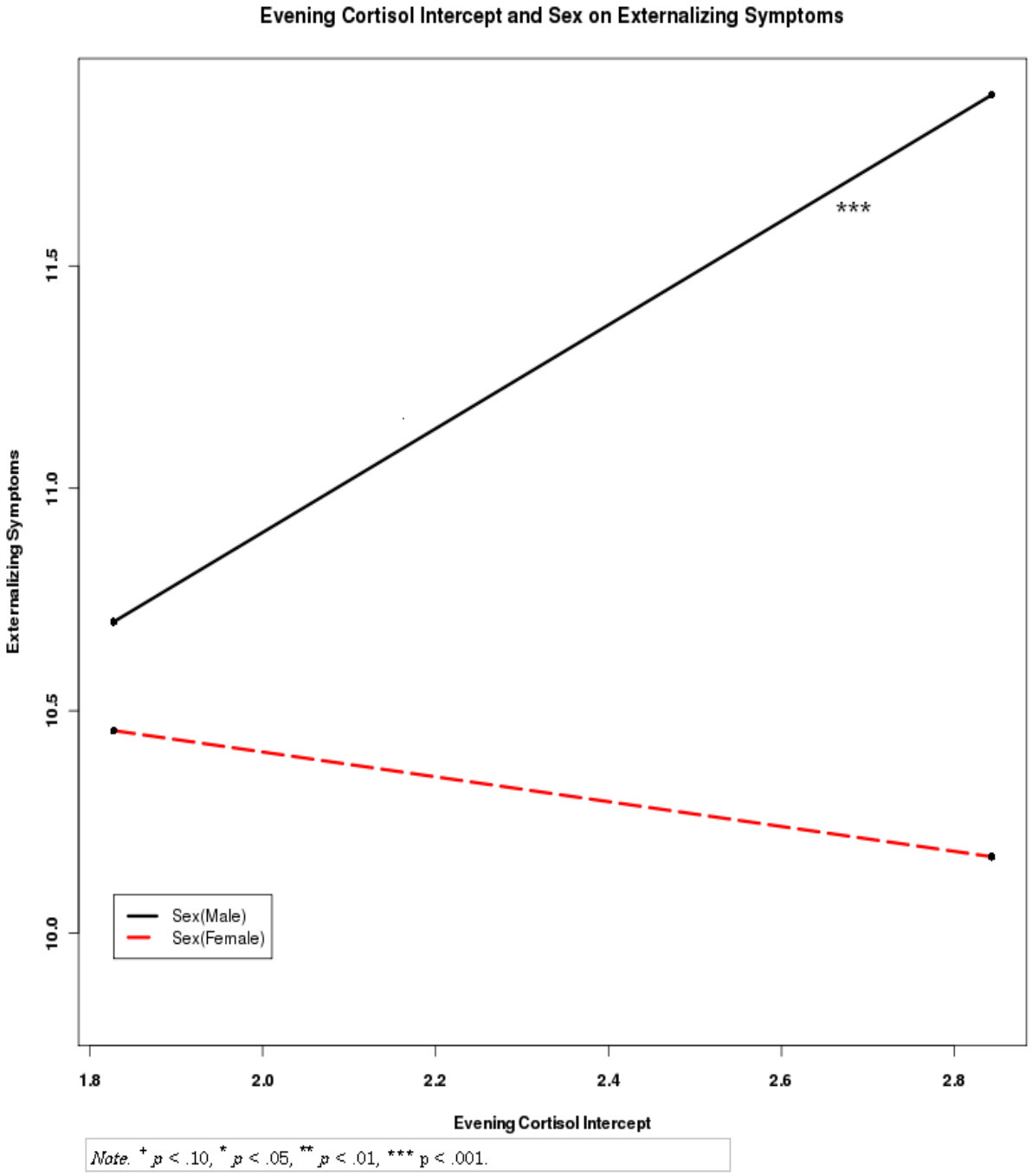
	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 2: DAT1 x Afternoon CORT Slope			
Intercept	8.163***	0.863	161.666
Sex	-0.985+	0.579	153.736
DISC Anxiety	0.451***	0.113	105.452
Afternoon CORT Slope	19.471**	6.600	180.157
DAT1-VNTR	-0.047	0.530	194.992
DAT1-VNTR*Afternoon CORT Slope	-14.627+	7.959	223.473
Afternoon CORT Slope*Sex	-24.179**	7.643	184.284
DAT1-VNTR*Sex	-0.332	0.681	234.352
DAT1-VNTR*Afternoon CORT Slope*Sex	22.147*	9.601	269.329
Hypothesis 2: DAT1 x Afternoon CORT Slope - Boys Only			
Intercept	8.075***	0.851	26.695
DISC Anxiety	0.587**	0.201	56.559
Afternoon CORT Slope	21.560**	6.181	43.594
DAT1-VNTR	0.132	0.591	22.546
DAT1-VNTR*Afternoon CORT Slope	-18.381*	8.393	66.113
Hypothesis 2: DAT1 x Afternoon CORT Slope - Girls Only			
Intercept	5.058***	0.646	207.997
DISC Anxiety	0.361**	0.116	170.501
Afternoon CORT Slope	-3.689	4.186	205.456
DAT1-VNTR	-0.435	0.440	207.950
DAT1-VNTR*Afternoon CORT Slope	7.172	5.442	194.092

Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.

