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Impact of Early Life Stress and 5-HTTLPR on Adulthood Stress Reactivity: Investigation of Changes in Cortisol, Gene Expression and DNA Methylation

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Abstract of the Dissertation

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Early life stress (ELS) is considered one of the important risk factors for adulthood psychopathology and has been associated with impairments in stress response systems such as the Hypothalamic-Pituitary-Adrenal axis (HPA). Over the last decade, studies on Gene-Environment interactions (GxEs) also suggested moderation of this relationship by genetic factors, such as the serotonin transporter polymorphism (5-HTTLPR). Although there are many studies investigating these associations, the underlying biopsychosocial mechanisms are not yet clear. The aim of this dissertation is to identify some of these mechanisms through the use of intermediate phenotypes such as cortisol reactivity, stress-related gene expression and DNA methylation. Healthy Caucasian men were recruited from the Stony Brook University and surrounding communities for participating in an experimental session that involved completion of questionnaires, a life events interview and an acute psychosocial stress paradigm called the Trier Social Stress Test (TSST). Saliva and blood samples were collected for genotyping, cortisol, gene expression and DNA methylation analyses. Results indicate an interaction between

ELS and 5-HTTLPR on cortisol reactivity to the TSST as well as differential expression and DNA methylation of the candidate genes. These results provide evidence for the impact of ELS and 5-HTTLPR on different intermediate phenotypes leading to altered stress reactivity in adulthood. Future studies with different gender, ethnicity and clinical groups would complement the results of this study and open up possibilities for behavioral and pharmacological interventions.

To my mother,

who was always aware of my enthusiasm in studying behavior and

gave me all the support and confidence I need to pursue and find my own way.

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List of Abbreviations

5-HT	5-hydroxytryptamine
5-HTT	5-hydroxytryptamine transporter
5-HTTLPR	5-hydroxytryptamine transporter linked polymorphic region
ACTH	Adrenocorticotropic hormone
ADR1	Alcohol dehydrogenase II synthesis regulator
AP-1	Activator protein-1
BDI	Beck Depression Inventory
Bp	Base pair
cDNA	Complementary DNA
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CV	Coefficient of variation
DEX-CRH	Dexamethasone-Corticotropin releasing hormone
DNA	Deoxyribonucleic acid
ELS	Early life stress
fMRI	Functional magnetic resonance imaging
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GCRC	General Clinical Research Center
GxE	Gene-environment interaction
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA	Hypothalamic-pituitary-adrenal axis
HPRT1	Hypoxanthine phosphoribosyltransferase
LINE-1	Long interspersed nuclear element-1
(m)RNA	(Messenger) Ribonucleic acid
MIST	Montreal Imaging Stress Test
NGFI-A	Nerve growth factor-inducible protein A
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
SLE	Stressful life event
SLE-5	Stressful life events during the first five years of life
SNS	Sympathetic nervous system
TICS	Trier Inventory of Chronic Stress
TF	Transcription factor
TFBS	Transcription factor binding site
TSST	Trier Social Stress Test
UNG	Uracil-N-glycosylase
VAS	Visual Analogue Scale

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Introduction

Early life stress (ELS) has serious detrimental effects on individuals' neurodevelopment, and physical and mental health in adulthood as demonstrated by various animal and human studies (for recent reviews, see Lupien et al., 2009; Heim et al., 2010; McCrory et al., 2012). However, the exact mechanisms by which ELS leads to these effects and possible moderators of this relationship are not yet fully understood. ELS has been suggested to impair stress response systems in the body, such as the Hypothalamic-Pituitary-Adrenal axis (HPA), leading to changes in stress reactivity and vulnerability to psychopathology (for a recent review, see Heim et al., 2010). Over the last decade, studies on gene-environment interactions (GxEs) have provided evidence for the moderation of the effects of ELS by genetic factors, such as the serotonin (5-hydroxytryptamine, 5-HT) transporter-linked polymorphic region (5-HTTLPR; Caspi et al., 2010). Recently, some studies aiming to understand the molecular basis of these interactions reported epigenetic mechanisms, such as DNA methylation, to be one of the ways in which genetic and environmental factors interact (Meaney, 2010).

Taking a biopsychosocial perspective, the aim of this study was to investigate the impact of ELS on individuals' stress reactivity in response to an acute psychosocial stressor, the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), based on the following three aims. *The first aim* was to investigate the effects of ELS and 5-HTTLPR on stress reactivity to the TSST by measuring salivary cortisol levels. *The second aim* was to investigate the effects of ELS at the molecular level, through changes in the expression of two stress-related candidate genes, which encode the serotonin transporter (5-HTT) and glucocorticoid receptor (GR), at the level of mRNA. Complementing the

effects of change in gene expression as a function of ELS, *the third aim* of this study is to investigate DNA methylation, a prominent epigenetic mechanism of gene regulation, as a putative way by which ELS modulates stress reactivity.

Early life stress

Considering the high prevalence rate and multitude of psychosocial, physical, and behavioral consequences of ELS on the individual, investigating the mechanisms underlying the effects of ELS becomes crucial. Several types of early life stressors have been reported, which can be categorized under abuse (physical, sexual, and emotional), neglect (emotional and physical), and other traumatic events, such as loss of a caregiver, or experiencing a life-threatening accident or disease (Barnett et al., 1993; Agid et al., 1999; Heim & Nemeroff, 2001). The impact of ELS on stress reactivity may be influenced by the type and timing of these stressors, together with genetic factors and adulthood stressors (Heim & Nemeroff, 2001; Heim et al., 2010; Taylor, 2010; Uher & McGuffin, 2010; Mueller et al., 2011). As a result, ELS can predispose individuals to various psychopathologies, including unipolar and bipolar depression, post-traumatic stress disorder (PTSD) and phobias, and increase the risk of suicide (for a review, see Heim et al., 2010).

The link between ELS and its detrimental consequences on the individual is suggested to be mainly through the alterations in the brain, leading to impairments primarily in the Hypothalamic-Pituitary-Adrenal axis (HPA) stress response system (for reviews, see Heim et al., 2010; Schmidt, 2010).

Stress and the Hypothalamic-Pituitary-Adrenal Axis

Reactivity to stress, defined as "the nonspecific response of the body to any demand" (Selye, 1974, p.14 as cited in Everly & Lating, 2002), varies widely between individuals due to their appraisal of the situations (Lazarus, 1966). This variability is also influenced by the individual's genetic make-up, environmental factors, and their interaction. Once a situation is appraised as stressful, the individual goes through a process called *allostasis,* which involves attempts to adapt to the stressor. The physiological cost of allostasis to the individual in the long run is referred to as the *allostatic load*, which leads to failures in the adaptive systems of the body (McEwen, 1998). These adaptive systems are the cardiovascular, metabolic, immune, and central nervous systems (CNS), that are in continuous interaction, mainly through the effects of hormones (McEwen, 1998). The alterations in these systems may make individuals more vulnerable to various physical and mental health problems.

The main stress response systems in the CNS are (1) the autonomic nervous system that triggers the immediate fight-or-flight response of the sympathetic nervous system (SNS) through the sympathetic-adrenal medullary axis, and (2) the HPA, which is an important part of the neuroendocrine system and is suggested to be activated in addition to the fight-flight system in case of a more prolonged or extreme stress that exceeds the levels that can be handled by an individual's resources and evokes a threat (Blascovich & Tomaka, 1996). In addition, HPA activation is strongly linked to psychological stress, both chronic and acute (Pacak & Palkovits, 2001). For both systems, the initiation of the stress response starts in the hypothalamus. In the fight-flight

response, this is followed by noradrenaline and adrenaline being secreted from the adrenal medulla.

In the HPA, stress leads to the release of corticotropin-releasing hormone (CRH) from the neurons in the paraventricular nucleus (PVN) of hypothalamus. The release of CRH leads to adrenocorticotropic hormone (ACTH) release from the anterior pituitary. ACTH release triggers the secretion of glucocorticoids (GCs; mainly *cortisol* in humans; corticosterone in rodents) from the adrenal cortex. GCs then regulate the CRH and ACTH release, in addition to their own release, through negative feedback loops (Bear et al., 2007; see Figure 1). Furthermore, GCs suppress the immune responses, such as the expression of inflammation-related cytokines, in order to prevent the damaging effects of these molecules on tissues (Gross & Cidlowski, 2008).

The PVN is an important site for the integration of stress-related signals and GC effects from other regions of the brain, such as the amygdala, hippocampus, and brain stem, and the initiation of the HPA response here is regulated by various molecules (for recent reviews, see Gunnar & Quevedo, 2007; Ulrich-Lai & Herman, 2009). One of these molecules is 5-HT, which is involved in the initiation of the HPA response by triggering CRH expression (Herman et al., 1996; Jørgensen et al., 2003; Charney, 2004). 5-HT was shown to play an important role in hypothalamic innervation, either through inputs directly from the raphe nucleus or through 5-HT-positive fibers that surround the PVN. The effects of 5-HT are mediated mainly through its transporter (5-HTT) and various receptors (Jørgensen et al., 2003; Lanfumey et al., 2008). 5-HTT is involved in the reuptake of excess serotonin from the synaptic cleft and mice deficient in 5-HTT show increased depressive-like behavior (Wellman et al., 2007). A genetic variant in the 5-

HTT gene in humans (5-HTTLPR) that leads to lower expression of the gene (Lesch et al., 1996; Bradley et al., 2005) was associated with neuroticism, altered brain activation to emotional stimuli, HPA reactivity, and increased depressive symptoms in the case of exposure to stressful life events (for reviews, see Canli & Lesch, 2007; Caspi et al., 2010). These studies underline the importance of the serotonergic system on regulating HPA reactivity and vulnerability to psychopathology.

In addition to 5-HTT, one other major regulator of HPA reactivity is the GR, which is mainly involved in the termination of the HPA response through negative feedback. GR is a nuclear receptor that can alter gene expression through binding to GCs. After GC-binding, GR is translocated to the nucleus of the cell, where it can induce or repress the expression of a great number of genes in different tissues. It was reported that the expression of 20% of the genes expressed in human leukocytes can be regulated by GR (Galon et al., 2002).

There are two mechanisms of gene expression regulation by GR: 1) binding to DNA sequences called glucocorticoid response elements (GREs) in the transcription-facilitating regions (*promoters*) of GC-responsive genes and interacting with the basic transcription machinery, 2) interacting directly with transcription factors (TFs), such as the activator protein-1 (AP-1; Jonat et al., 1990), altering their effects on gene expression (Jonat et al., 1990). GR's influence on gene expression in relation to HPA reactivity depends on the proper functioning, availability and translocation of the GR (Nicolaides et al., 2010) and any influence disrupting this mechanism may alter HPA reactivity.

During stress, HPA activation and the release of GCs is beneficial in the short term for alerting the brain, dampening the immune responses and maintaining the homeostasis. However, if the stressful situation is beyond the limits of control, such as when it is prolonged, repeated or intense (as ELS can be), then the system may be disrupted and individuals may show altered stress responses to acute stressors, as reflected in the markers of HPA activity, such as cortisol levels (McEwen, 2007). Various acute psychosocial stress paradigms have been developed to investigate HPA activation in laboratory settings and one of the most reliable paradigm among those is the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), which is the stressor utilized in this study.

The Trier Social Stress Test

The TSST is a standardized acute psychosocial stressor that was reported to reliably activate the HPA better than many other acute stressor tests due to its induction of psychological unpredictability and social-evaluative threat component (Dickerson & Kemeny, 2004). The paradigm consists of a short preparatory period followed by the testing period that consists of public speaking and mental arithmetic components, and a recovery period. During the preparatory period, the participant is informed that he/she will give a speech in front of a committee to convince them that he/she is the best candidate for a job and that his/her verbal and non-verbal behavior will be evaluated. While the participant is allowed to take notes during the preparatory period, he/she is informed that these notes will not be available for later use. The public speech lasts 5 minutes, during which time the participant faces a two-person committee that provides no feedback on any kind. This speech is then followed by a mental arithmetic task, which

involves counting backwards from a high number in uncommon increments for 5 minutes (e.g., counting back in increments of 17 from the number 1342). Participants are told to start from the beginning every time they make a mistake. The continuous lack of verbal and non-verbal feedback from the committee during the speech and negative feedback during the arithmetic task creates the perception of the TSST as uncontrollable and threatening to the social self. The TSST was shown to produce a 2-3 fold increase in salivary cortisol levels in 70-80% of the participants within 10-20 minutes (Kudielka et al., 2007). Moreover, it triggers additional systems like the SNS and the immune system (Kudielka & Wüst, 2010).

The activation of the HPA in response to the TSST was studied widely in order to investigate the effects of various genetic and environmental factors, and their interactions on HPA reactivity (for a review, see Kudielka & Wüst, 2010). The main environmental factor of interest in this study was ELS.

Early life stress and HPA reactivity

The effect of ELS on HPA reactivity has been investigated in both animal and human studies (for a recent review, see Heim et al., 2010). In animals, ELS has been modeled using paradigms such as prenatal stress, poor maternal care, and maternal separation, and is generally associated with hyperactivation of the HPA and increased depression-like behavior in adulthood (Levine, 1967; Plotsky & Meaney, 1993; Liu et al., 1997; Francis et al., 1999; Ladd et al., 2000; Liu et al., 2000; Schmidt, 2010). Similarly, in humans CRH hypersecretion was reported to be an indicator of depression (Risbrough & Stein, 2006) and CRH mRNA levels were shown to be increased in the PVN of depressed

patients (Raadsheer et al., 1995). However, our understanding of the role of ELS in these alterations is not clear due to possible confounding effects of depression. In a series of studies, Heim et al. (reviewed in Heim et al., 2010) attempted to clarify this point by utilizing depressed and non-depressed individuals with or without ELS. Comparing women who had experienced ELS to women who had not, these investigators reported greater ACTH responses to the TSST as a function of ELS (Heim et al., 2000). In another study, similar effects were observed in men (Heim et al., 2008) in response to the dexamethasone-CRH (DEX-CRH) test, an indicator of CRH hypersecretion. In both of these studies, the depressed group with ELS had the highest HPA reactivity, followed by non-depressed individuals with ELS. It is important to note that while the ACTH response to the TSST was increased in ELS women, non-depressed women with ELS showed similar cortisol responses as did controls (i.e., individuals who had neither a history of ELS nor depression). This led the authors to suggest that the women who have not (yet) developed depression may be at increased risk for depression through the effects of additional negative life events that disrupt this control mechanism (Heim et al., 2002). Interestingly, depressed individuals without ELS had much lower reactivity, similar to the controls, suggesting the increase in HPA reactivity to be related to ELS, rather than depression *per se*. Contradicting Heim et al.'s studies, however, others have reported decreased ACTH (Carpenter et al., 2007) and cortisol (Carpenter et al., 2007; MacMillan et al., 2009) responses to the TSST in healthy individuals with ELS. Additional studies suggested potential reasons for these conflicting results, such as differences in gender (DeSantis et al., 2011), prior history of or current depression (Rao et al., 2008), the type

of ELS (Carpenter et al., 2009), and exposure to additional negative events and chronic stress in adulthood (Rao et al., 2008).

In addition to these factors, studies on GxEs suggest moderation of the relationship between ELS and HPA reactivity by genetic factors. One of the widely studied polymorphisms in this respect is the 5-HTTLPR, which will be discussed in the next section.

GxE studies of 5-HTTLPR and ELS

5-HTTLPR is one of the most widely studied polymorphisms in GxE studies, mainly in the context of its interaction with Stressful Life Events (SLEs) on predicting phenotypes related to stress reactivity and vulnerability to psychopathology (for reviews, see Canli & Lesch, 2007; Caspi et al., 2010). This polymorphism refers to a short (S) and a long (L) variant (*allele*) of the gene, with the short allele producing lower expression of the gene (Lesch et al., 1996). The traditional system of grouping the three types of carriers of these alleles (SS, SL, and LL) has been "biallelic", such that carriers of the S-allele (SS and SL) would often be grouped together because their gene expression values are similar, and they are compared to the homozygous L-allele carriers. In more recent work, grouping has become "triallelic", based on evidence for another polymorphism located within the L-allele (rs25531) that is an A/G Single Nucleotide Polymorphism (SNP), such that there are now considered to be these three alleles: S, L with a G-SNP (denoted L_G), and L with an A-SNP (denoted L_A). The L_G allele is suggested to yield expression levels similar to the S-allele (Hu et al., 2005). Therefore, grouping of genotypes on the basis of the triallelic system usually compares carriers of the S-allele or its functional

equivalent L_G allele (SS, SL_G, L_GL_G) to homozygous L_A allele carriers (L_AL_A). It is important to note that the L_G allele is relatively uncommon (6.5% as reported in Wendland et al., 2006), which means that most LL subjects of unknown A/G SNP genotype will be L_AL_A.

Following the initial association study of 5-HTTLPR with neuroticism (Lesch et al., 1996), there have been numerous studies investigating the effect of 5-HTTLPR on various phenotypes. However, these studies generally explained a very small portion of the phenotypic variance (Lesch et al., 1996) and often yielded mixed results. In relation to HPA-reactivity to the TSST, there have been conflicting findings as well. Some studies reported heightened cortisol reactivity to the TSST or similar paradigms in S-homozygotes (Jabbi et al., 2007; Gotlib et al., 2008; Way & Taylor, 2010), while others reported no differences by genotype (Alexander et al., 2009; Armbruster et al., 2009; Wüst et al., 2009; Bouma et al., 2010; Mueller et al., 2011).

One of the potential reasons for these results was suggested to be the influence of environmental factors, such as ELS. Caspi et al. (2003) were the first to report such an interaction between 5-HTTLPR and SLEs in a cohort of individuals from a large longitudinal study. They have indicated that S-carriers who have experienced SLEs showed an increased risk for depressive symptoms, diagnosis of clinical depression and suicidality in comparison to L-homozygotes with the same number of SLEs. Multiple investigations have emerged following this seminal study, some replicated the original findings, while others only replicated partially (such as in a single gender or age group) or failed to replicate at all (for reviews, see Uher, 2008; Uher & McGuffin, 2010). In order to investigate the significance of this GxE, results of these studies were combined

and analyzed in two separate meta-analyses, both of which reported no or negligible effects of this interaction on depression (Munafò et al., 2009; Risch et al., 2009). However, critical reviews of these meta-analyses pointed out that the selection of included studies was biased and thus, investigated this interaction through systematic reviews (Uher, 2008; Caspi et al., 2010; Uher & McGuffin, 2010). These analyses concluded that the age of the participants and measures of SLEs were potential reasons for non-replications of the original study. More specifically, the interaction was not replicated in studies utilizing adolescents and those measuring SLEs through subjective self-reports rather than structured interviews or objective measures, suggesting that age and the way SLEs are measured may be important (Uher, 2008; Uher & McGuffin, 2010). In addition, the use of endophenotypes, such as HPA reactivity or gene expression, rather than different measures of depression was suggested to be a more enhanced way of investigating these interactions (Caspi et al., 2010).

The points that are raised by these reviews were considered in some of the recent studies. The first study that measured the HPA reactivity as an endophenotype to investigate this GxE was by Alexander et al. (2009), who utilized a public speaking test similar to the TSST in healthy males. They reported a significant GxE, such that that the highest cortisol reactivity was observed in S-carriers with high SLEs. Similarly, Mueller et al. (2011) reported a significant interaction of 5-HTTLPR and SLEs in younger adults who had experienced a high number of SLEs during the first five years of life (SLE-5). However, they did not find evidence for this GxE interaction in children or in older adults. Furthermore, they observed that cortisol reactivity in L-homozygotes was much higher than in S-carriers in the absence of SLE-5 and that this reactivity was negatively

correlated with the number of SLE-5, while it was positively correlated in S-carriers. This pattern of opposite correlations with life stress as a function of 5-HTTLPR genotype was strikingly similar to imaging data obtained from the amygdala and hippocampus and from self-report data on rumination reported by Canli et al. (2006). Considering these findings together with some other studies (such as Taylor et al., 2006), the effect of the S-allele on stress reactivity was suggested to interact with the environment. In addition, this study nicely illustrated the importance of age and timing of SLEs in investigating potential GxEs related to the HPA reactivity. The influence of these factors imply that the effects of SLEs may be reflected in the genome differentially through developmental processes leading to alterations in gene expression patterns, possibly through epigenetic mechanisms.