Table 11: Interactions with DAT1 and Cortisol Measures on Externalizing Symptoms

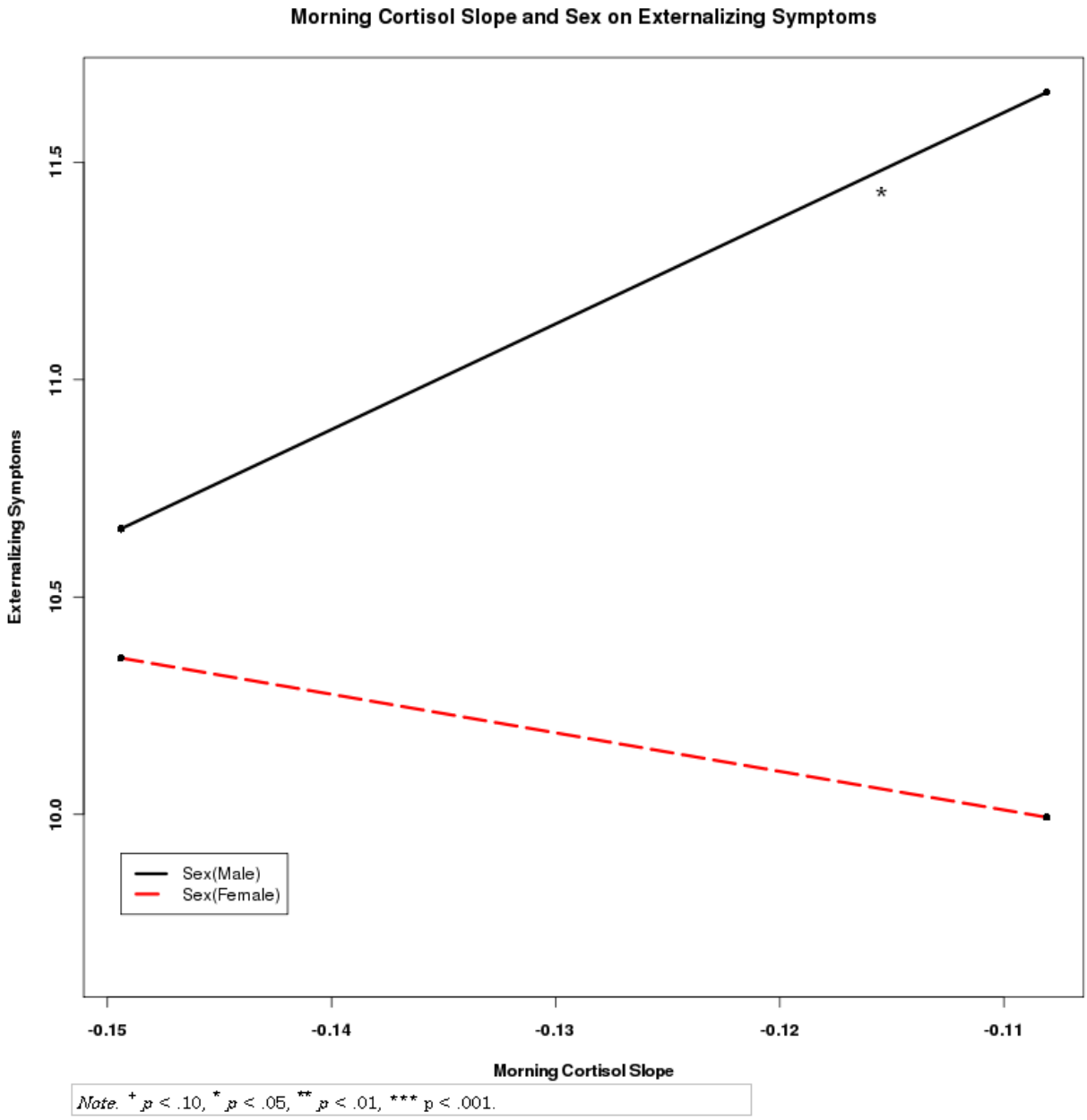
	Externalizing Symptoms		
	B	S.E.	df
Hypothesis 2: DAT1 x Evening CORT Intercept			
Intercept	5.723***	1.198	355.371
Sex	-1.024*	0.455	137.810
DISC Anxiety	0.457***	0.110	100.246