DNA methylation and changes in gene expression

In addition to the effects of genetic variations in the DNA sequence on gene expression, there are also epigenetic alterations in the DNA that do not change the DNA sequence but can also modify gene expression. One of the important epigenetic changes is DNA methylation, which involves addition of methyl groups to cytosine nucleotides, mostly in the context of adjacent cytosine-guanine dinucleotides (CpG sites). This methylation of the CpG site inhibits gene expression through either preventing the binding of certain TFs or attracting methyl-binding proteins that block the transcription initiation sites (Meaney & Ferguson-Smith, 2010). In this respect, both methylation at specific CpG sites and methylation across longer regions might influence gene expression. While most of the gene promoters include regions rich in CpG sites (called *CpG islands*), these regions are usually too hypomethylated compared to the rest of the genome to allow transcription of

the gene. Increased methylation in these regions is suggested to be related to the influence of environmental factors on the genome, providing a basis for GxEs (Zhang & Meaney, 2010).

The first study to explain this type of a mechanism of the influence of early environment on the genome was conducted by Weaver et al. (2004a), who reported that the HPA response of adult rats who had experienced good vs. poor maternal care as pups was different due to DNA methylation differences in the hippocampal GR promoter. More specifically, methylation of a CpG site that the nerve growth factor-inducible protein A (NGFI-A) binds was shown to be increased in pups with poor maternal care. The NGFI-A is a transcription factor whose expression in the hippocampus was suggested to be associated with increased serotonergic activity by maternal care leading to increased GR expression (Meaney et al., 2000; Laplante et al., 2002; Weaver et al., 2004bb). In addition to this site, there were also some additional CpG sites throughout the GR promoter that showed a similar pattern of methylation by poor maternal care. This differential methylation was shown to alter GR gene expression and feedback sensitivity, leading to differences in CRH expression and HPA reactivity (Weaver et al., 2004a). Interestingly, in a recent study Belay et al. (2011) reported that a genetic variation in 5-HTT in rats, similar to 5-HTTLPR in humans, may interact with prenatal stress and alter the expression of hippocampal GR, as well as corticosterone reactivity in adulthood. Therefore, GR expression might be regulated as a function of the interaction between ELS and 5-HTTLPR.

In humans, similar methylation differences were measured in hippocampal GRs in *postmortem* human brains of suicide victims but only if they had a history of childhood

abuse (McGowan et al., 2009). There were no differences in methylation between controls who died of natural causes and suicide victims who were not exposed to childhood abuse, suggesting the importance of differential effects of ELSs and psychopathology on HPA reactivity (Heim & Nemeroff, 2001). In addition to the *postmortem* human brain tissue, a similar methylation pattern in the same region was also observed in the cord blood GR promoters of newborns of depressed, relative to non-depressed, mothers (Oberlander et al., 2008). Interestingly, this pattern was also associated with the infants' cortisol response at 3 months (Oberlander et al., 2008). This finding is promising since the availability of brain tissue is limited and most of the studies depend on the measures from blood. Recently, Tyrka et al. (2012) investigated the same region in relation to ELS and cortisol responses to the DEX-CRH test. For the putative NGFI-A binding site, there was an increase in methylation with increased maltreatment. There were also significant correlations between other CpG sites in the promoter region and cortisol responses of the individuals.

In addition to studies considering the methylation of GR, there is also a growing literature on the methylation of a CpG island upstream of the 5-HTT (Philibert et al., 2007). The 799 bp long CpG island consists of 81 CpG sites and is located upstream of the gene and downstream of 5-HTTLPR, including an untranslated exon identified by Mortensen et al. (1999). Methylation throughout the CpG island was reported to increase in human lymphoblast cell lines towards this exon and on (Philibert et al., 2008). In a longitudinal study with twins, methylation in this region was suggested to be less heritable and influenced more by environmental factors (Wong et al., 2010).

Studies investigating the effect of methylation on gene expression showed both overall methylation (Philibert et al., 2008; Kinnally et al., 2010) and methylation of specific CpG sites (Olsson et al., 2010) might be involved in gene expression. Initially, this relation was suggested to be different as a function of 5-HTTLPR genotype (Philibert et al., 2007), although this observation was not replicated (Philibert et al., 2008). With respect to the moderating effects of 5-HTTLPR genotype, some studies reported higher methylation in S-carriers (Philibert et al., 2007; Kinnally et al., 2010) and that genotype may interact with methylation to increase the effects of ELS (Beach et al., 2011), although some reported the reverse pattern (van IJzendoorn et al., 2010).

With respect to ELS, several human and primate studies have reported associations between overall or CpG-specific methylation and childhood abuse, maternal separation and experience of traumatic life events on the risk for depression and PTSD, antisocial behavior, behavioral reactivity and unresolved trauma or loss (Philibert et al., 2008; Beach et al., 2010; Kinnally et al., 2010; Olsson et al., 2010; van IJzendoorn et al., 2010; Beach et al., 2011; Kinnally et al., 2011). Beach et al. (2010; 2011) reported increased methylation associated with childhood abuse and a mediating effect of 5-HTT methylation on that the relation between childhood sexual abuse and symptoms of antisocial behavior. In addition, they reported that 5-HTTLPR moderates this relationship and individuals with increased number of S-alleles had higher associations between methylation and antisocial behavior symptoms. In contrast, another study investigating the relationship between unresolved trauma or loss and 5-HTT methylation, investigators reported that the unresolved trauma or loss was associated with higher methylation in the L-homozygotes, but not in S-carriers (van IJzendoorn et al., 2010). Apart from humans,

Kinnaly et al. (2010) showed that Rhesus macaques experiencing ELS (maternal separation) exhibited positive correlations between behavioral activity and overall methylation of the 5-HTT gene in peripheral blood mononuclear cells (PBMCs), while there was no association for controls without ELS. In a follow-up study, they reported that the ELS group also had a higher behavioral stress response with increased methylation in both 5-HTT and throughout the whole-genome (Kinnally et al., 2011). This study is therefore also important in underlining the importance of investigating global measures of DNA methylation together with the methylation of candidate genes as a function of ELS.

These experiments suggest that changes in gene expression may occur as a result of environmental factors that may alter DNA methylation at specific regulatory regions of genes or throughout the genome.

Aims of the study

Based on the literature summarized above, there is evidence that ELS leads to impairments in HPA reactivity and that this relationship may be moderated by 5-HTTLPR. However, there is conflicting data on the direction of the effect of ELS on HPA reactivity, possibly due to factors such as age, gender, history of or current psychopathology and adulthood stressors/chronic stress. Moreover, these factors may also influence the effects of genetic factors, as well as gene expression and DNA methylation. A study design that would control for all these confounding variables would require a massive sample size that is beyond the scope of this dissertation and available lab funding. Therefore, this dissertation aimed to remove some of the most significant

confounds, at the cost of more limited generalizability. For example, in order to avoid genetic confounds due to ethnic stratification, only Caucasian individuals were included. In order to avoid confounds due to mental illness, only individuals without diagnosed mental illness (based on self-report) were included. In addition, subclinical ongoing depressive symptoms and chronic stress were measured and controlled for. Finally, in order to avoid confounds due to fluctuating sex hormone levels in women, which have been shown to affect cortisol reactivity (Kirschbaum et al., 1999; Kudielka & Kirschbaum, 2005) and gene expression (Heninger, 1997; Cosgrove et al., 2007), only males were recruited.

Based on the findings from previous studies, I hypothesized under Aim 1 that ELS would correlate positively with HPA reactivity to the TSST in the S-carriers of the 5-HTTLPR and correlated negatively with HPA reactivity in the LL homozygotes. Under Aim 2, I wanted to investigate whether differential HPA reactivity would also be reflected in the expression of 5-HTT and GR that are key regulators of HPA. Because the stress literature suggests that the gene expression at baseline might be different than the gene expression profile in response to stress (Morita et al., 2005; Nater et al., 2009; Tsolakidou et al., 2010), I aimed to compare the change in gene expression of these genes before and after the TSST and investigate whether this relationship would differ as a function of 5-HTTLPR genotype. To my knowledge, there are no studies to date that have investigated gene expression of 5-HTT and GR in response to the TSST and the possible role of 5-HTTLPR in this relation. I predicted that ELS would be correlated negatively with the expression of both GR and 5-HTT, and that this decrease might be more pronounced for the S-carriers, considering the lower transcriptional efficiency of

the S-allele. Finally, under Aim 3, I wanted to investigate the differences in the methylation of candidate regions of 5-HTT and GR. Based on prior evidence, I predicted that ELS would be associated with increased 5-HTT and GR methylation, both overall and at specific CpG sites. I predicted that this relation might be differ as a function of 5-HTTLPR, considering the studies reporting higher methylation and less expression of the S-allele. In addition, I predicted that there might be specific CpG sites that would be more affected by ELS due to being located on specific TFBSs, such as the case for the GR NGFI-A binding site. In sum, these investigations may yield data to identify some of the mechanisms of individual differences on the effect of ELS on stress reactivity in adulthood.

Methods

Participants

Prospective study participants were recruited from Stony Brook University and surrounding communities through flyers and newspaper and online advertisements. Participants were screened via phone interviews for eligibility. Exclusion criteria included: being female, smoking, habitual substance and/or alcohol abuse in the last 6 months, high (>30) or low (<18) BMI, use of any kind of mood-altering medication, thyroid disease, diabetes, or previous diagnosis of mental health problems, due to the effects of these factors on HPA reactivity. In addition, individuals who reported to be under immense stress or utilized any medication that alters HPA reactivity (such as asthma medications) were excluded. Finally, individuals were asked about their fear of having their blood drawn on a scale from 1 (no fear) to 7 (extreme fear). Only individuals with scores lower than 4 were recruited.

All of the participants (N = 105) completed questionnaires to assess early life and chronic stress and provided blood samples for genotyping and methylation analyses. Of these participants, seventy-one healthy Caucasian males aged 18-77 (M = 29.79, SD = 15.24) performed the TSST and provided cortisol samples to assess stress reactivity, and blood samples before and after the TSST to assess change in candidate gene expression. The remaining participants (n = 34) performed a similar stress test in the fMRI scanner, called the Montreal Imaging Stress Test (MIST; Dedovic et al., 2005). As a result, gene expression and cortisol analyses were only carried out with the TSST participants, while 5-HTTLPR genotype and DNA methylation data were available for both TSST and MIST participants. Individuals participating in the TSST and MIST were compensated $100 and $50, respectively, plus public transportation costs. The flowchart that summarizes the number of participants included in each analysis is given in Figure 2.

Self-report measures

Demographics. Age, gender and ethnicity were assessed with a self-report demographics form.

Early life stress. Early life stress was assessed with the Childhood Trauma Questionnaire (CTQ; Bernstein et al., 1994), which is a 28-item self report of childhood maltreatment with subscales that cover physical, sexual, and emotional abuse and physical and emotional neglect. Each subscale consists of 5 items, while 3 items are utilized to control for denial of maltreatment. The items are rated on a 5-point Likert scale (1-5) with higher scores indicating higher maltreatment. The relevance of subscales to different forms of childhood trauma was confirmed in a large cohort (Scher et al.,

2001) and the scale was reported to have high internal consistency, test-retest reliability and convergent validity (Bernstein et al., 1994; Bernstein et al., 1997).

Chronic stress. Chronic stress during the last three months was assessed with the Trier Inventory of Chronic Stress (TICS; Schulz & Schlotz, 1999; Schulz et al., 2004). TICS is a 12-item self report measure that assesses the frequency of experiences related to chronic stress, such as having too many duties to fulfill or having worries that overwhelm one self. Items are on a scale from 0-4 and added up to obtain a measure of chronic stress. Higher scores indicate higher chronic stress. The scale was recently applied to a large sample and reported to have good internal consistency (Petrowski et al., 2012).

Depressive symptoms. Considering that the participants were only prescreened for previously *diagnosed* psychopathology (based on self-report), I aimed to control for the influence of potential ongoing and undiagnosed depressive symptoms by using the Beck Depression Inventory II (BDI-II; Beck et al., 1996). The BDI-II is a 21-item self-report measure of current depressive symptoms such as sadness, hopelessness, and self-blame. Each item is on a scale from 0-3. Higher scores indicate higher depressive symptoms and individuals who score 20 or above are considered to experience moderate to severe depression (Beck et al., 1996) BDI-II has good internal consistency, $\alpha = .93$ (Beck et al., 1996). Considering the potential influence of ongoing depressive symptoms on stress reactivity, individuals who scored at 20 or higher ($n = 8$) were excluded from analysis.

Experience of the TSST. Experience of the TSST was assessed using a self-report Visual Analogue Scale (VAS) that was administered right after the TSST. The scale

consists of 8-items that are related to the participants' perception of the TSST, such as being threatening, stressful, or challenging. For each item, participants reported percentages by placing an X over a line that ranges from 0 to 100.

Experimental procedure for the TSST

After the screening over the phone, eligible participants were invited. The experimental sessions were carried out in the same way either at the Stony Brook University Hospital General Clinical Research Center (GCRC) or at the Stony Brook University Psychology Department. All of the sessions started between 12-2pm, in order to keep consistency in the diurnal rhythm of cortisol across all study participants. Participants were instructed to refrain from eating, drinking (other than water) and exercise for at least an hour before their arrival.

The total procedure, which took about 4 hours, included consenting, completion of questionnaires, the TSST, a life events interview and debriefing. Participants also provided blood samples for genotyping and methylation analyses, one at the beginning of the session (at least 45min before the TSST) and one at the end (105 min after the TSST). Cortisol levels were assessed via the saliva samples collected throughout the session. The consent and debriefing forms are given in Appendices A and C, respectively.

Following consenting, participants provided the first saliva (*not* used in analyses) and baseline blood sample and filled out various questionnaires. After 45 minutes from the first blood draw, participants provided the second saliva sample (used as baseline) and were taken to the TSST room by the instructions given in Appendix B.

The Trier Social Stress Test was performed as described in Kirschbaum et al. (1993). Briefly, the task consisted of a preparation phase (5 min) followed by a public speech (5 min) on why the participant would be the best candidate for his or her dream job and a backward-counting task (5 min) in front of a two-person committee that provided no verbal or non-verbal feedback. The active committee member, who gave instructions to the subject during the TSST, was always of the opposite sex (female) and the inactive committee member, who did not communicate with the participant, was always of the same sex (male) as the participant. After the TSST, participants returned to initial testing room and provided a saliva sample (2 min after the TSST) and filled out the VAS. Additional saliva samples were collected at 10, 20, 30, 45, 60, 90 and 105 minutes after the TSST. Following the TSST, an interview about the participants' life events was carried out followed by completion of additional questionnaires. The session ended by providing the second blood sample (105 min after the TSST) and debriefing.

Saliva sampling and cortisol analysis

For each saliva sampling, participants chewed a small polyester roll called a *salivette* (Sarstedt, Rommelsdorf, Germany) for a minute to collect saliva samples at 10 time points during the experiment: right after consenting, 2 minutes before the TSST and 2, 10, 20, 30, 45, 60, 90, and 105 minutes after the TSST. There were at least 45 minutes between the first blood draw and the saliva sampling 2 minutes before the TSST (baseline saliva sample) to eliminate the effects of possible stress generated by the blood draw. The salivettes were stored at -20^{o}C immediately after the session until being shipped to Brandeis University, Boston, for the analysis of cortisol concentration. Each sample was assayed in duplicates using a commercially available chemiluminescence immunoassay

(RE62019) with a sensitivity of 0.16 ng/ml (IBL International, Canada). Inter- and intra-assay coefficients of variation (CV; equals 100*SD/M) were less than 7% and 4%, respectively.

For investigating the *cortisol reactivity* to the TSST, the difference in cortisol between baseline and the highest level after the TSST was calculated, as was done in previous studies reporting an interaction between ELS and 5-HTTLPR (Alexander et al., 2009; Mueller et al., 2011). For all participants the highest response after the TSST was observed within 10-20 minutes after the TSST (see Figure 5). This reactivity measure was selected due to representing the response to the TSST, which is potentially more closely associated with changes in gene expression, rather than the use of area under the curve response over time that is associated more closely with the overall hormonal output in time (Pruessner et al., 2003).

Processing blood samples

Blood consists of various cell types that may differ from one another with respect to their gene expression and methylation profiles. In order to start with a uniform group of cells for subsequent analyses related to gene expression and methylation, mono-nuclear cells from the peripheral blood (PBMCs; monocytes and lymphocytes) were isolated from blood. The extraction of the PBMCs from whole blood was performed immediately after the blood draw using Leucosep® tubes (Greiner Bio-One Inc., NC) and Ficoll-Paque (GE Healthcare, PA) separation medium according to manufacturer's protocol. Use of these tubes along with ficoll allows the separation of the PBMC layer as a buffy coat after

centrifugation. The isolated PBMC pellets were stored at -80°C for subsequent DNA and RNA extraction procedures.

DNA and RNA extraction

DNA and RNA extractions from PBMC pellets were carried out by the AllPrep DNA/RNA/Protein Mini kit (Qiagen, CA) according to manufacturer's instructions. Quantity and quality of the DNA and RNA were assessed through NanoDrop ND-1000 (Thermo Scientific, DE) and all samples were stored at -20°C.

Genotyping

5-HTTLPR genotype was determined through amplification by the polymerase chain reaction (PCR) with the primers used by Wendland et al. (2006). PCR was carried out with 25 ng DNA at annealing temperature of 67.5^{o}C in Eppendorf Mastercycler gradient (Eppendorf, Germany). PCR products were run on 2.5% agarose gels stained with ethidium bromide and visualized using the Gel Doc EZ system (Biorad, CA). A subset of samples was processed twice to double-check genotypes along with positive and negative controls. As a result, individuals were genotyped as S/S, S/L or L/L.

For genotyping the A/G SNP (rs25531), 6 µl of the 5-HTTLPR PCR products were digested with 5 Units of *Hpa*II restriction enzyme (New England Biolabs, MA) for 3 hours at 37^{o}C followed by an inactivation step of 20 min at 65^{o}C. Digestion products were run on 2.5% agarose gels stained with ethidium bromide and visualized using the Gel Doc EZ system (Biorad, CA) to determine genotypes. As a result, individuals were genotyped as S/S, S/L_A, S/L_G, L_A/L_A, L_A/L_G, L_G/L_G. Since expression of L_G-allele was suggested to be similar to the S-allele (Hu et al., 2005), while performing triallelic (S, L_A,

L_G) classification, S/L_G and L_G/L_G individuals were grouped as S/S and L_A/L_G individuals were grouped as S/L.

Bisulfite treatment of DNA samples

Bisulfites are compounds that are commonly used in DNA methylation analyses due to their ability to convert the unmethylated cytosine nucleotides into uracils in the DNA, leaving the methylated cytosines unchanged. Using the Epitect Bisulfite kit (QIAGEN, CA), 500 ng DNA from each participant was bisulfite converted according to the manufacturer's instructions and stored at -20^{o}C until used in methylation analyses. In addition, 500 ng unmethylated (0%) and fully-methylated (100%) human DNA (Zymo Research, CA) were bisulfite treated along with the samples to be utilized as bisulfite conversion controls in all methylation analyses.

Methylation analysis by Sequenom Epityper MassArray

The Sequenom Epityper MassArray system is designed by Sequenom (San Diego, CA) for the quantification of methylation percentages in DNA fragments of 200-600 bp length. With this technique, DNA fragments of interest are bisulfite-converted, PCR-amplified and then cleaved into smaller units called *CpG Units* that contain one or more CpG sites. The methylation level for each of these CpG Units is the average methylation percentage of the CpG sites in that CpG Unit. In addition, the methylation percentages of each CpG Unit can be averaged to obtain methylation percentage of the whole amplified product (*amplicon*). Considering that different CpG Units contain different number of CpG sites, a weighted methylation average for each amplicon was calculated.

For the amplification of the regions of interest in the 5-HTT and GR genes, primers were designed using the Epityper software (Sequenom, CA), which creates primers specific to the bisulfite converted DNA sequence. One of the primers also includes a T7-promoter tag, which enables *in vitro* transcription of the product for sequence read out. The other primer includes a 10mer tag to balance differences in melting temperature between primer pairs. Following the PCR with bisulfite-converted DNA and these primers, the non-methylated cytosines are converted to thymines (C/T conversion). This change is utilized to detect methylation in the subsequent steps, mainly through the mass differences of methylated and non-methylated cytosines analyzed in the mass spectrum (Coolen et al., 2007). In order to select the primer set with the maximum CpG coverage, the amplicons with the candidate primer sets were visualized by using an R-script developed by Coolen et al. (2007). As a result, two primer sets covering 71 CpG sites of the 5-HTT CpG island (named as HTT3 and HTT6) and one primer set covering the exon 1F promoter of the GR (named as GR2) were selected (see Figure 3 for the fragmentation of each amplicon).

Primers with the Sequenom tags were ordered through the Stony Brook University DNA Sequencing Facility. For each amplicon, PCRs were carried out utilizing the Qiagen HotStar Taq polymerase kit (Qiagen, CA) with 2 µl bisulfite treated DNA and 160 nM from each primer in a total reaction volume of 40 µl. The primer sequences and optimized PCR conditions for each amplicon is given in Table 1. To assure successful amplification, PCR products for each amplicon of each participant were run on 2% agarose gels stained with ethidium bromide and visualized using the Gel Doc EZ system (Biorad, CA). Afterwards, PCR products were aliquoted in triplicates into 384-well plates

(Axygen, CA) and shipped on dry ice to Genomics Facility of Albert Einstein School of Medicine, New York, where the *in vitro* RNA transcription and base specific cleavage was performed and the samples were analyzed through MALDI-TOF mass spectrometry (Ehrich et al., 2005).

Gene-specific methylation analysis by Pyrosequencing

According to the results of preliminary experiments and literature on 5-HTT and GR methylation, one CpG Unit for each gene (HTT6 amplicon CpG9.10.11.12 and GR2 amplicon CpG10.11) was selected to be further investigated by pyrosequencing (Ronaghi et al., 1998). Pyrosequencing is based on DNA sequencing that allows CpG-site-specific methylation analysis of short DNA fragments that might be related to specific TFBS important in the regulation of gene expression. Similar to Sequenom Epityper MassArray, pyrosequencing also requires the PCR amplification of bisulfite-converted DNA with primers designed for the region of interest. In pyrosequencing. one of the PCR primers is tagged with biotin from its 5' end to allow binding to streptavidin-coated Sepharose beads (Qiagen, CA) and sequencing of single-stranded DNA. Primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, CA) and ordered through Stony Brook University DNA Sequencing Facility. PCR conditions were optimized using the Qiagen HotStar Taq polymerase kit (Qiagen, CA) for each amplicon with 1 µl bisulfite-converted DNA and 160 nm of each primer in a 25 µl total reaction volume. The PCR products generated were run on 2.5% agarose gels stained with ethidium bromide and visualized using the Gel Doc EZ system (Biorad, CA).

The pyrosequencing reactions were prepared with 5 µl of PCR product according to manufacturer's protocol and assayed in triplicates in the PyroMark Q96 Platform (Qiagen, CA) located in the Stony Brook University Genomics Core Facility. A single sequencing primer was utilized for each amplicon that spanned about 50 bp of the PCR product that includes the CpG sites of interest. Artificially unmethylated (0%) and fully-methylated (100%) samples of human DNA (Zymo Research, CA) were used as controls. In addition, samples with no template and with no sequencing primer were used as negative controls. The primer sequences and PCR conditions for each amplicon are given in Table 2.

Global methylation analysis by Pyrosequencing

The methylation of Long Interspersed Nuclear Element-1 (LINE-1) was used as a measure of global methylation both for investigation of associations with ELS (similar to Kinnally et al., 2011) and for controlling for global methylation when investigating gene-specific methylation (similar to performed by Oberlander et al., 2008). LINE-1 is among the repetitive sequences in the genome and is normally highly methylated. Decreased methylation of LINE-1 was reported by aging and in relation to genomic instability and carcinogenesis (Chalitchagorn et al., 2004; Kim et al., 2009; Irahara et al., 2010).

LINE-1 methylation was quantified by the PyroMark Q96 CpG LINE-1 kit (Qiagen, CA) in PyroMark Q96 MD system according to manufacturer's protocol using the commercial primers provided with the kit. The region that is analyzed includes 4 CpG sites. Modified PCR conditions are given in Table 2. Artificially unmethylated (0%) and fully-methylated (100%) samples of human DNA (Zymo Research, CA) were used as

controls. In addition, samples with no template and with no sequencing primer were used as negative controls. The PCR products generated were run on 2.5% agarose gels stained with ethidium bromide and visualized using the Gel Doc EZ system (Biorad, CA). The pyrosequencing reactions were prepared with 20 µl of PCR product according to manufacturer's protocol and assayed in duplicates in the PyroMark Q96 Platform (Qiagen, CA) located in the Stony Brook University Genomics Core Facility.

RNA integrity, reverse transcription and gene expression analysis

For reliable gene expression analysis, RNA sample should be of good integrity (meaning they are not degraded). The integrity of the RNA samples extracted from PBMCs was assessed with the Agilent 2100 BioAnalyzer (Agilent Technologies, CA) located in the Stony Brook University Genomics Core Facility. All of the samples had RNA integrity numbers (RIN) higher than 5 (most of the samples > 8), suggesting good integrity of RNA samples.

1 µg of RNA from each time point (at baseline and 105 min after the TSST) was converted to complementary DNA (cDNA) through the use of QuantiTect Reverse Transcription Kit according to the manufacturer's protocol (Qiagen, CA). The cDNA samples were then diluted five times with molecular grade water (Sigma-Aldrich, MO). 1 µl of the diluted cDNA samples was used for the gene expression analysis of the candidate genes at the mRNA level by quantitative PCR (qPCR), using the Qiagen SYBR Green PCR + UNG kit (Qiagen, CA) and gene specific primers. Roche Universal Probe Library website (http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) was utilized to design all primers and primer sequences are given in Table 3.

The qPCR reactions were carried out in triplicates in the Roche 480 LightCycler system (Roche Applied Science, IN) following uracil-N-glycosylase (UNG) treatment to eliminate possible PCR carryover contamination. All amplicons were optimized at the following PCR conditions: UNG treatment of 2 min at 50^{o}C, initial denaturation of 15 min at 95^{o}C and 45 cycles of 15 sec 95^{o}C and 1 min 60^{o}C. For all reactions, melting curve analysis was conducted to ensure successful amplification without any non-specific products or primer-dimers. Samples with no cDNA and no reverse transcriptase were used as negative controls. In addition, after the optimization of the PCR conditions of each amplicon, the qPCR products were run on 2.5% agarose gels stained with ethidium bromide and visualized using the Gel Doc EZ system (Biorad, CA) to assure amplification of the products with the expected size. Gene expression is measured as C_T (threshold cycle) values that is the number of cycles required for the signals from the PCR product to exceed the background signal. The more a gene is expressed, the earlier the threshold cycle will be reached; therefore, C_T values are inversely related to gene expression. C_T values that are obtained by the qPCR were then used to assess gene expression change between baseline and 105 min after the TSST samples through the delta-delta-C_T method (Livak & Schmittgen, 2001). The gene expression change for each sample is shown as fold-change values, representing the fold-change in the 5-HTT and GR after the TSST in comparison to baseline.

Selection of the best reference genes in PBMCs. For gene expression analyses, reference (*housekeeping*) genes that have relatively stable expression in time and across different tissues are used in order to normalize the expression of genes of interest (5-HTT and GR). To find out the best reference genes in PBMCs, the expression of six candidate

reference genes were analyzed from baseline and 105 min after the TSST RNA samples of five individuals. The candidate reference genes and their primer sequences are given in Table 3.

For the analysis of results, GenEx software (MultiD Analyses AB, Göteborg, Sweden) was used that has reference gene selection tools implemented within (Genorm by Vandesompele et al., 2002; NormFinder by Andersen et al., 2004) and that utilizes a modified delta-delta-C_T method with multiple reference genes for assessing gene expression. According to NormFinder analysis shown in Figure 4a, the number of housekeeping genes to be used is selected as 2, considering the small difference in *SD* values compared to using 5 genes. Both GeNorm and NormFinder results indicated HPRT1 (hypoxanthine phosphoribosyltransferase 1) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as the best reference genes in PBMCs (Figure 4b). C_T values of these genes were used to normalize GR and 5-HTT expression in all samples.

Statistical analysis

All statistical analyses were performed using SPSS for Windows version 16.0 (Chicago, IL). Significance level was taken as $\alpha = .05$ for all analyses. All variables were checked for outliers, normality and assumptions necessary for the statistical tests used.

Cortisol analysis. Prior to all analyses, cortisol data were tested for normal distribution by the Kolmogorov-Smirnov test. In case of violation of normality ($p > .05$), log-transformations were used. For investigating whether the TSST successfully evoked cortisol response, repeated measures ANOVA was used for the nine saliva samples collected throughout the experiment. Greenhouse-Geisser corrections were applied when

sphericity was violated. For the cortisol reactivity, outliers were defined as those whose cortisol values exceeded the group mean of the study sample by 2.5 z-scores, who were excluded from analysis. There were 3 such outliers, all of whom had extremely high baseline cortisol levels that decreased as the participant went through the TSST, leading to extreme negative values for cortisol reactivity. In addition, 1 participant had extreme cortisol values for all of the time points (z-score > 2.5) and was excluded from analysis.

Sequenom Epityper MassArray analysis. For each amplicon, results for each CpG Unit are investigated for quality control. As shown in Figure 3, CpG Units in gray were not analyzed in the mass spectrum due to their relatively high or low mass. Some CpG Units within the same amplicon were duplicates of each other, meaning they have the same mass, and thus yielded identical methylation levels. Since it was not clear from which CpG Unit the methylation levels come, these Units were also excluded from analysis. Duplicate CpG Unit pairs for each amplicon were as follows: HTT3 CpG3 – HTT3 CpG25.26, HTT6 CpG17.18 – HTT6 CpG25.26 and GR2 CpG26 – GR2 CpG34. Finally, two CpG Units (HTT6 CpG1.2 and GR2 CpG35) were excluded from analysis due to high *SD* (>10%) between triplicates in more than 1/3 of the participants. For the remaining CpG Units, if the *SD* across the triplicates was lower than 10% (cut-off as used by Godfrey et al., 2011; Izzi et al., 2012), their average was taken as the methylation percentage. If the *SD* was higher than 10%, then the extreme value within that triplicate was replaced with the mean of the triplicate. Across all the samples (TSST and MIST) and all CpG Units analyzed, there were three such cases for the GR2, eleven for the HTT3 and six for the HTT6 amplicon.

Pyrosequencing analysis. Following the pyrosequencing run, PyroMark software provides information on each sample, indicating whether the run was successful ('pass'), needs to be checked ('check') or failed ('fail'). All failed samples were repeated. For 'check' samples, pyrograms that show the peaks for each nucleotide of the amplicon were further investigated. If the dispensation order was correct and there was less than 2% *SD* between the replicates, average of the replicates were taken as the methylation level. If the *SD* across the replicates was higher than 2%, those samples were repeated.

For 5-HTT and GR amplicons, the CpG sites of interest were both significantly intercorrelated (all *p*-values < .05) and correlated with Sequenom CpG Unit methylation percentages that they belong to (all *p*-values < .05). Therefore, Sequenom CpG Unit methylation results were used in all analyses. For LINE-1 amplicon, the average of the 4 CpG sites analyzed was used as the LINE-1 methylation percentage for each sample as a measure of global DNA methylation.

Gene expression analysis. A cut-off of 0.5 *SD* in C_T difference between triplicates was used to detect outliers and there were no outliers in the sample analyzed as indicated by GenEx. In order to control for variability between different runs, the same cDNA sample was utilized in every plate (inter-plate CV < 5%).

Regression. A multiple regression was performed between the cortisol reactivity as the dependent variable and age, BDI total, TICS total, CTQ total and 5-HTTLPR genotype group as independent variables. CTQ scores were log-transformed for normality and were centered. Centered log-CTQ total scores were used to create an interaction term with 5-HTTLPR genotype group.

Correlations. For investigating correlations between stress, cortisol and methylation measures, Pearson's correlation coefficient was used. If necessary, partial correlations were used controlling for age, BDI, TICS and LINE-1 methylation.

Results

Aim 1: Is ELS associated with higher HPA reactivity to the TSST, specifically in the S-carriers of the 5-HTTLPR?

ELS, 5-HTTLPR and cortisol reactivity

Participant characteristics. A total of 59 healthy Caucasian men aged 18-77 were included in the final analysis of the effect of ELS and 5-HTTLPR on cortisol reactivity to the TSST. There was only one participant experiencing sexual abuse (scoring 7 out of 25), who also scored high on neglect subscales of the CTQ. Apart from sexual abuse, all CTQ subscale scores were significantly correlated with each other (r = .27 - .72) and with the CTQ total score (r = .67 - .88). Means and SDs of age, CTQ total, TICS total, BDI total and baseline cortisol and distribution of the 5-HTTLPR genotype is presented in Table 4.

5-HTTLPR genotypes were in Hardy-Weinberg equilibrium using both biallelic and triallelic classification systems ($p > .05$). Independent samples t-tests revealed no differences in age, CTQ or BDI total scores by 5-HTTLPR genotype group (all p-values > .05). LL participants had higher TICS scores ($p < .05$).

TSST and cortisol response. To determine whether the TSST was a successful stressor in triggering a cortisol response, a repeated measures ANOVA was used with

log-transformed cortisol measures due to non-normality (Kolmogorov-Smirnov test $p <$ .05). Due to violation of sphericity ($p < .05$), Greenhouse-Geisser corrected values were used. Results showed a significant change in cortisol in response to the TSST (F(2.53, 58) = 59.05, p = .000, partial η^2= .50; Figure 5).

After ensuring that the TSST worked as a successful stressor, cortisol reactivity in response to the TSST was calculated as the difference between baseline cortisol and the highest cortisol level after the TSST. This value was used in the subsequent analyses as the 'cortisol reactivity' of each participant. For 75% of the participants there was a positive cortisol reactivity, meaning an increase in cortisol levels in response to the TSST, while 25% of the participants had a decrease in cortisol levels following the TSST.

ELS and 5-HTTLPR on cortisol reactivity. A multiple regression was used to investigate the effect of ELS and 5-HTTLPR genotype on cortisol reactivity. Considering significant bivariate correlations between cortisol reactivity and age (r(57) = .36, p = .005) and BDI total scores (r(57) = -.34, p = .009), both of these variables were added as covariates in the regression model. In addition, in order to control for chronic stress, TICS score was added as a third covariate. Results indicated a significant interaction between ELS and 5-HTTLPR (F(1, 52) = 4.19, p = .046), such that S-carriers had higher cortisol reactivity with higher ELS (β = .49, t = 2.05, p = .046), while l-homozygotes showed the opposite pattern (β = -.30, t = -2.05, p = .046; Figure 6). There were no significant main effects of ELS or 5-HTTLPR. Controlling for the same covariates, individuals with higher cortisol reactivity reported feeling more threatened by the TSST (pr(54) = .29, p = .033; Figure 7).

Aim 2: Is differential HPA reactivity reflected in the expression of 5-HTT and GR?

ELS, 5-HTTLPR and gene expression. In order to understand the molecular mechanisms of the interaction between ELS and 5-HTTLPR on cortisol reactivity, change in *5-HTT* and *GR* expression between baseline and 105 min after the TSST were investigated. The fold-change in gene expression between the time points ranged from .25 to 2.14 for *5-HTT* (M = 1.05, SD = .43) and from .41 to 1.80 for *GR* (M = 1.07, SD = .30). There were 3 outliers for *GR* and 4 outliers for *5-HTT* (z-scores > 2.5) that were excluded from analysis.

Independent-samples t-test revealed a significant difference in *5-HTT* expression fold-change in response to the TSST, such that the S-group had lower *5-HTT* expression than the LL group ($t(53) = 2.74$, $p = .010$; see Figure 8). Furthermore, the S-group showed a negative correlation between ELS and gene expression ($r(31) = -.34$, $p = .053$), whereas the LL group showed no correlation ($r(20) = .011$, $p = .963$; see Figure 9).

Aim 3: Methylation of candidate regions of 5-HTT and GR

ELS, 5-HTTLPR and DNA methylation

Descriptives. In order to investigate the patterns between ELS and DNA methylation and possible changes as a function of 5-HTTLPR genotype, bivariate correlations on age, stress and methylation measures were run for the whole sample (N = 105), as well as for each 5-HTTLPR genotype group. 5-HTTLPR genotypes were determined according to triallelic classification, in which S-allele and L_G allele carriers constituted the "S-group", (n = 70) and L_AL_A-homozygotes constituted the "LL" group (n = 35). Methylation measures included individual CpG Units of all amplicons and overall

methylation of GR, 5-HTT (*HTT3 and HTT6 combined*) and LINE-1. Stress measures included CTQ total and subscales and TICS. BDI scores were not included in the analysis of the total sample due to lack of BDI scores from MIST participants.

Means and SDs for age, stress and DNA methylation measures for the whole sample, as well as for each genotype group are summarized in Table 5. None of the DNA methylation measures differed significantly by 5-HTTLPR genotype ($p > .05$).

Methylation levels of each CpG Unit across *5-HTT* and *GR* amplicons as a function of 5-HTTLPR are shown in Figures 10 and 11, respectively. For *5-HTT*, methylation was lower at the 5'- end of the CpG island (HTT3 amplicon) and increased towards the 3'-end (HTT6 amplicon), similar to a previous report (Philibert et al., 2008). In a region that was previously associated with ELS (Beach et al., 2010; Beach et al., 2011), HTT6 CpG27-41, methylation percentage was significantly higher than the overall 5-HTT CpG island methylation (Figure 12). In addition, a single CpG Unit in this region (HTT6 CpG27.28) was the only CpG Unit that was differentially methylation as a function of 5-HTTLPR genotype ($t(103) = 2.36$, $p = .020$).

For the GR2 amplicon, the highest methylation was observed in GR2 CpG10.11, which corresponds to the putative NGFI-A binding site of the Exon 1F promoter that was associated with childhood abuse (McGowan et al., 2009). None of the CpG Units were differentially methylated as a function of 5-HTTLPR.

Age and DNA methylation. Bivariate correlations between age and methylation measures are investigated and significant correlations ($p < .05$) are summarized in Table 6. Consistent with the literature (Fraga et al., 2007; Kim et al., 2009) age was negatively

correlated with LINE-1 methylation for the whole sample and the S-group. LL subjects also had a negative correlation, but not significant ($r(33) = -.23$, $p = 191$). Age was positively correlated with overall *5-HTT* and HTT6 CpG27-41 methylation for all groups. Age was also correlated with several CpG Units of *5-HTT*, mostly in the HTT6 amplicon, some of which were common to all groups, while some were specific to each genotype group. In addition, S-group had more CpG Units correlated with age. There was no correlation between age and any of the CpG Units of GR (all p-values > .05).

ELS and DNA methylation. In order to identify associations between ELS and *5-HTT*, *GR* and LINE-1 methylation, bivariate correlations between CTQ total and subscale scores and methylation measures were run and significant correlations ($p < .05$) were summarized in Table 7. *5-HTT* overall methylation was not correlated with any of the CTQ scores. However, when individual CpG Units were investigated, there was an interesting pattern in some CpGs of the HTT6 CpG27-41 (such as HTT6 CpG27.28) that ELS was associated with higher methylation of this region in the S-group ($r(68) = .26$, $p < .05$) and with lower methylation in the LL participants ($r(33) = -.33$, $p < .05$; see Figure 13). For methylation of other CpG sites in the 5-HTT, there were both positive and negative correlations with CTQ and subscales, mostly specific to each genotype group.

In relation to ELS and GR methylation, there was only a single CpG site (GR2 CpG9) that was associated with emotional abuse ($r(103) = .23$, $p < .05$; Figure 14). Finally, LINE-1 methylation was only associated with ELS in the LL group, having positive correlations with CTQ total (Figure 15) and all CTQ subscales except emotional abuse.

Chronic stress and DNA methylation. In order to investigate whether chronic stress in the last three months would be associated with changes in methylation, bivariate correlations between TICS total scores and methylation measures were run and significant correlations were summarized in Table 8. Chronic stress was associated with methylation of various CpG Units from all amplicons.

Chronic stress was associated with increased LINE-1 methylation in both genotype groups (Figure 16). For *5-HTT*, mostly CpG Units in the HTT6 CpG27-41 region were associated with chronic stress. Specifically, higher chronic stress was associated with decreased HTT6 CpG27.28 methylation for both genotype groups (Figure 17). Outside of HTT6 CpG27-41, some CpGs of the HTT3 amplicon were also correlated positively with TICS.

Associations between 5-HTT and LINE-1 methylation. For the whole sample, LINE-1 methylation was correlated with *5-HTT* overall methylation ($r(103) = .39$, $p < .001$), but not with HTT6 CpG27-41 ($r(103) = -.094$, $p = .339$). Most of the CpG Units of *5-HTT* were correlated positively with LINE-1 methylation ($r = .20$-$.62$) and the ones that did not correlate were mostly in HTT6 CpG27-41 region. Genotype groups had similar patterns except for HTT6 CpG27.28. This Unit was negatively correlated with LINE-1 in LL participants ($r(33) = .370$, $p < .05$), but not in S-group ($r(68) = -.042$, $p = .727$; Figure 18).