Evening CORT Intercept	0.228	0.502	324.028
DAT1-VNTR	-0.312	0.356	336.666
DAT1-VNTR*Evening CORT Intercept	0.081	0.600	351.056
Hypothesis 2: DAT1 x Morning CORT Slope			
Intercept	7.094***	1.697	334.488
Sex	-1.035*	0.456	138.147
DISC Anxiety	0.456***	0.109	98.374
Morning CORT Slope	6.549	12.338	323.301
DAT1-VNTR	-0.330	0.356	329.966
DAT1-VNTR*Morning CORT Slope	-5.512	15.685	326.091
Note. $^{+}$ $p < .10$, * $p < .05$, ** $p < .01$, *** $p < .001$.			

Figure 1



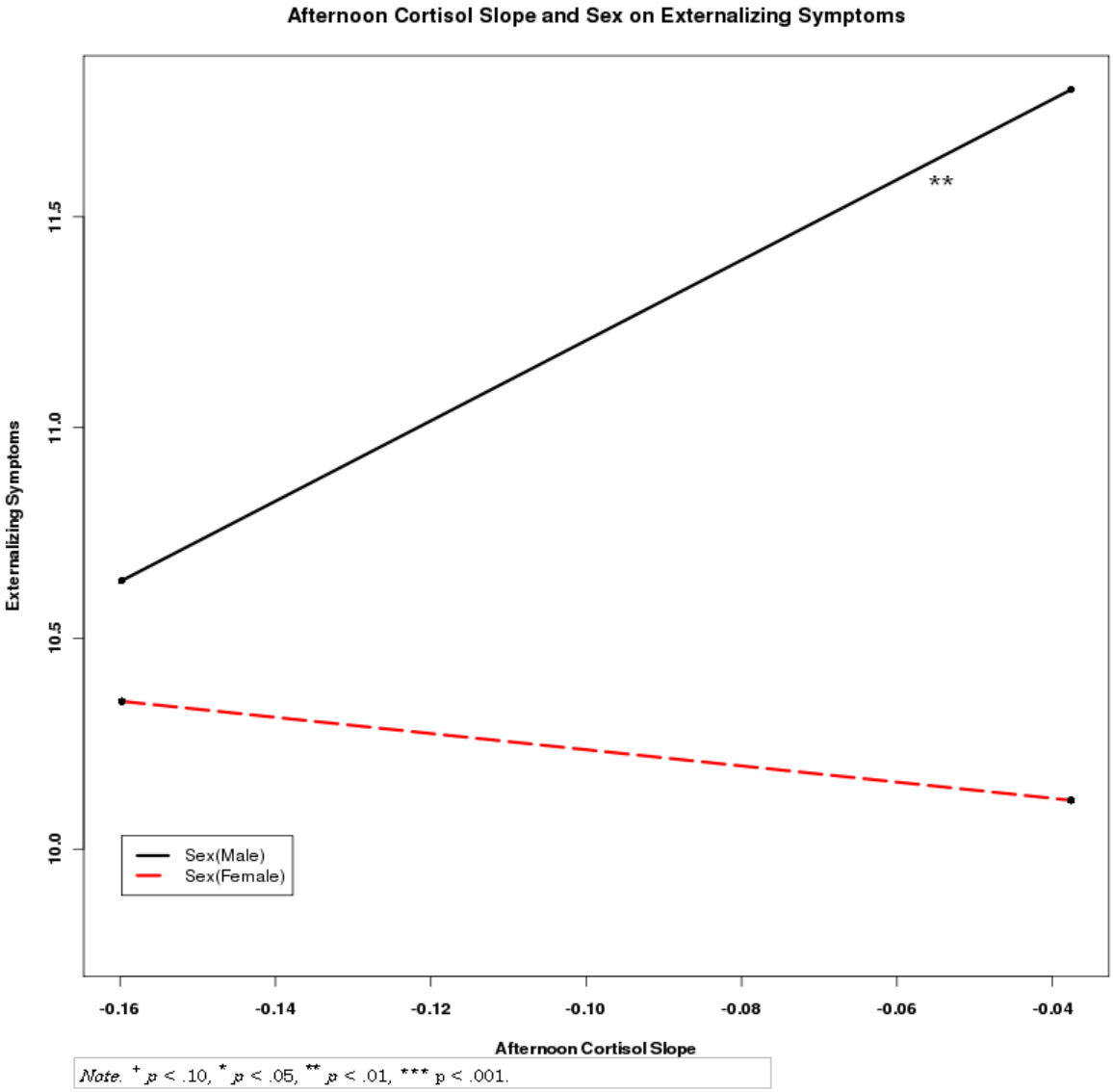
Note. $^{+}p < .10$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Figure 2