Associations between GR and LINE-1 methylation. Overall *GR* methylation (Figure 19) and all GR2 CpG Units, except GR2 CpG10.11 and CpG12.13, were correlated with LINE-1 methylation ($r = .28$-68). All of these correlations were similar

for both 5-HTTLPR genotype groups, except CpG9, which was only correlated with LINE-1 methylation in the S-group ($r(68) = .29$, $p = .016$).

Associations between 5-HTT and GR methylation. Most of the CpG Units and overall methylation of *5-HTT* and *GR* were positively correlated for the whole sample (r =.27-75; Figure 20). *5-HTT* CpG Units that were not correlated with *GR* were mostly in the HTT6 CpG27-41 region. HTT6 CpG40.41 was the only Unit that was correlated negatively with *GR* methylation in LL participants ($r(33) = -.49$, $p = .003$; Figure 21). For GR2 CpG Units, all were correlated with *5-HTT* methylation (r = .28-.66) except GR2 CpG12.13.

ELS, 5-HTTLPR and 5-HTT methylation in the TSST participants

Considering information from the initial analyses mentioned above, 5-HTT overall and HTT6 CpG27-41 methylation as well as methylation at HTT6 CpG27.28 were investigated in relation to ELS and 5-HTTLPR. A summary of the DNA methylation measures for the TSST participants are given in Table 9. Similar with the initial analyses, *5-HTT* overall methylation was not associated with CTQ. However, 5-HTTLPR genotype groups exhibited different methylation patterns as a function of ELS within the HTT6 CpG27-41 region, specifically at HTT6 CpG27.28. Therefore, this CpG Unit was investigated in detail.

Methylation at HTT6 CpG27.28. Although differentially methylated by 5-HTTLPR genotype, methylation of this Unit significantly correlated with HTT6 CpG27-41 region in both genotype groups (r = .57-.62, $p < .001$; Figure 22). However, in relation to ELS, S-group participants exhibited a positive correlation between HTT6

CpG27.28 methylation and ELS ($r(33) = .39$, $p = .021$), whereas the LL group showed an opposite trend ($r(22) = -.39$, $p = .063$; see Figure 23).

Methylation at HTT6 CpG40.41. Similar to the results of the initial analysis, methylation at HTT6 CpG40.41 was negatively associated with ELS for the LL participants, while there was no correlation for the S-group (Figure 24).

ELS, 5-HTTLPR and GR methylation in the TSST participants

GR methylation, ELS and cortisol reactivity. Results of the initial methylation analysis indicated a relation between emotional abuse and GR2 CpG9 methylation in the S-group individuals. In order to examine whether this would be reflected in cortisol response, the same relationship was also investigated in the TSST sample. There was again a significant correlation between emotional abuse and CpG9 methylation only in the S-group ($r(33) = .42$, $p = .012$; Figure 25) and higher methylation of this site was also associated with higher cortisol increase in the S-group ($r(33) = .39$, $p = .019$; Figure 26), while an opposite pattern was observed in LL participants.

Discussion

The aim of the present study was to investigate whether ELS would have an impact on stress reactivity, stress-related gene expression and DNA methylation, and whether these relationships would be influenced by 5-HTTLPR. In order to investigate these relations without the influence of known confounders of HPA reactivity, all of the participants were healthy Caucasian men prescreened for factors influencing HPA reactivity. Findings of this study suggest that ELS influences cortisol reactivity, candidate gene expression and DNA methylation and these relationships can be moderated by 5-HTTLPR.

Aim 1. ELS, 5-HTTLPR and cortisol reactivity

The first aim of this study was to investigate the effect of ELS and 5-HTTLPR on cortisol reactivity to the TSST. Results indicate an interaction between ELS and 5-HTTLPR such that ELS increased cortisol reactivity in S-allele carriers, but decreased cortisol reactivity in homozygous L-allele carriers (Figure 6), controlling for the potential confounders of cortisol reactivity (age, depressive symptoms and chronic stress). LL participants with low ELS had higher cortisol reactivity than the S-group with low ELS and this pattern was reversed as ELS increased. For LL participants, cortisol reactivity decreased by higher ELS, while it increased for the S-group. This pattern between ELS and 5-HTTLPR on cortisol reactivity is similar to what was reported in the two previous studies investigating the same interaction (Alexander et al., 2009; Mueller et al., 2011). In addition, studies investigating the same interaction with different outcomes also reported similar results. For example, a similar pattern was observed in studies investigating the interaction between 5-HTTLPR and family environment on the risk of depression (Eley et al., 2004; Taylor et al., 2006; Wilhelm et al., 2006). Moreover, Canli et al. (2006) have reported a similar interaction between 5-HTTLPR and SLEs on amygdala and hippocampus activation in healthy adults.

Considering the results of these studies together with my results, the evidence illustrates that individuals with different 5-HTTLPR genotypes respond differentially towards environmental stimuli, rather than only the S-group being susceptible/plastic to changes in the environment as suggested by others (Belsky & Pluess, 2009). Thus, S-carriers of this polymorphism tend to function better in favorable environments and worse in unfavorable environments, whereas the opposite pattern is true for the LL group.

This interaction may partially explain the inconsistencies between the studies investigating only the effect of ELS (Heim et al., 2000; Heim et al., 2002; Carpenter et al., 2007; Heim et al., 2008; MacMillan et al., 2009) or 5-HTTLPR on HPA reactivity (Jabbi et al., 2007; Gotlib et al., 2008; Alexander et al., 2009; Mueller et al., 2009; Wüst et al., 2009; Way & Taylor, 2010). In addition to providing a more comprehensive thinking about individual differences in stress reactivity, this idea of differential genetic sensitivity may also influence the design and follow up of behavioral and pharmacological interventions. For example, consideration of ELS in addition to 5-HTTLPR genotype would improve the findings of previous studies suggesting differential responses to behavioral and pharmacological therapies for psychopathology by 5-HTTLPR (Smits et al., 2008; Bryant et al., 2010; Porcelli et al., 2012).

Aim 2. ELS, 5-HTTLPR and gene expression

Considering the interaction between ELS and 5-HTTLPR on cortisol reactivity, I was interested in the corresponding changes in 5-HTT gene expression. I found that participants in the S-group had significantly lower 5-HTT gene expression in response to the TSST than did LL participants (Figure 8). This finding is consistent with the reports associating the S-allele with lower expression of the 5-HTT (Lesch et al., 1996; Bradley et al., 2005). Previous reports considering the effect of ELS in relation to 5-HTT expression reported lower 5-HTT expression (Kinnally et al., 2010b) or 5-HTT binding potential (Miller et al., 2009) independent of 5-HTTLPR genotype. According to the results of this study, S-group individuals had a negative correlation between ELS and 5-HTT expression, indicating even less 5-HTT expression with ELS (Figure 9), whereas LL individuals were not influenced by ELS. It was unexpected to see no association between

5-HTT expression and ELS in the LL participants. Considering their cortisol reactivity pattern, I would expect a positive correlation between 5-HTT expression and ELS for these participants. Looking at Figure 9, LL participants seem to have a positive correlation between ELS and 5-HTT expression when they are at the lower end of CTQ scale, while this pattern changes as CTQ scores increase. However, due to low number of LL participants with higher CTQ scores, it is difficult to drive a conclusion. For future studies, it would be interesting to investigate the same relationship with higher number of participants across the CTQ scale.

Considering the fold-changes in gene expression, the amount of gene expressed between baseline and after the TSST is not very high (around 2-fold). One of the reasons for this might be related to the timing of the samples. It is possible that 105 minutes is still early to see the effects of the TSST in the expression of our candidate genes. Studies that investigate the influence of stressors in animals usually consider longer time frames, such as 4-8 hours (Tsolakidou et al., 2010) or even longer as is reported in a study in humans (Morita et al., 2005). Actually, initially I have tried two time points to test gene expression changes (one 45 minutes after and one 105 minutes after the TSST) and preliminary results indicated that the change in expression was higher in the samples collected later. Therefore, it is possible that the changes will be more pronounced in a longer time frame. On the other hand, having blood samples from the same individuals at different time points is potentially very useful in controlling for inter-individual factors that would influence gene expression and might be one of the reasons we had significant results even with subtle fold-changes in 5-HTT expression.

In relation to GR, there was no effect of ELS on gene expression. In addition, GR expression was not correlated with 5-HTT expression. According to the studies suggesting the interaction between serotonergic pathways and GR within the HPA (Mitchell et al., 1990; Mitchell et al., 1990b; Mitchell et al., 1992; Weaver et al., 2004b), I expected a correlation between the expression of these genes. However, considering that GR is found abundantly in various tissues and can influence the regulation of many genes, it is possible that 5-HTT and GR expression are not linked to each other in the periphery and that there is no change as a function of 5-HTTLPR. On the other hand, it is possible that their effects on each other might be regulated by other mechanisms, such as through DNA methylation, which will be discussed in the next section.

Aim 3. ELS, 5-HTTLPR and DNA methylation

Differences in methylation by age. Consistent with the literature suggesting increases in promoter methylation by age (Richardson, 2003), there was a positive correlation between 5-HTT and GR methylation for most of the CpG Units investigated. On the contrary, LINE-1 methylation is suggested to decrease by age (Kim et al., 2009), which was also the pattern in our sample set.

Differences in LINE-1 methylation. In relation to global methylation, I found that LINE-1 methylation was correlated with ELS, but only in the LL participants. Strikingly, in a recent study of Rhesus macaques with the LL genotype (Kinnally et al., 2011), it was also reported that ELS was associated with global methylation changes. In order to control for 5-HTTLPR genotype effects, these researchers reported only using LL-homozygotes and concluded that global methylation may be one of the ways that also

affects the LL participants. Considering the results of our study, we can extend these findings saying that global methylation changes as a function of ELS is only observed in the LL participants, but not in the S-group. This suggests that ELS may have differential effects on DNA methylation as a function of 5-HTTLPR. Considering that global methylation changes by ELS only affects LL participants, it would be interesting to question whether this background risk would contribute to the high correlations between early life and chronic stress observed only in these individuals. On the other hand, it is possible that ELS also influences methylation of specific genes, which will be discussed next.

Differences in 5-HTT methylation. The 5-HTT CpG island methylation pattern was similar to what was reported by Philibert et al. (2008), with intercorrelations between CpG Units being higher in the 5' end of the CpG island, while the overall methylation increased towards the 3' end. A region at the 3' end, HTT6 CpG27-41, was methylated significantly higher than the overall 5-HTT methylation in both genotype groups. In two recent studies, the same region was selected to represent the overall methylation of the CpG island (Beach et al., 2010; Beach et al., 2011) and was associated with ELS. Consistent with these studies, methylation in this region was correlated with overall 5-HTT methylation. However, in relation to ELS, methylation pattern in this region was not similar with 5-HTT overall methylation. Therefore, it is important to point out that the association between ELS and methylation of this region may not represent the association with the overall 5-HTT methylation.

Examination of CpG Units across the CpG island as a function of 5-HTTLPR revealed a difference in only a single CpG Unit, HTT6 CpG27.28, although this

difference was before multiple comparison corrections. On the other hand, in addition to differential methylation by 5-HTTLPR genotype, this same CpG Unit was also differentially methylated by ELS. For S-group participants, methylation of this CpG Unit was increased by ELS, while the opposite pattern was observed in LL participants. Since methylation of this Unit with the region that was previously associated with ELS (HTT6 CpG27-41) was similar in both genotype groups (Figure 22), it was possible to suggest that differential methylation of this CpG Unit might be involved in the differential regulation of 5-HTT expression through TF binding. In this respect, I investigated the potential TFBSs close to this CpG Unit through an online tool for TFBS search (TFSearch; http://www.cbrc.jp/research/db/TFSEARCH.html). Results indicated that there are many potential TFBSs for the TF ADR1 (alcohol dehydrogenase II synthesis regulator) within the HTT6 CpG27-41 region, one of which overlaps with CpG27.28 Unit, as well as the other CpG sites that were associated with differential methylation by ELS (HTT6 CpG38, 39 and 40.41). ADR1 is a zinc-finger TF that can activate or repress gene transcription (Kacherovsky et al., 2008) and mutations in the binding sites of this TF, similar to NGFI-A, was reported to alter DNA binding specificity (Thukral et al., 1991; Swirnoff & Milbrandt, 1995). Therefore, it is possible that methylation of the binding sites of these TFs may alter the binding efficiencies of these TFs to DNA and influence gene expression. Although the differential effect of methylation at these sites by 5-HTTLPR genotype is not clear, it is possible that the deletion in the S-group may change the interaction between certain TF interactions and affect S-group differently than the LL participants. One other possibility is that the potential effect of methylation of these CpG Units on gene expression is significant for the S-group, considering that they

have lower expression of 5-HTT, but not for the LL participants. Interestingly, GR CpG sites also had binding sites for the same TFs and methylation at 5-HTT and GR amplicons were mostly correlated significantly with each other. Thus, it would be of great interest to investigate whether methylation of these sites for the same TFs would influence the expression of these genes that are known to influence the expression of each other and cortisol response (Glatz et al., 2003; Weaver et al., 2004b; Belay et al., 2011).

Differences in GR methylation. Considering the role of serotonergic input in the regulation of GR expression and HPA reactivity (Mitchell et al., 1990; Mitchell et al., 1990b; Mitchell et al., 1992; Weaver et al., 2004b) and differential GR expression and HPA reactivity in rat hippocampus by a polymorphism in 5-HTT (Belay et al., 2011), similar to 5-HTTLPR, it is possible that the effects of ELS on GR methylation might change as a function of 5-HTTLPR. In the GR amplicon, there were no CpG Units that was differentially methylated as a function of 5-HTTLPR. However, increased methylation of the GR CpG9 Unit was associated with increased emotional abuse and cortisol reactivity only in the S-group. This CpG site is near TFBSs for the TF HSF (heat shock transcription factor), which is involved in the regulation of heat shock proteins that play a role in activating GR (Yu et al., 2010). Interestingly, the expression of one of the HSPs involved in GR regulation, HSP70, was suggested to be influenced by serotonin reuptake inhibitors used as antidepressants (Yu et al., 2010). Considering the relation between CpG9 methylation and cortisol increase in the S-group, it is possible to speculate that the decreased binding of HSF due to methylation limits the expression of GR and thus leads to increased cortisol reactivity, although empirical studies investigating this possibility should be performed. Interestingly, in a recent study investigating the effect of

GR methylation in response to DEX-CRH test also reported an association with this same CpG site with cortisol area under the curve (Tyrka et al., 2012). Considering these findings together, future studies should investigate the relation between methylation at this site and cortisol response in relation to the functioning of GR.

Limitations of the current study

One of the limitations of this study is the sample size. With a larger sample size, it would be possible to investigate each genotype separately and also investigate three-way interactions between ELS, 5-HTTLPR and factors such as chronic stress. On the other hand, investigating the associations regarding DNA methylation in a larger sample size helped in investigating methylation at target sites.

One other limitation of the study was the range and quantification of ELS as determined by CTQ scores. As pointed out before, the type, timing and number of the early life stressors may be important on their effects on stress reactivity. It is possible that in order to eliminate individuals with psychopathology, we lost the possibility of recruiting individuals with higher CTQ scores. However, it is promising that even within the range of CTQ scores we have (25-66 out of 25-125 possible), we still observed changes in different endophenotypes as a function of ELS. In addition to the severity of ELS, it may also be important to investigate different types of ELS. For example, we had only one participant with sexual abuse and thus were not able to investigate the effect of sexual abuse separately.

Finally, considering studies that report differences in the association of ELS (and 5-HTTLPR) with HPA reactivity by age and gender (Kudielka et al., 2004; Carpenter et

al., 2009; Danese et al., 2009; DeSantis et al., 2011; Mueller et al., 2011), investigating the same relationship in different age groups, such as children, and in both genders, would also complement the results of this study.

Conclusion and future directions

The results of this study suggests that ELS may influence HPA reactivity as a function of 5-HTTLPR, such that participants in the S-group have higher cortisol response with increased ELS, while an opposite pattern is observed in the LL participants. Similar effects of genotype were observed in the expression of 5-HTT in response to the TSST such that the S-group had lower 5-HTT expression with increased ELS. Although the overall methylation of 5-HTT did not differ by ELS, 5-HTTLPR or their interaction, for specific CpG sites, such as CpG27.28, S-group had higher methylation by ELS, while LL participants showed the reverse pattern. Future studies would benefit from using animal models or cell culture to test the neuroanatomical bases of this GxE interaction (similar to Jasinska et al., 2012) and the consequences of methylation of this CpG Unit on gene expression and cortisol response. In addition, statistical analysis methods such as Structural Equation Modelling, may provide better causal insights about the relationships between these factors.

In conclusion, the results of this study indicate that the impact of ELS might be reflected in different levels of phenotype and have profound effects on stress reactivity in adulthood, which may be moderated by the 5-HTTLPR genotype.

Table 1.

Primer sequences, amplicon length and PCR conditions of Sequenom Epityper MassArray amplicons

Name	5' to 3' primer sequence (Sequenom tags in lower case)	Length	PCR conditions
HTT3	F: aggaagagagGGGTTTTTATATGGTTTGATTTTTAGA	335 bp	95°C 15 min; 50 cycles of 94°C 30 sec, 59.5°C 45 sec, 72°C 10 sec; 72°C 1 min
	R: cagtaatacgactcactatagggagaaggctCACCTACTCCTTTATACAACCTCCC		
HTT6	F: aggaagagagGGTTATTTAGAGATTAGATTATGTGAGGG	424 bp	95°C 15 min; 50 cycles of 94°C 30 sec, 58.5°C 45 sec, 72°C 12 sec; 72°C 1 min
	R: cagtaatacgactcactatagggagaaggctCTACAACAATAAACAAAAAAACCCC		
GR2	F: aggaagagagTAATTTTTTAGGAAAAAGGGTGG	400 bp	95°C 15 min; 46 cycles of 94°C 30 sec, 53°C 45 sec, 72°C 10 sec; 72°C 1 min
	R: cagtaatacgactcactatagggagaaggctCCTAAAACCTCCCCAAAAAAC		

Table 2.

Primer sequences, amplicon length and PCR conditions of Pyrosequencing amplicons

Name	5' to 3' primer sequence (5' Biotin tag denoted as BIO)	Length	PCR conditions
HTT6 CpG9-12	F: BIO-AGAGATTAGATTATGTGAGGGTT R: AACCCACCTACCAAACTAC Seq: AAACAAACCAACTAACCTC	152 bp	95°C 15 min; 50 cycles of 94°C 30 sec, 57.5°C 45 sec, 72°C 12 sec; 72°C 2 min
GR2 CpG10.11	F: AGTTTTAGAGTGGGTTTGGAG R: BIO-AAACCACCCAATTTCTCCAATTTCTT Seq: GAGTGGGTTTGGAGT	86 bp	95°C 15 min; 50 cycles of 94°C 30 sec, 57.5°C 45 sec, 72°C 12 sec; 72°C 2 min
LINE-1	PyroMark Q96 CpG LINE-1 kit commercial primers used.	146 bp	95°C 15 min; 45 cycles of 94°C 30 sec, 50°C 30 sec, 72°C 20 sec; 72°C 4 min

Table 3.

qPCR primer sequences for reference genes and genes of interest.

Gene symbol	5' to 3' primer sequence
B2M	F: TGCTGTCTCCATGTTTGATGTATCT
	R: TCTCTGCTCCCCACCTCTAAGT
GAPDH	F: CGCTGAGTACGTCGTGGAG
	R: GCAGAGATGATGACCCTTTTG
HPRT1	F: TGACACTGGCAAAACAATGCA
	R: GGTCCTTTTCACCAGCAAGCT
PPIA	F: GCATACGGGTCCTGGCATCTTG
	R: TGCCATCCAACCACTCAGTCTTG
SDHA	F: TGGGAACAAGAGGGCATCTG
	R: CCACCACTGCATCAAATTCATG
YWHAZ	F: ACTTTTGGTACATTGTGGCTTCAA
	R: CCGCCAGGACAAACCAGTAT
NR3C1 (GR)	F: GAAGGAAACTCCAGCCAGAACTG
	R: GATGATTTCAGCTAACATCTC
SLC6A4 (5-HTT)	F: TGTCTGAGGTGGCCAAAGA
	R: GTTGGCTATCGCTTCTGCAT

Table 4.

Characteristics of the TSST participants

Measure	All
	N = 59
Age, *Mean* (*SD*)	30.29 (15.34)
CTQ Total, *Mean* (*SD*)	34.82 (10.17)
TICS Total, *Mean* (*SD*)	15.95 (8.72)
BDI Total, *Mean* (*SD*)	5.57 (4.67)
Baseline cortisol, *Mean* (*SD*)	9.10 (6.52)
5-HTTLPR genotype, *n* (%)	
S-group	35 (59)
LL	24 (41)

Table 5.

Means and SDs of age, stress and DNA methylation measures.

Measure	All ($N = 105$)	S-Group ($n = 70$)	LL ($n = 35$)	p
Age, Mean (SD)	28.51 (13.82)	27.39 (13.12)	30.77 (15.07)	.238
CTQ Total, Mean (SD)	34.83 (9.57)	34.82 (9.90)	34.86 (9.00)	.986
PSS, Mean (SD)	14.10 (7.45)	13.89 (7.65)	14.51 (7.11)	.688
TICS, Mean (SD)	17.42 (9.70)	17.30 (10.17)	17.66 (8.82)	.860
5-HTT, Mean (SD)	8.85 (1.67)	8.77 (1.68)	8.99 (1.67)	.288
HTT6 CpG27-41, Mean (SD)	15.52 (2.16)	15.36 (2.30)	15.83 (1.81)	.545
GR, Mean (SD)	5.30 (1.30)	5.29 (1.36)	5.31 (1.18)	.929
LINE-1, Mean (SD)	74.77 (2.38)	75.00 (2.41)	74.41 (2.31)	.270

Table 6

Bivariate correlations of age and methylation measures

	All ($N = 105$)	S-Group ($n = 70$)	LL ($n = 35$)
Measure	Age	Age	Age
LINE-1	-.25*	-.24*	
5-HTT	.33**	.28*	.40*
HTT6 CpG27-41	.42**	.45**	.35*
HTT3 CpG1.2	.21*		
HTT3 CpG16.20			.39*
HTT6 CpG9.10.11.12	.39**	.28*	.57**
HTT6 CpG13.14	.46**	.40**	.52**
HTT6 CpG15	.34**	.33**	.34*
HTT6 CpG27.28	.46**	.40**	.54**
HTT6 CpG30	.25*	.24*	
HTT6 CpG31.32.33	.39**	.48**	
HTT6 CpG36	.29**	.38**	
HTT6 CpG39		.30*	

Note. *$p < .05$; **$p < .01$.

Table 7.

Bivariate correlations of CTQ total, subscales and methylation measures

	All (N = 105)					S-Group (n = 70)					LL (n = 35)				
Measure	TOT	EA	EN	PA	PN	TOT	EA	EN	PA	PN	TOT	EA	EN	PA	PN
LINE-1											.45**		.34*	.40*	.45**
HTT6 CpG27-41													-.35*		
HTT3 CpG1.2	.20*				.24*									.50**	
HTT3 CpG3							.27*								
HTT3 CpG12.13										-.28*					
HTT3 CpG14.15														.38*	
HTT3 CpG16-20												.38*			
HTT3 CpG21										-.26*					
HTT3 CpG22.23														.35*	
HTT6 CpG16	.21*	.22*	.21*			.24*		.27*							
HTT6 CpG22									.31**						
HTT6 CpG27.28						.26*				.25*	-.33*		-.38*		
HTT6 CpG30						.30*	.26*	.25*	.27*				-.35*		
HTT6 CpG39		.22*					.24*								
HTT6 CpG40.41											-.42*		-.37*	-.39*	-.38*
GR2 CpG9		.23*					.28*								

Note. *$p < .05$; **$p < .01$.

Table 8.

Bivariate correlations of TICS and methylation measures

	All (N = 105)	S-Group (n = 70)	LL (n = 35)
Measure	TICS	TICS	TICS
LINE-1	.30**	.24*	.48**
HTT6 CpG27-41	-.31**	-.33**	
HTT3 CpG22.23	.19*		
HTT3 CpG25.26	.22*	.32**	
HTT6 CpG27.28	-.36*	-.40**	-.34*
HTT6 CpG38	.24*	.33*	
HTT6 CpG39			.36*
HTT6 CpG40.41	-.33*	-.35**	
GR2 CpG19	.26**		.39*
GR2 CpG36	.21*		

Note. *p < .05; **p < .01.

Table 9.

DNA methylation measures of the TSST participants

Measure	All
	N = 59
5-HTT, Mean (SD)	9.25 (1.76)
HTT6 CpG27-41, Mean (SD)	16.01 (2.11)
GR, Mean (SD)	5.37 (1.27)
LINE-1, Mean (SD)	74.54 (2.57)

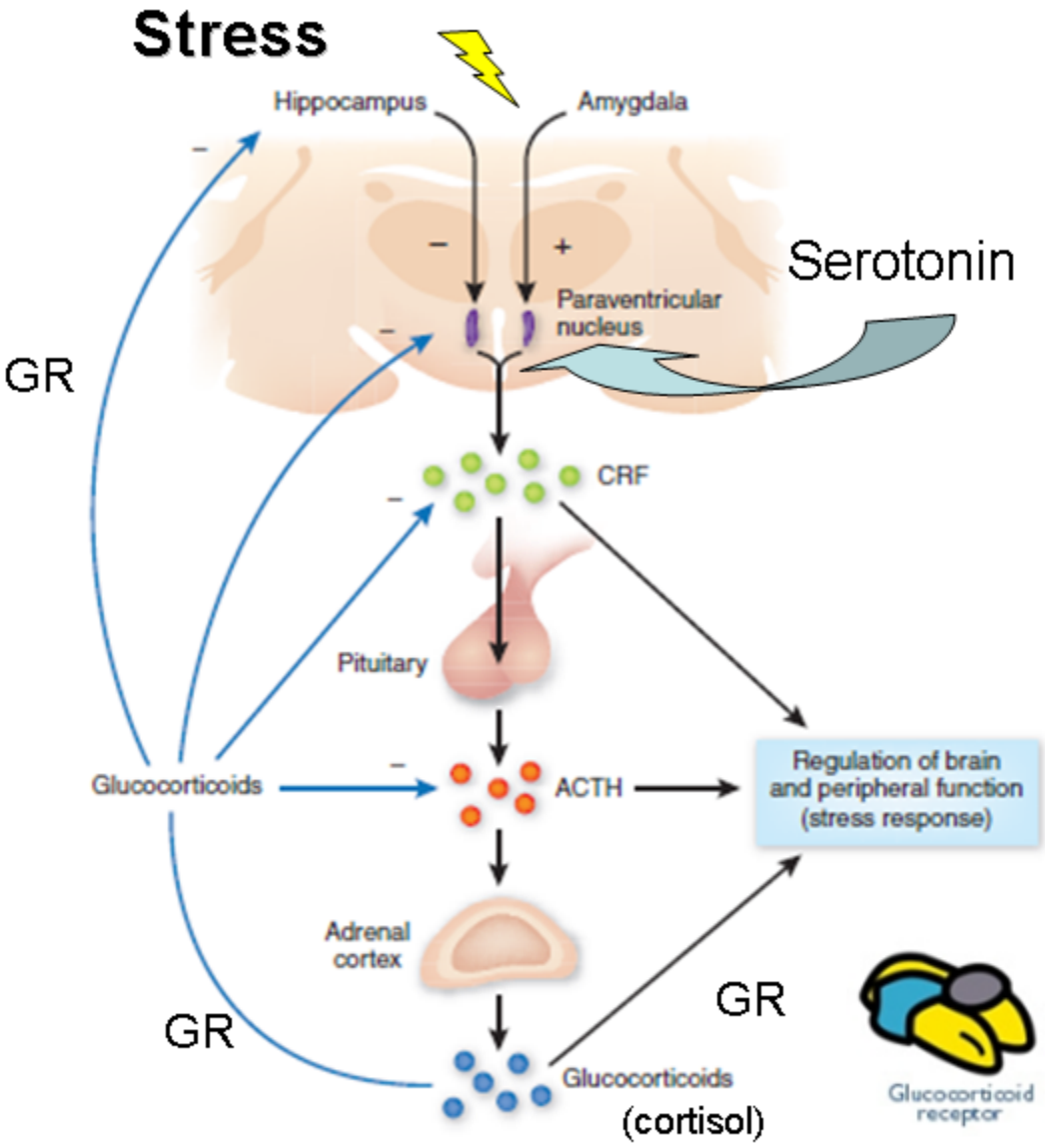


Figure 1. The Hypothalamic-Pituitary-Adrenal axis (HPA).

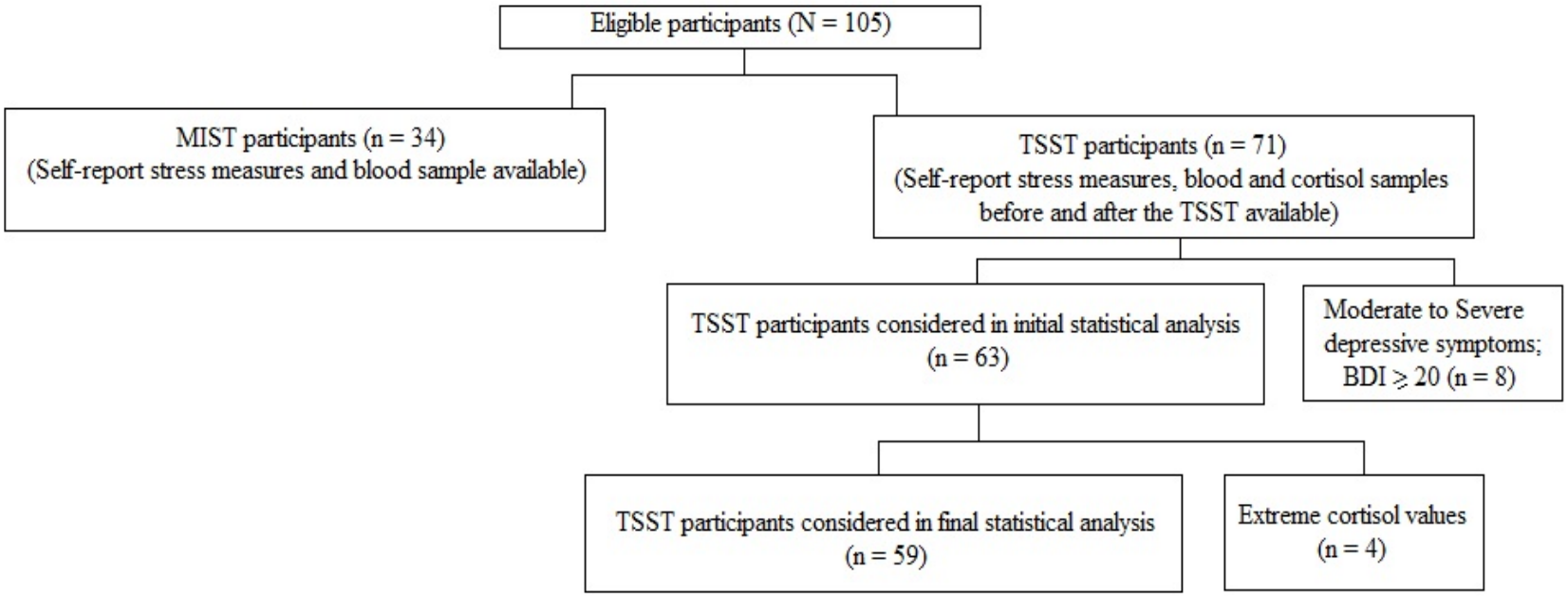
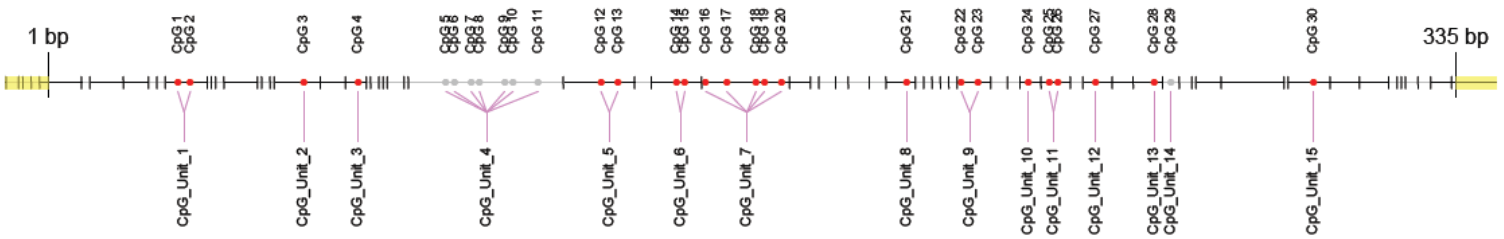
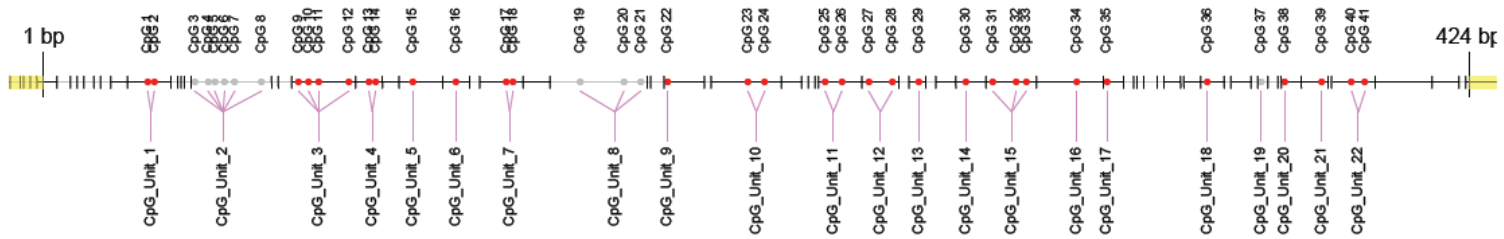


Figure 2: Flowchart of the participants included in the analysis. A total of 105 participants from TSST (n = 71) and MIST (n = 34) were used in the exploratory analyses of stress measures, 5-HTTLPR and DNA methylation. A subset of the TSST participants (n = 59) were also used in cortisol and gene expression analyses.

a) HTT3 amplicon



b) HTT6 amplicon



c) GR2 amplicon

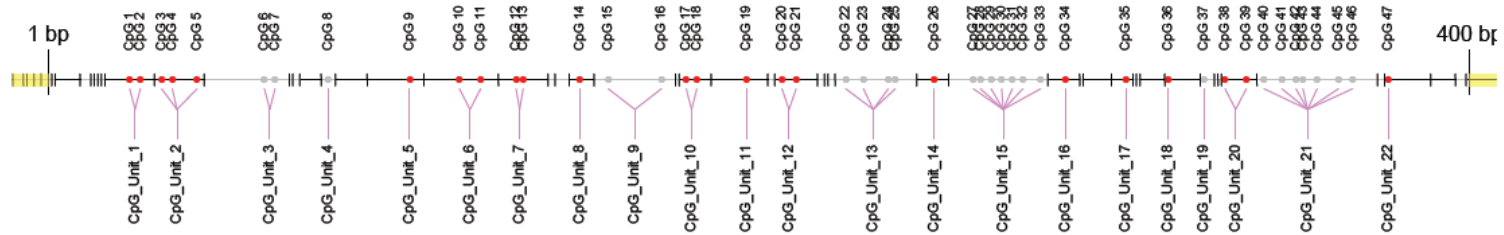


Figure 3. Fragmentation and CpG Units of each Sequenom Epityper MassArray amplicon. CpG Units in red denote the CpG Units that can be analyzed by mass spectrometry, while the ones in gray are the ones that cannot be analyzed.

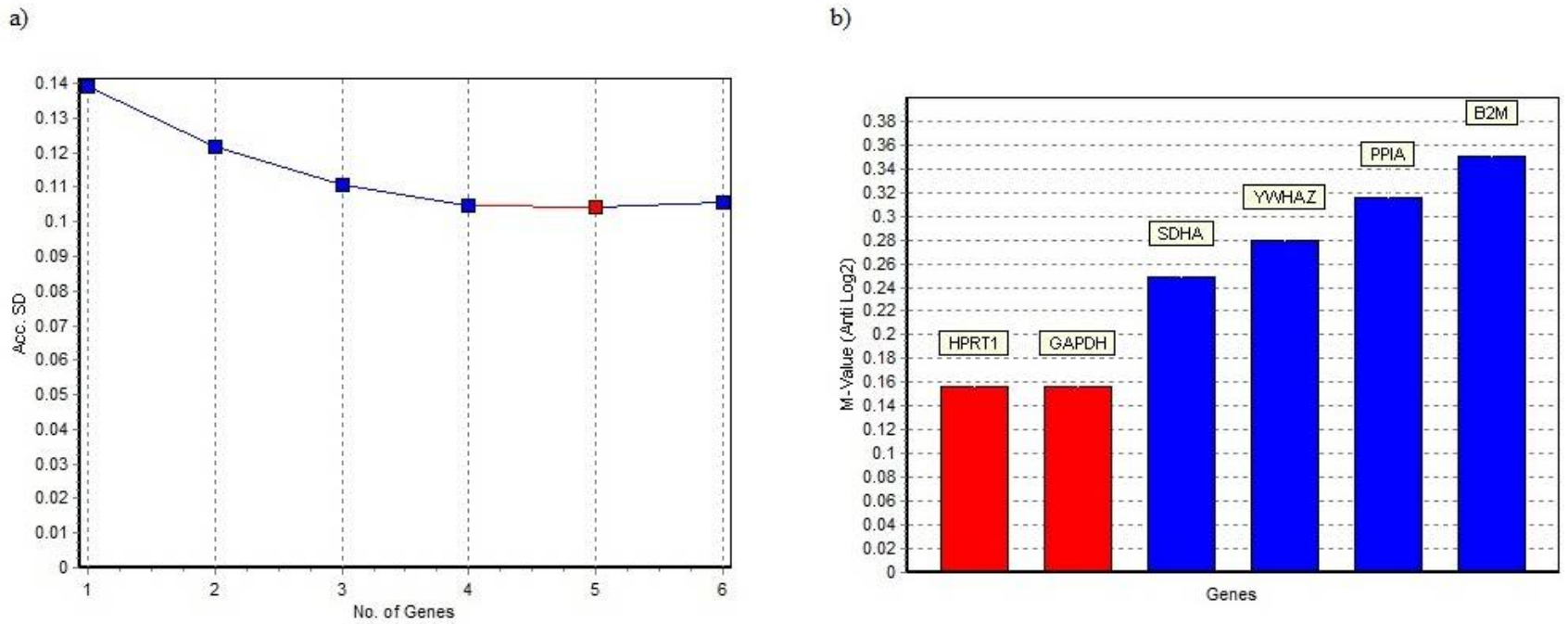


Figure 4. Determination of the optimum reference genes in PBMCs for qPCR analysis of samples at baseline and in response to the TSST. a) NormFinder results for the effect of number of reference genes on standard deviation. b) GeNorm results of the best reference genes tested, shown in red.

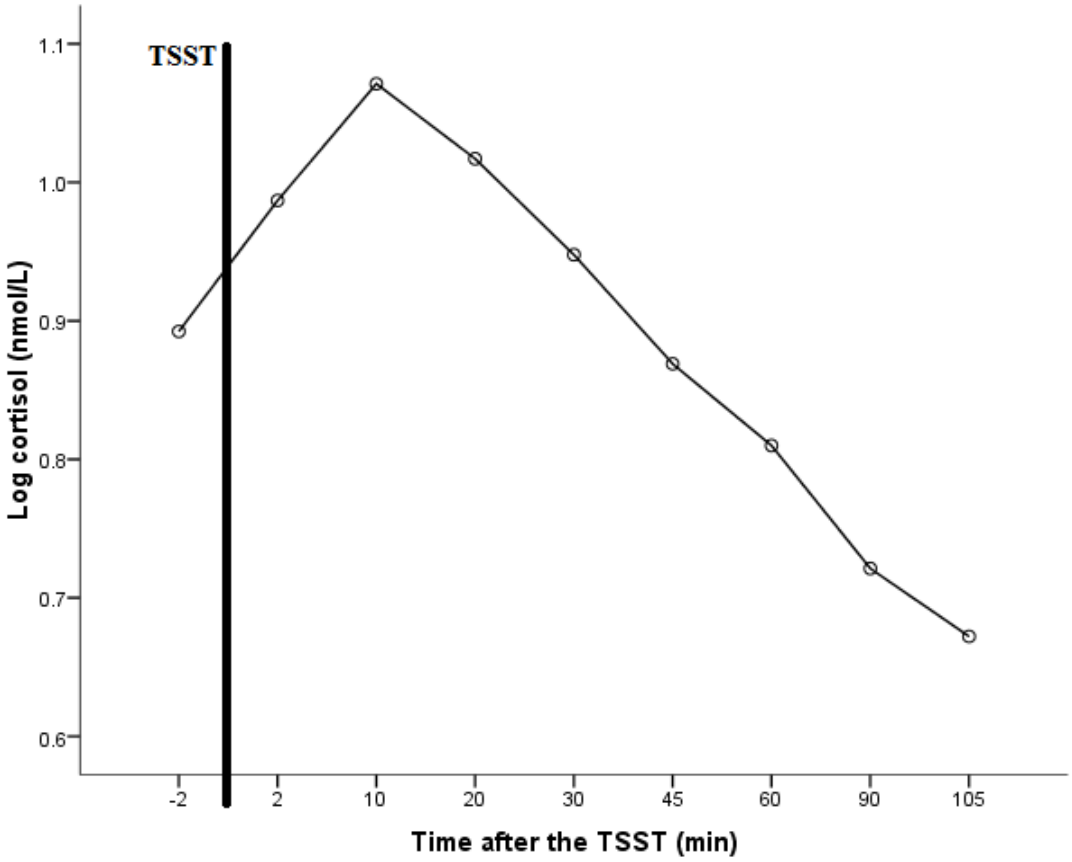


Figure 5. Change in cortisol in response to the TSST by time.