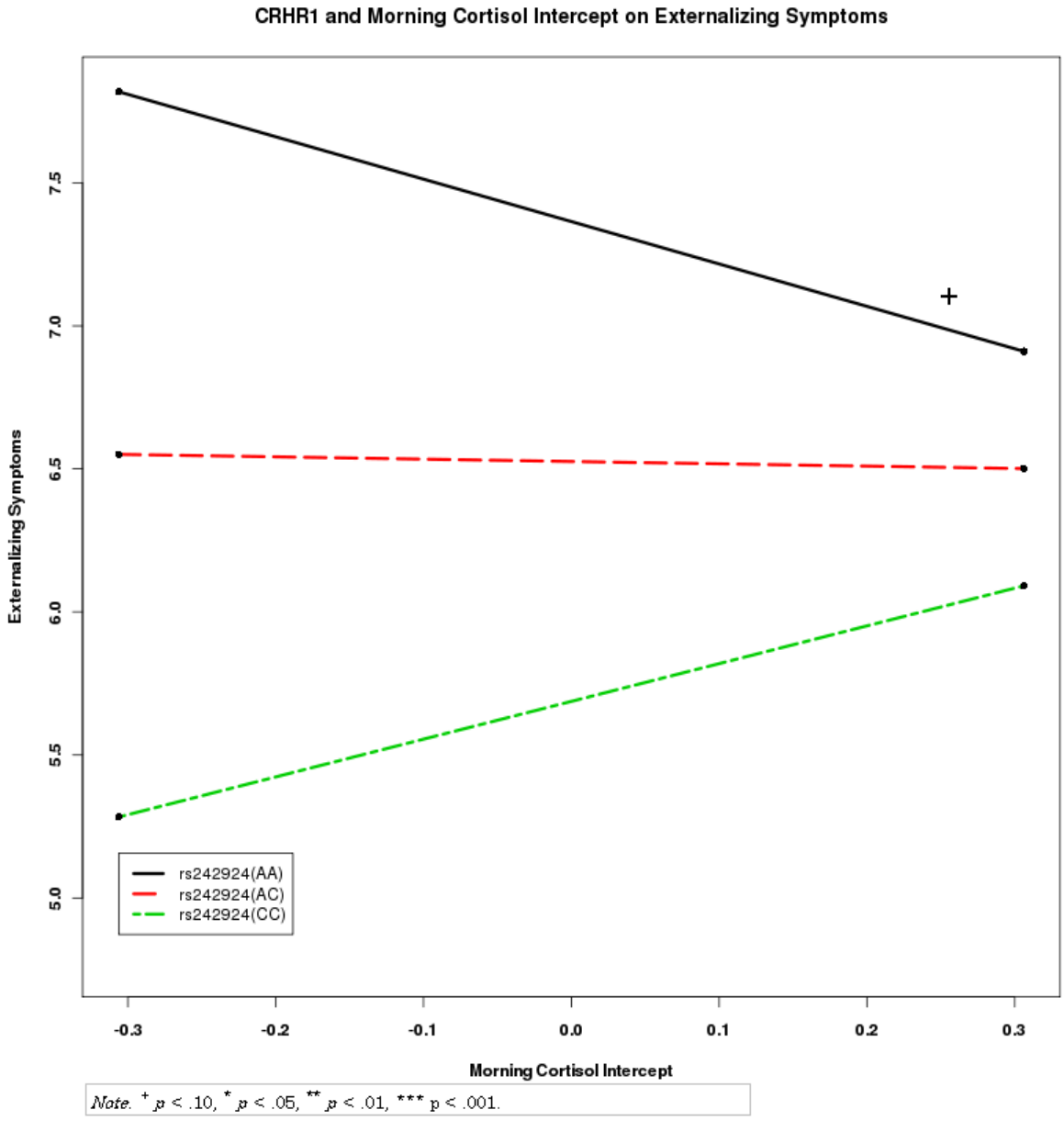
Note. $^{+}p < .10$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Figure 3



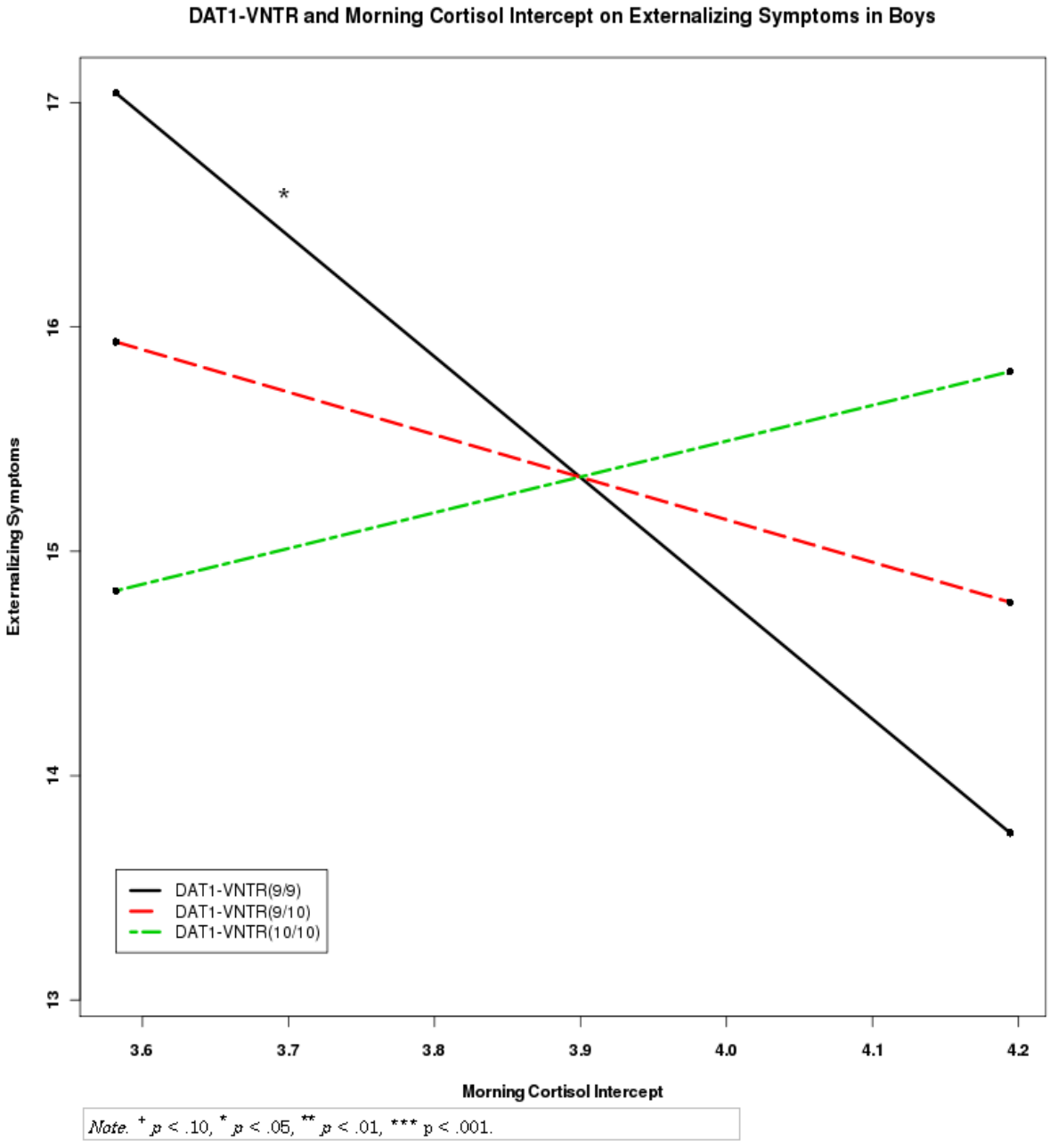
Note. $^{+}p < .10$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Figure 4