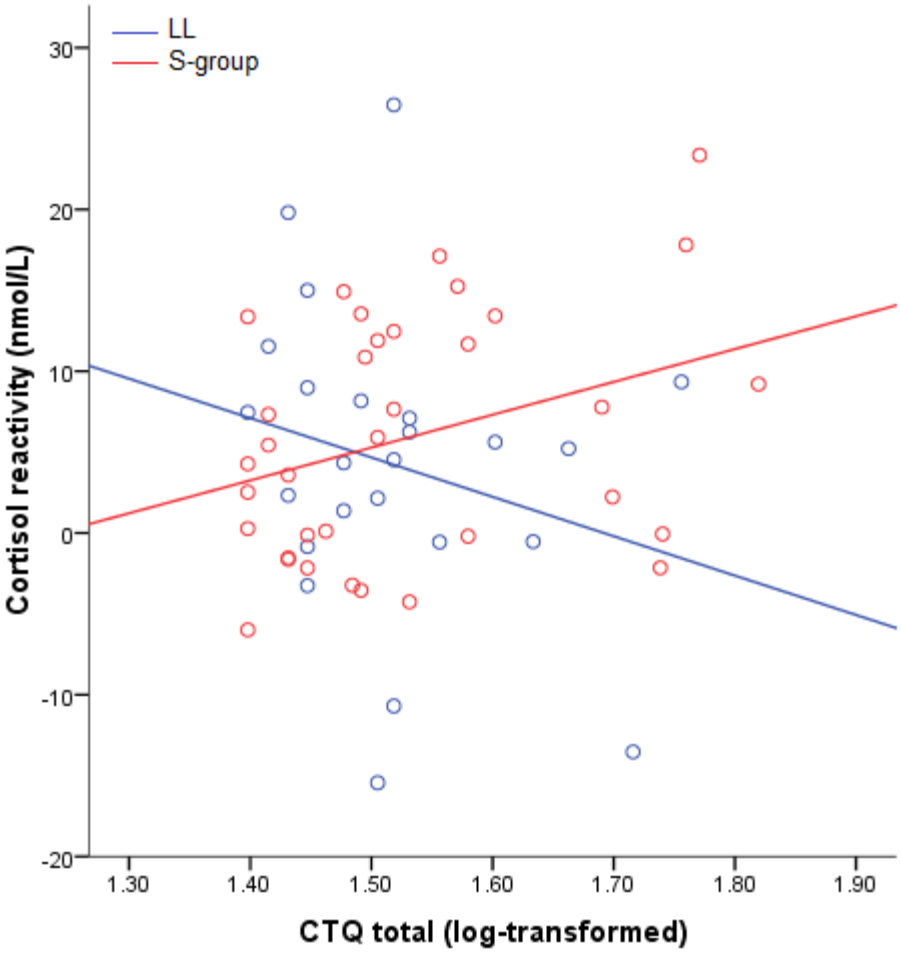


Figure 6. Interaction between early life stress and 5-HTTLPR genotype on cortisol reactivity.

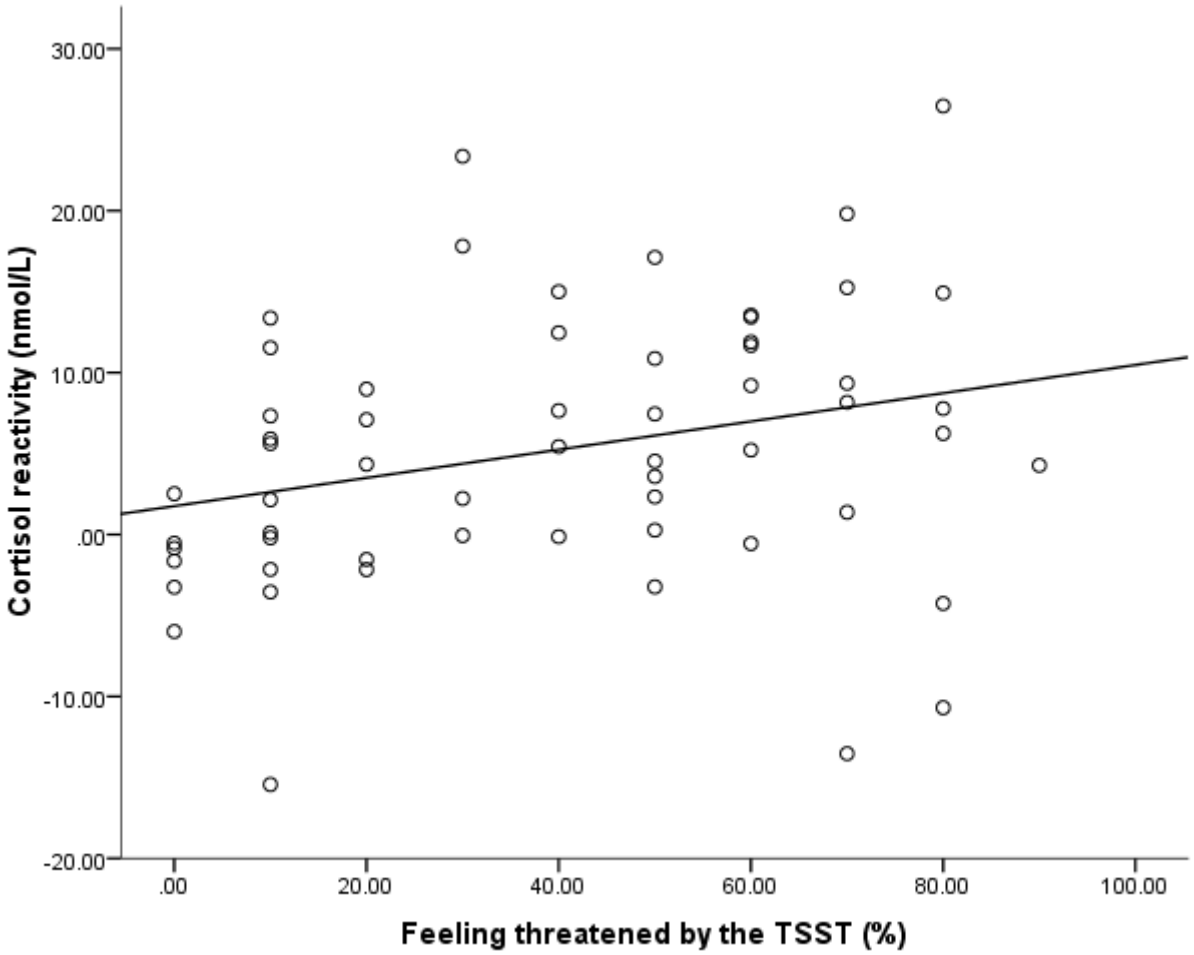


Figure 7. Association between cortisol reactivity and self-report of feeling threatened by the TSST.

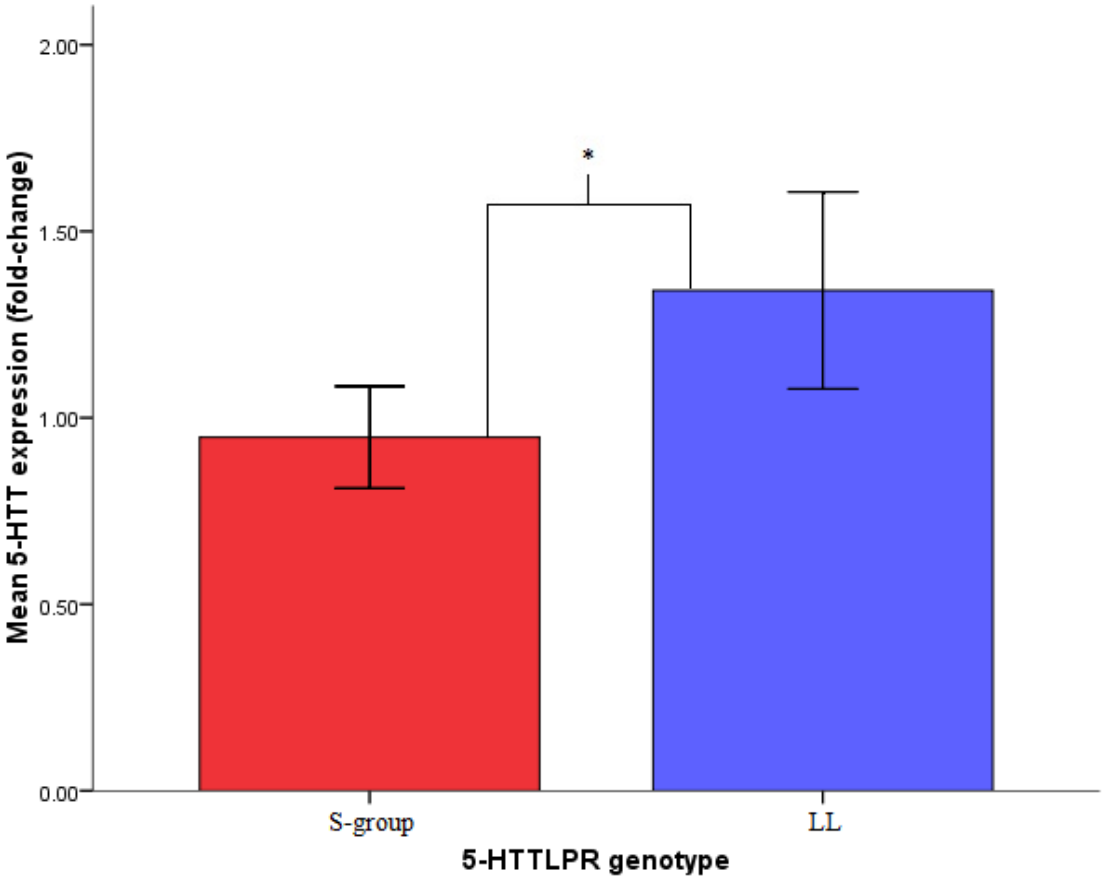


Figure 8. Mean 5-HTT expression by 5-HTTLPR genotype. Individuals with LL genotype had higher 5-HTT expression fold-change in response to the TSST than individuals in the S-group.

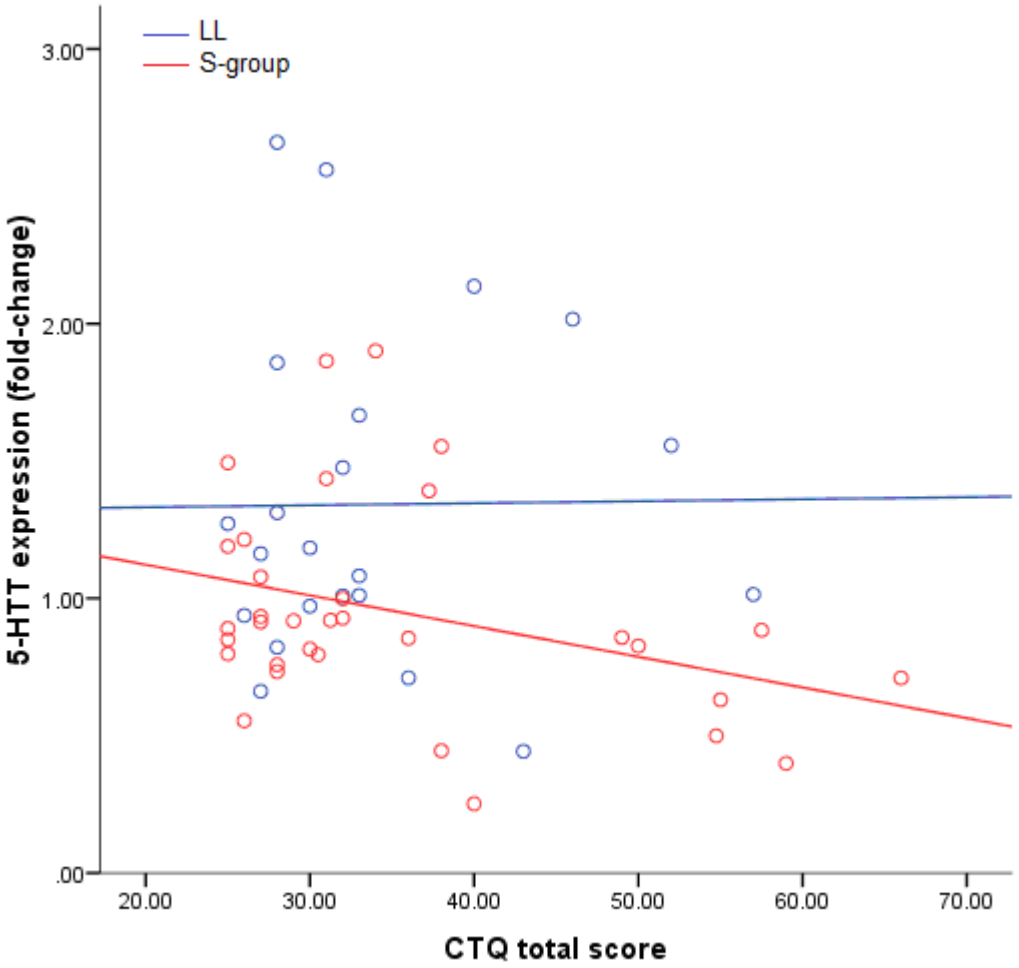


Figure 9. Association between 5-HTT expression and ELS by 5-HTTLPR genotype.

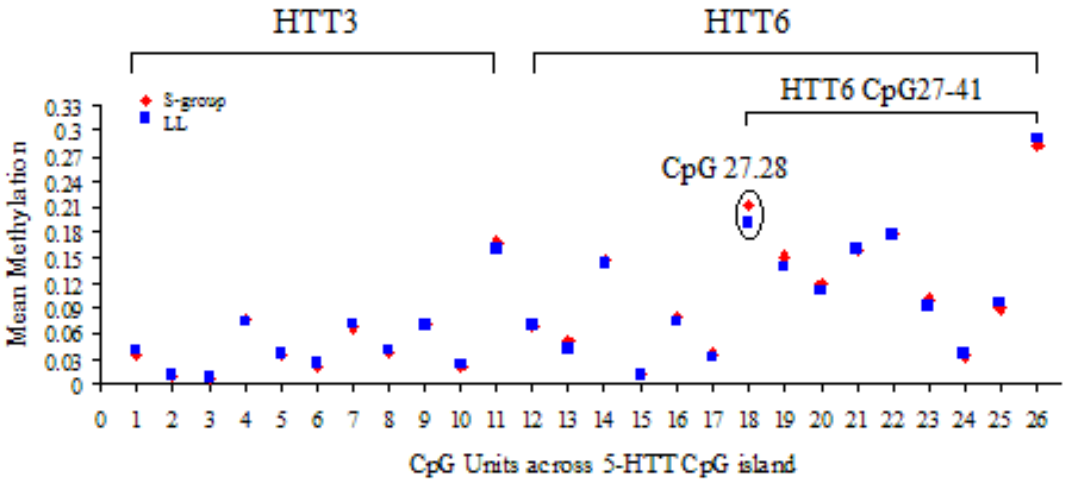


Figure 10. Methylation across the CpG Units of 5-HTT CpG island by genotype. Total of 26 CpG Units covering 44 CpG sites are included in analyses. HTT3 amplicon covers CpG Units 1-11 and HTT6 amplicon covers CpG Units 12-26. HTT6 CpG27-41 includes CpG Units 18-26. S-group and LL participants only differed in methylation at HTT6 CpG27.28 Unit, as shown in circle ($t(103) = 2.36$, $p = .020$).

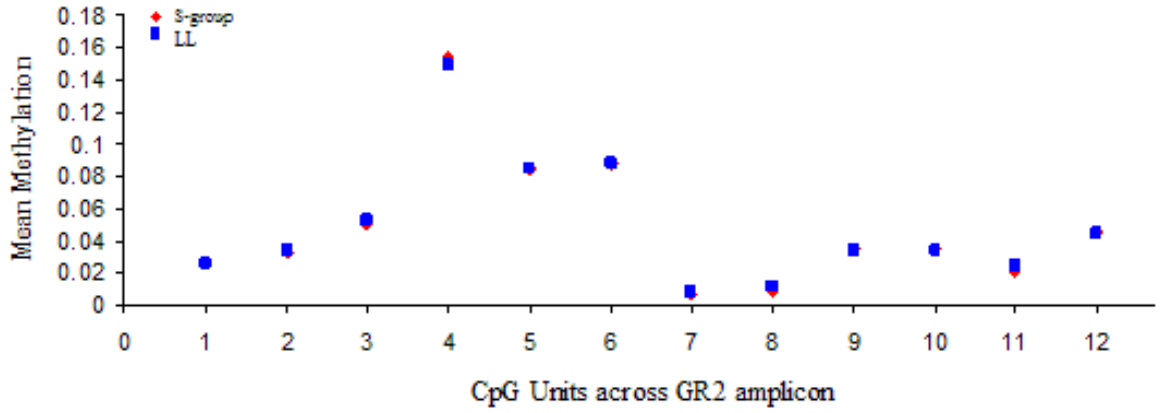


Figure 11. Methylation across the CpG Units of GR2 amplicon by genotype. Total of 12 CpG Units covering 20 CpG sites are included in analyses. S-group and LL participants did not differ in methylation in any of the CpG Units. The highest methylation is observed in CpG Unit 4 that includes GR2 CpG10.11.

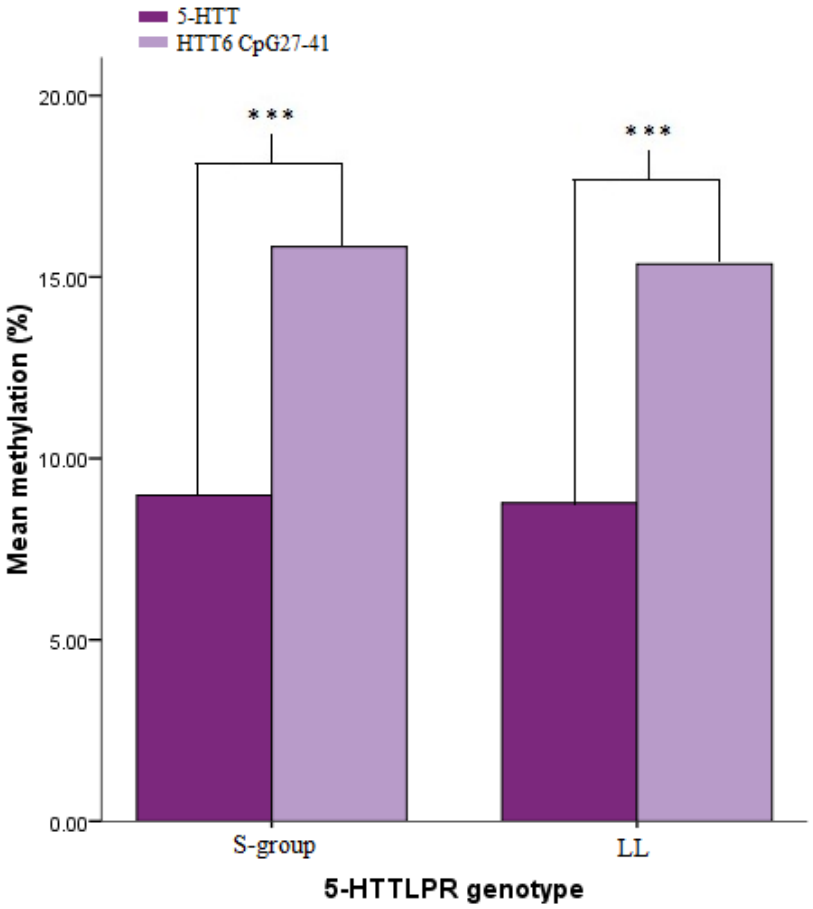


Figure 12. Comparison of 5-HTT overall methylation with methylation at HTT6 CpG27-41. Methylation of the region covering HTT6 CpG27-41 was significantly higher than the 5-HTT overall methylation ($p < .001$).