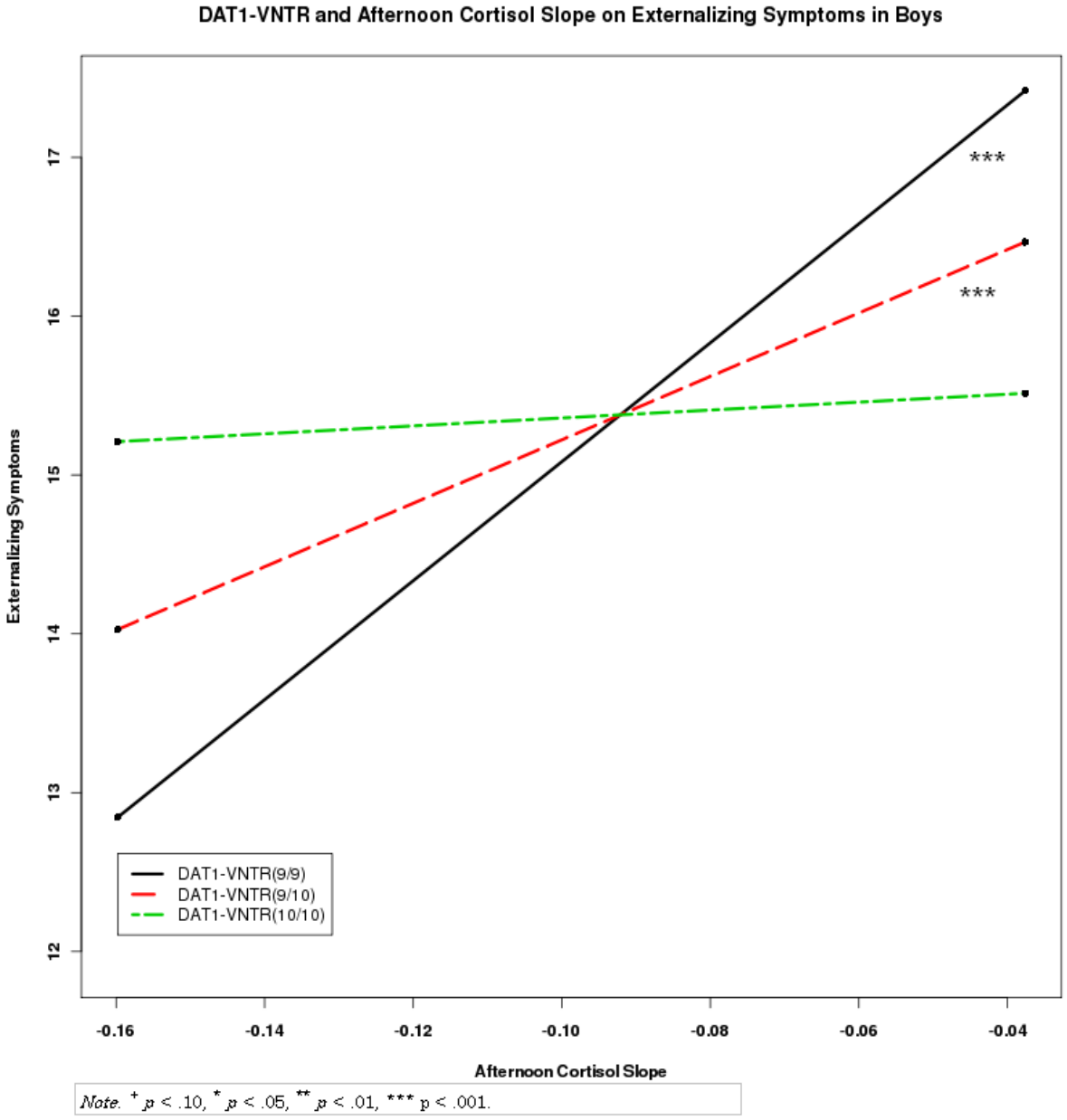
Note. $^{+}p < .10$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Figure 5



Note. $^{+}p < .10$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Figure 6



Note. $^{+}p < .10$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.