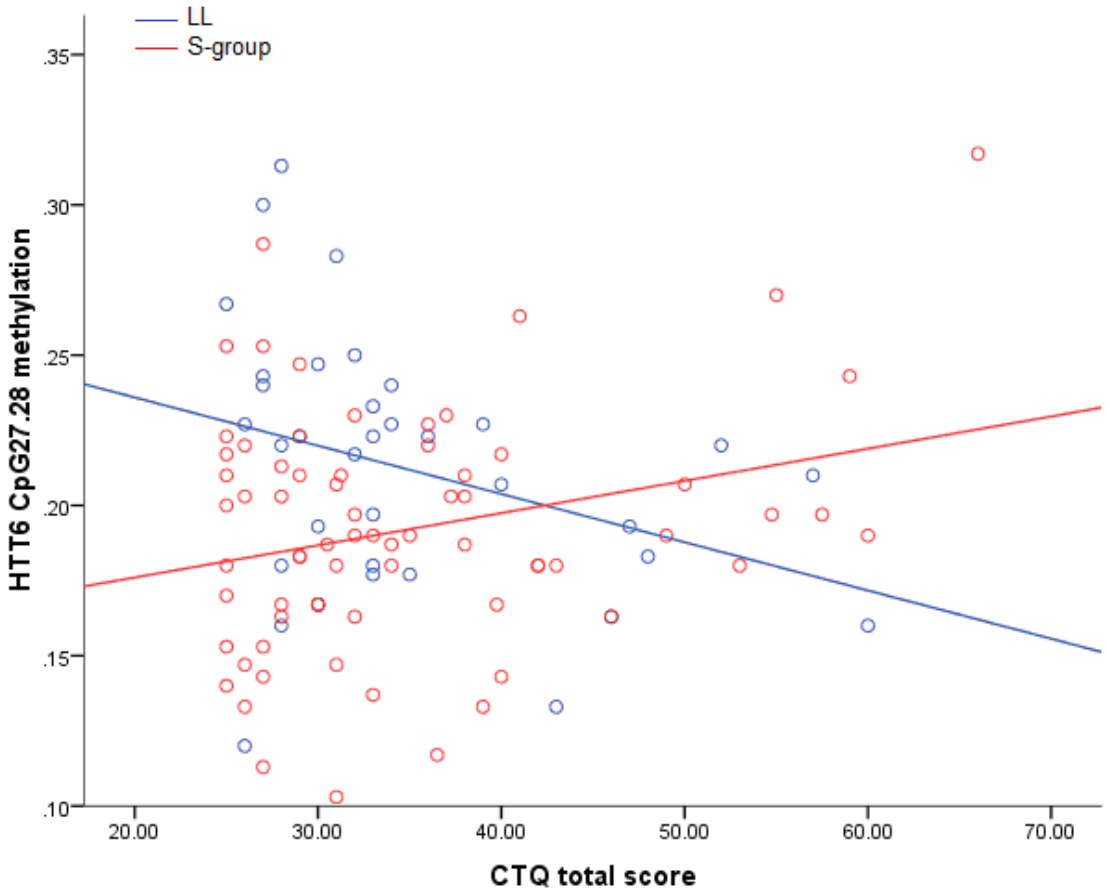


Figure 13. Association between ELS and HTT6 CpG27.28 methylation by 5-HTTLPR genotype for the TSST and MIST samples combined.

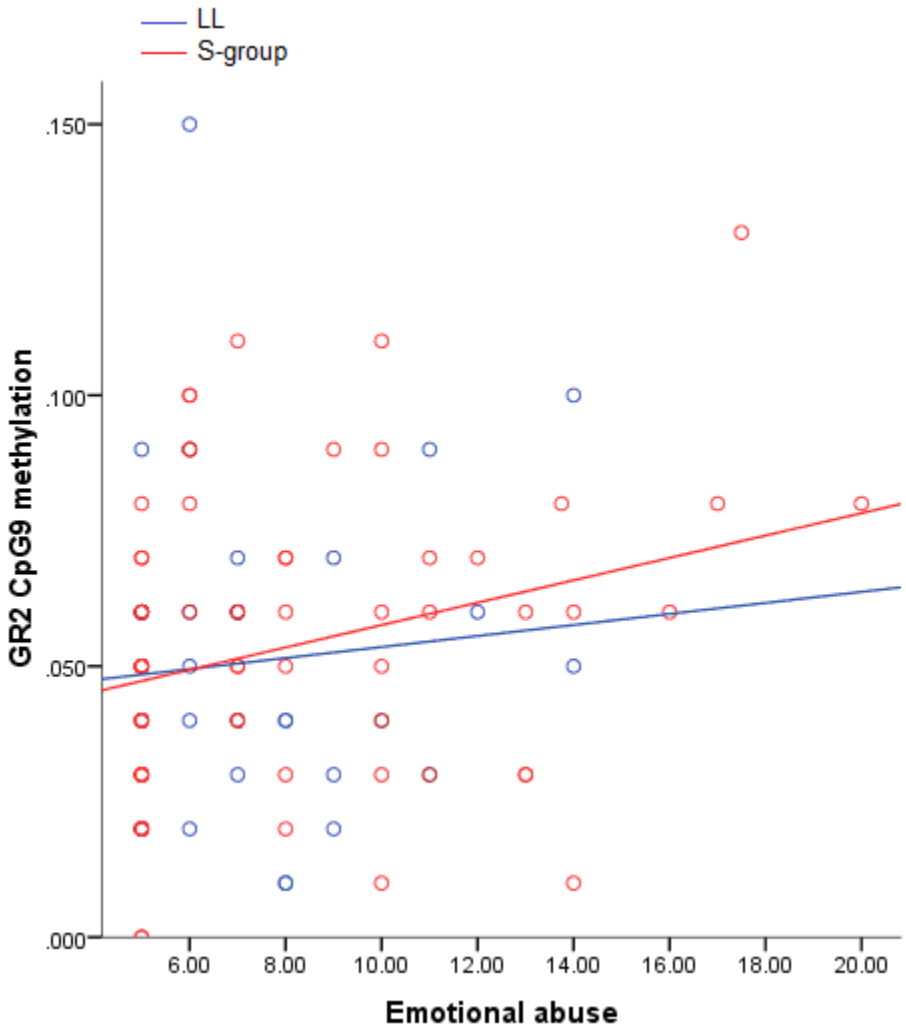


Figure 14. Association between emotional abuse and GR2 CpG9 methylation by 5-HTTLPR genotype.

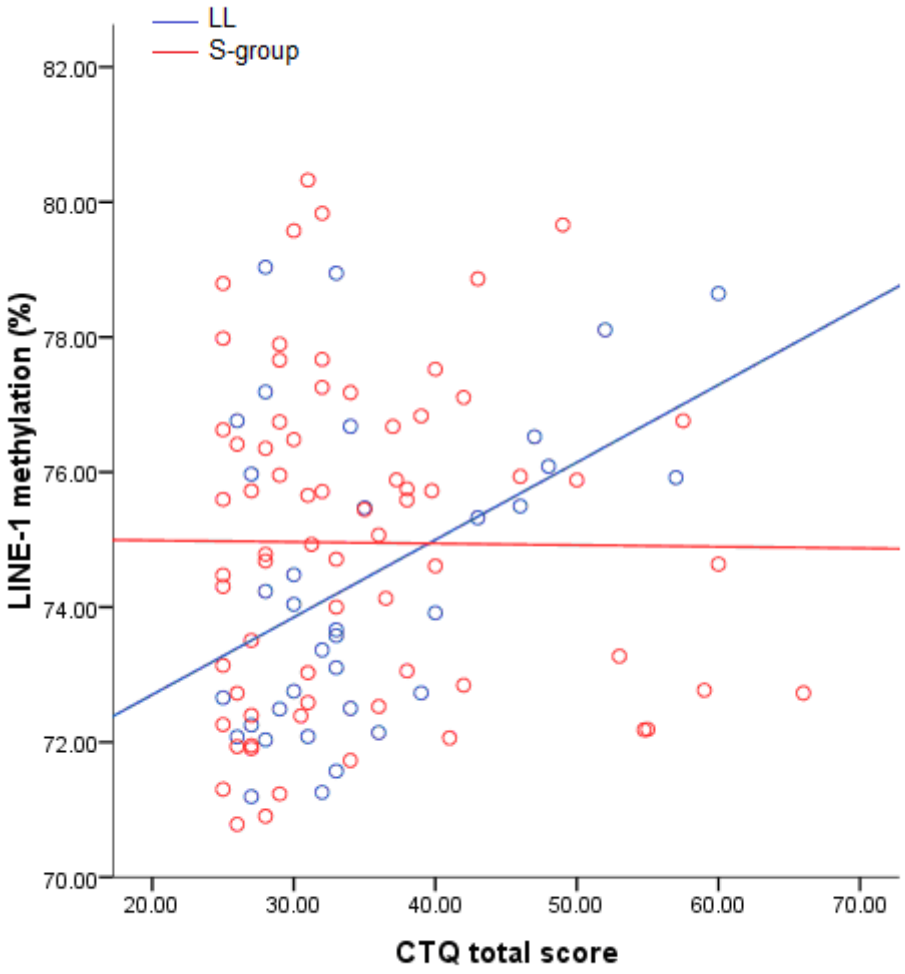


Figure 15. Association between ELS with LINE-1 methylation by 5-HTTLPR genotype.

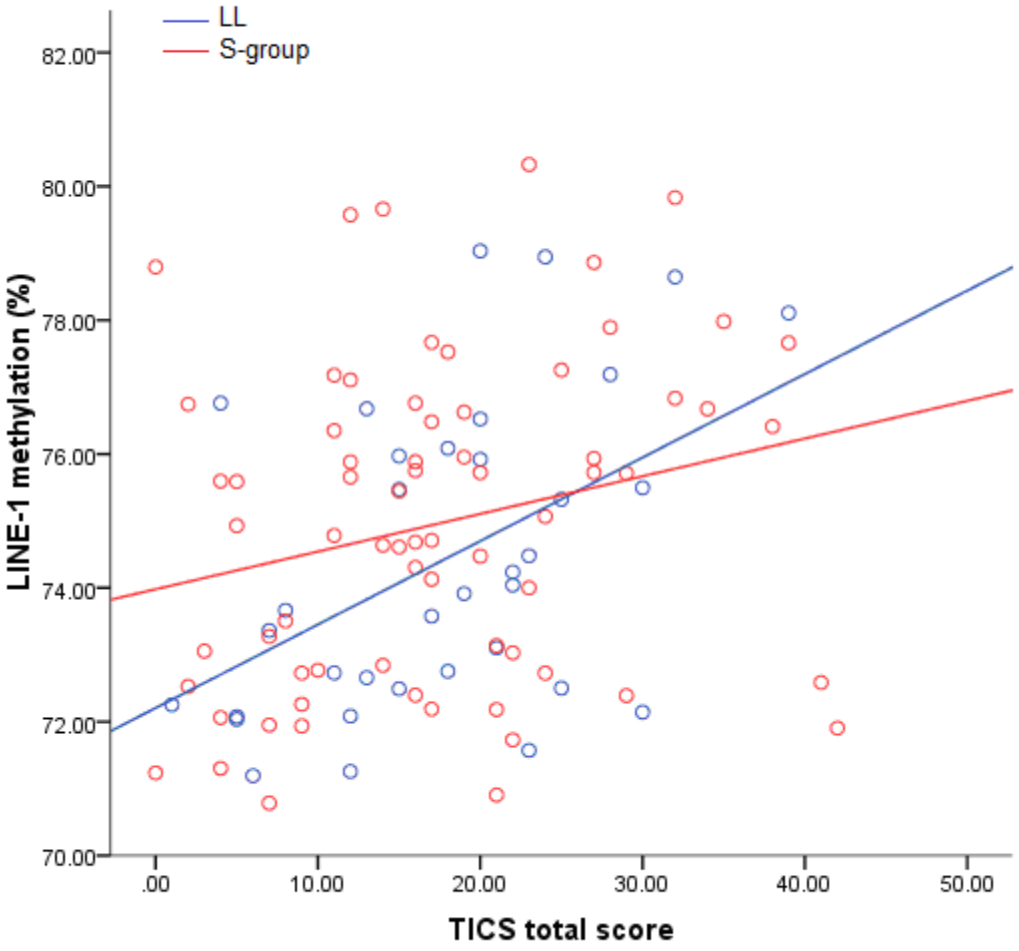


Figure 16. Association between chronic stress and LINE-1 methylation by 5-HTTLPR genotype.

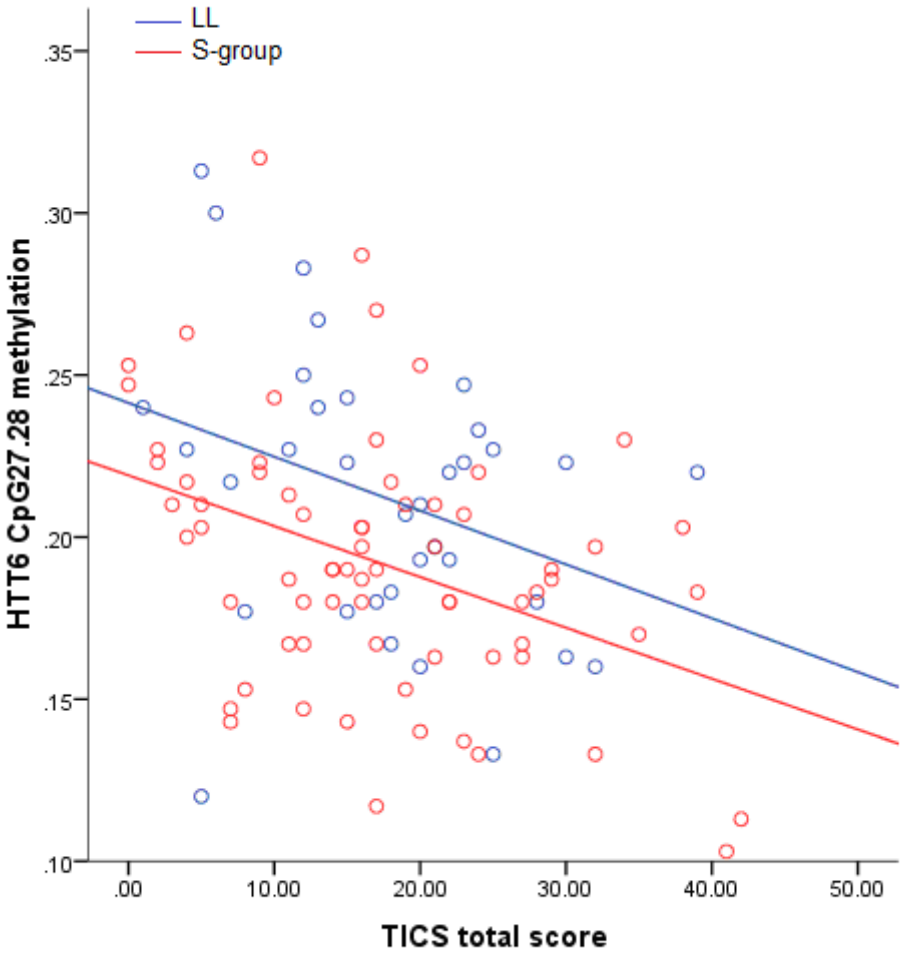


Figure 17. Association between chronic stress and HTT6 CpG27.28 methylation by 5-HTTLPR genotype.

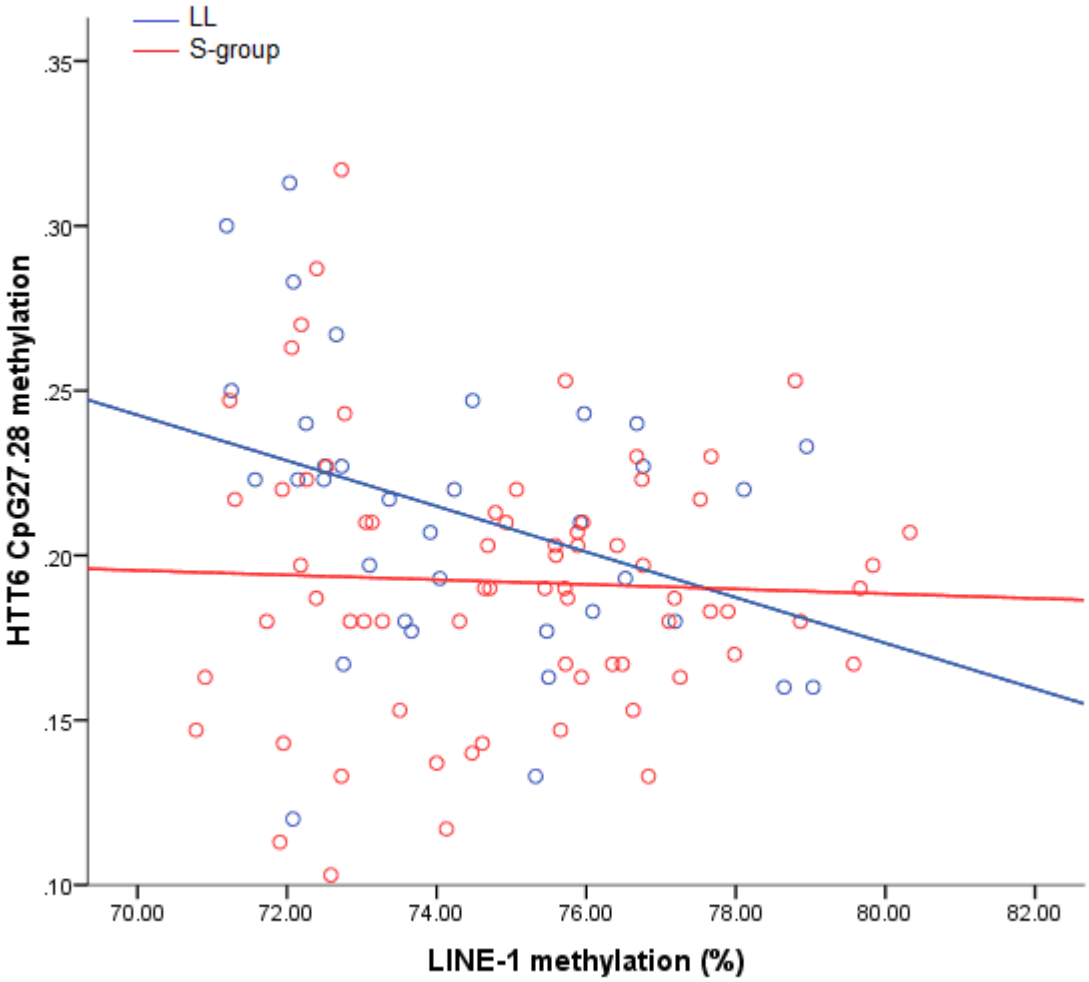


Figure 18. Association between HTT6 CpG27.28 and LINE-1 methylation by 5-HTTLPR genotype.

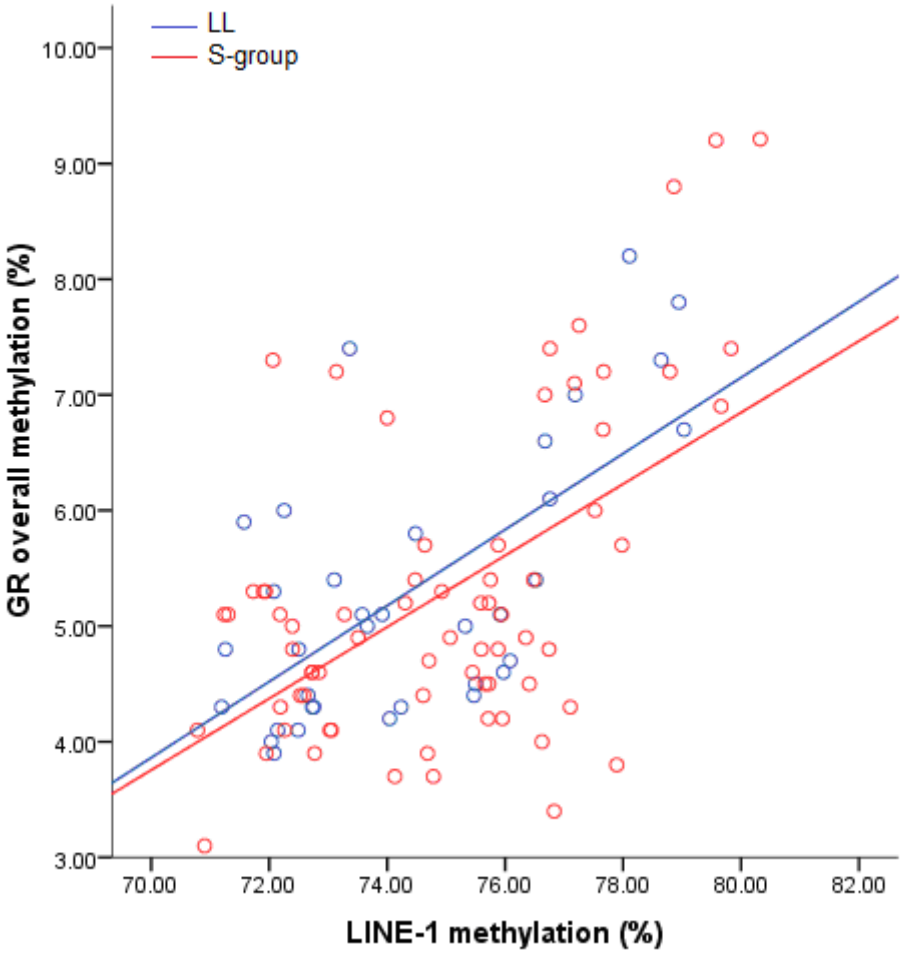


Figure 19. Association between GR overall and LINE-1 methylation by 5-HTTLPR genotype.

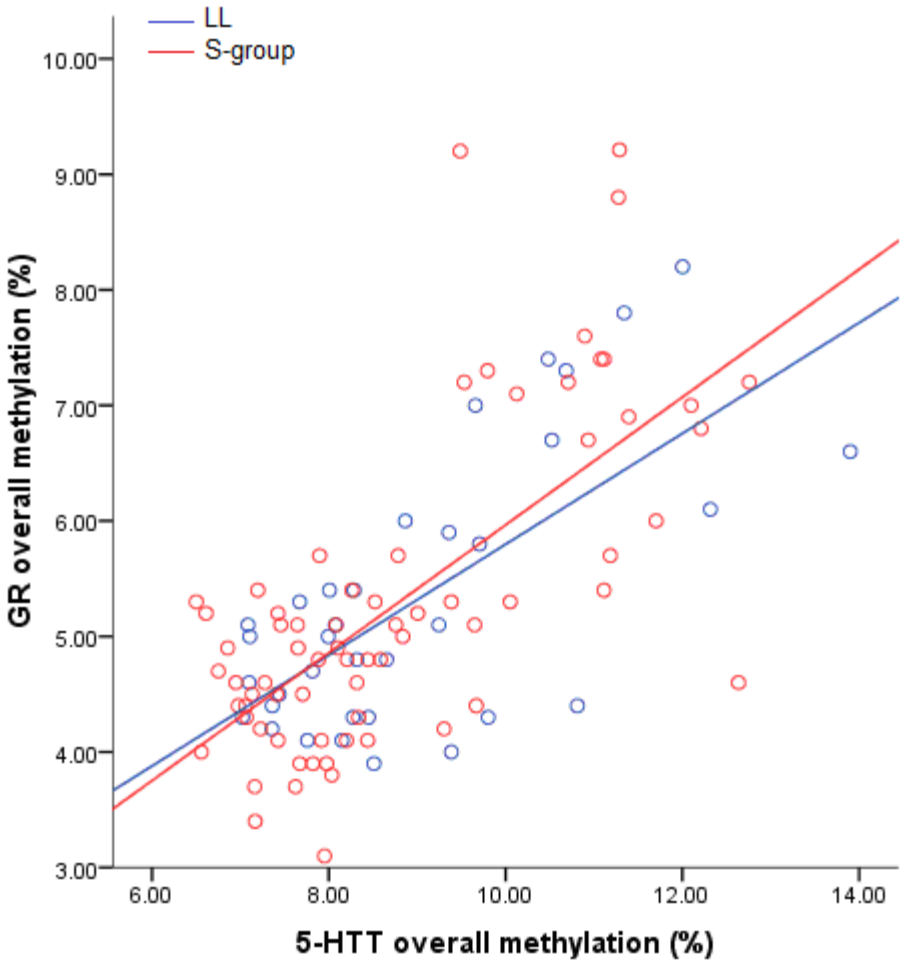


Figure 20. Association between 5-HTT and GR overall methylation by 5-HTTLPR genotype.

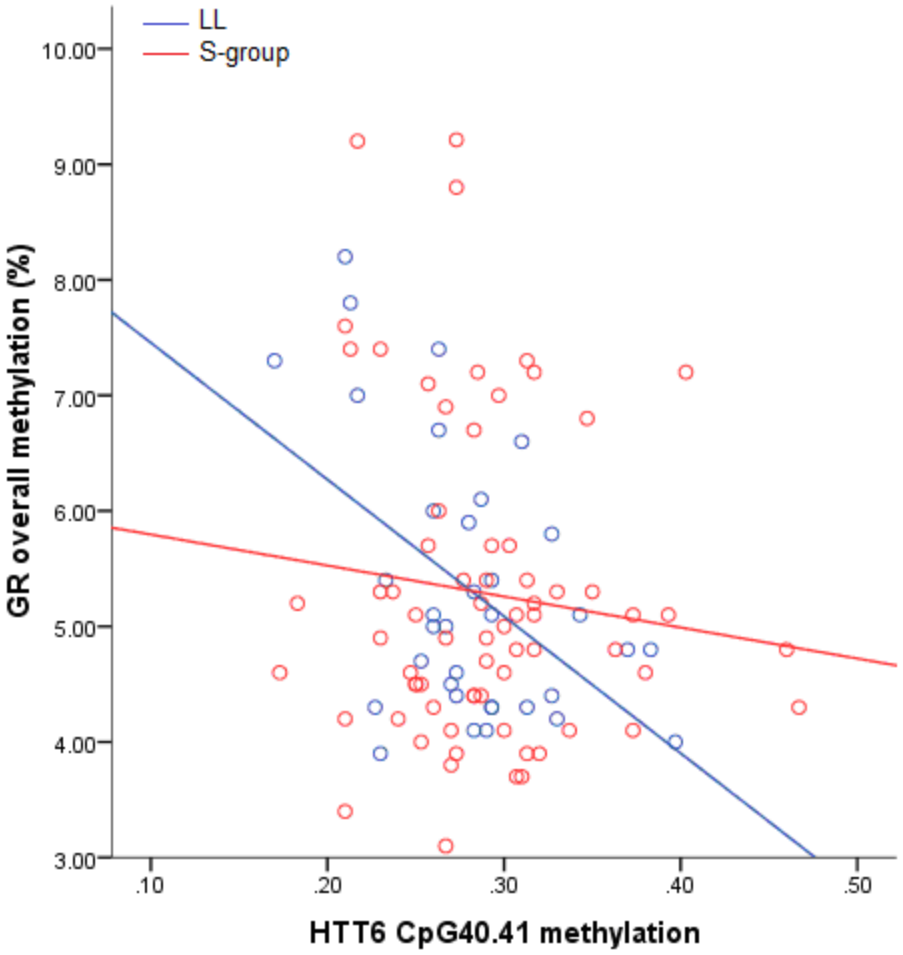


Figure 21. Association between GR overall methylation and HTT6 CpG40.41 methylation by 5-HTTLPR genotype.

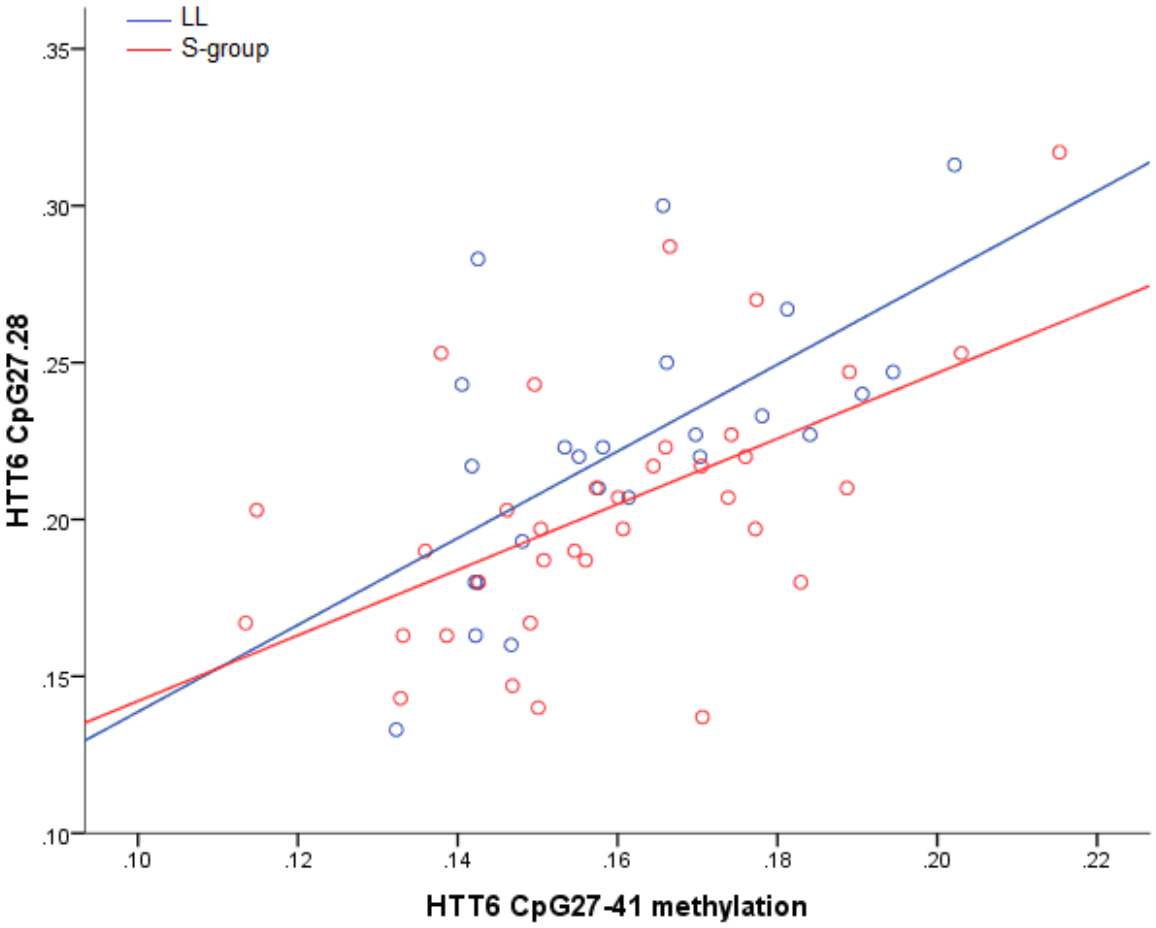


Figure 22. Association between HTT6 CpG27.28 and HTT6 CpG27-41 methylation by 5-HTTLPR genotype.

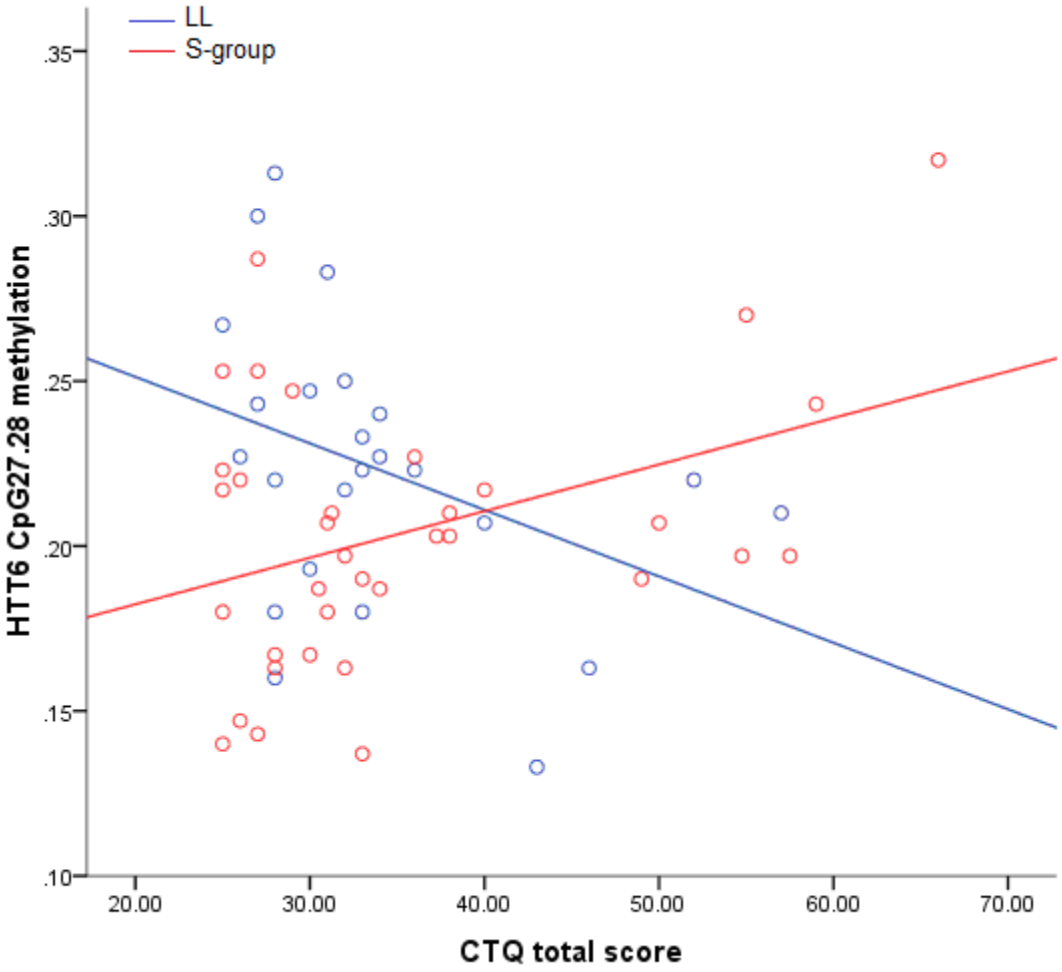


Figure 23. Association between HTT6 CpG27.28 methylation and ELS by 5-HTTLPR genotype.

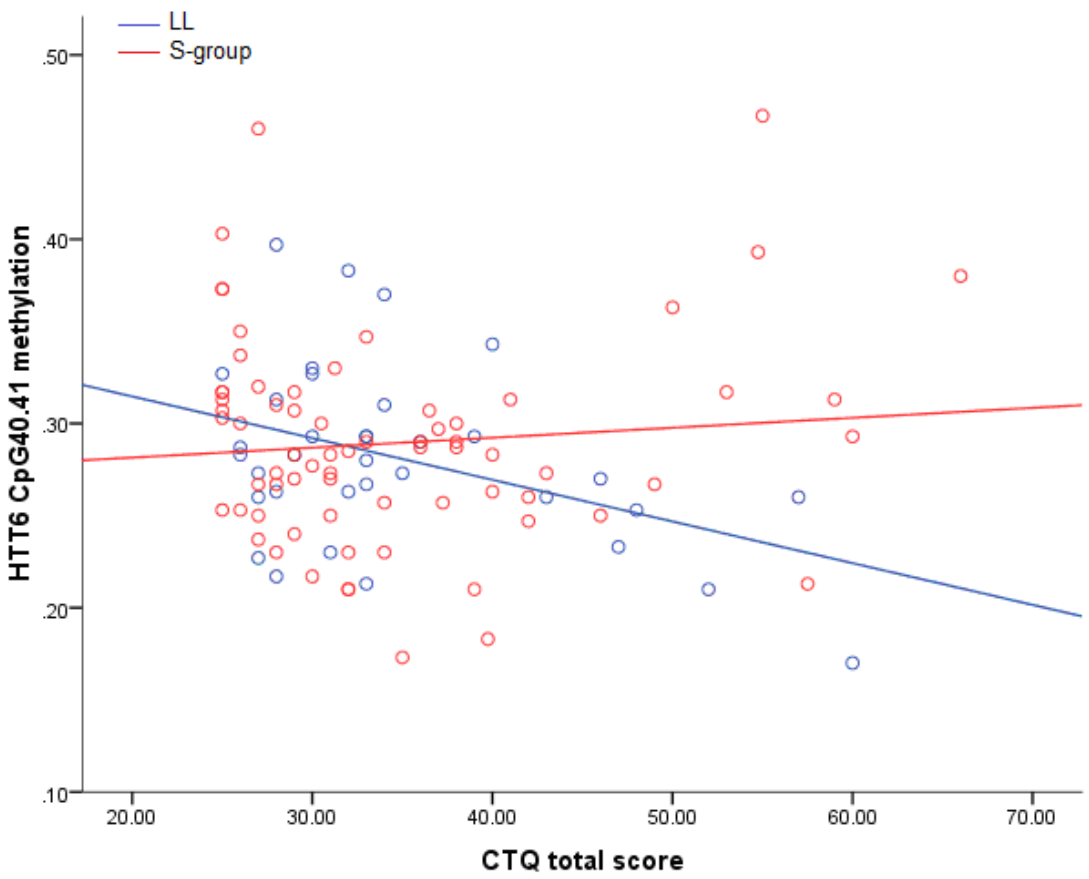


Figure 24. Association between HTT6 CpG40.41 methylation and ELS by 5-HTTLPR genotype.

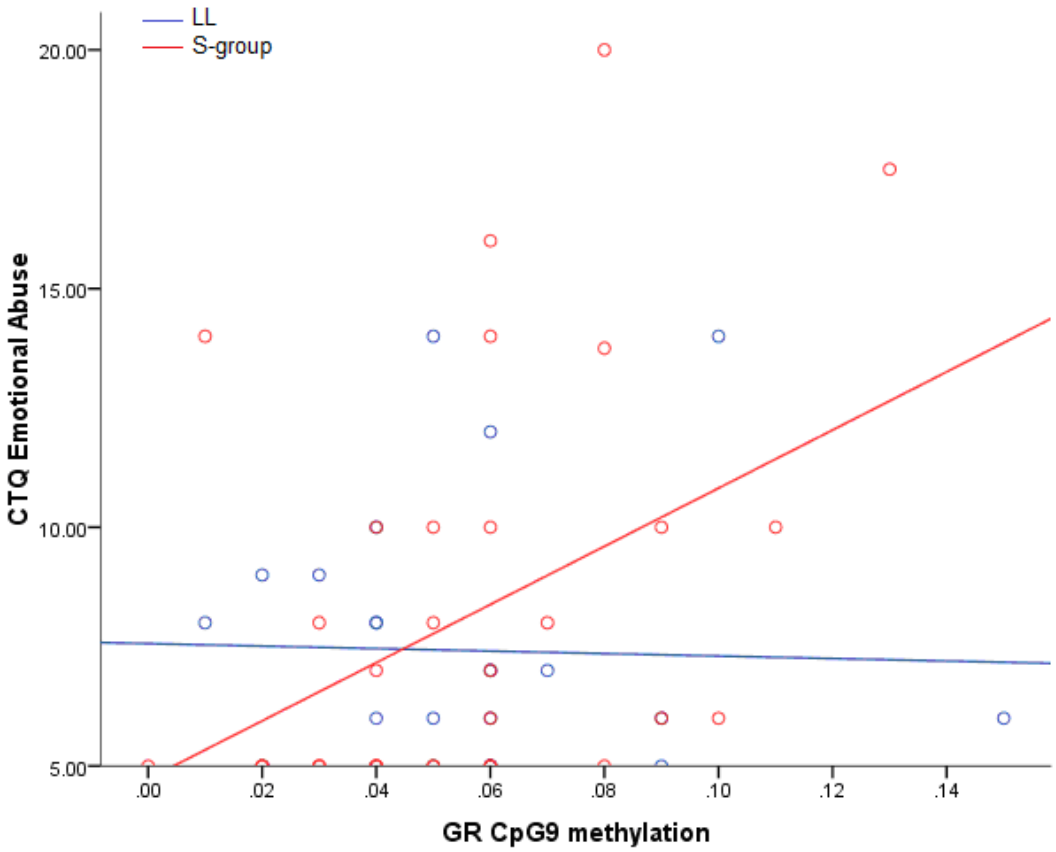


Figure 25. Association between emotional abuse and GR CpG9 methylation by 5-HTTLPR genotype.

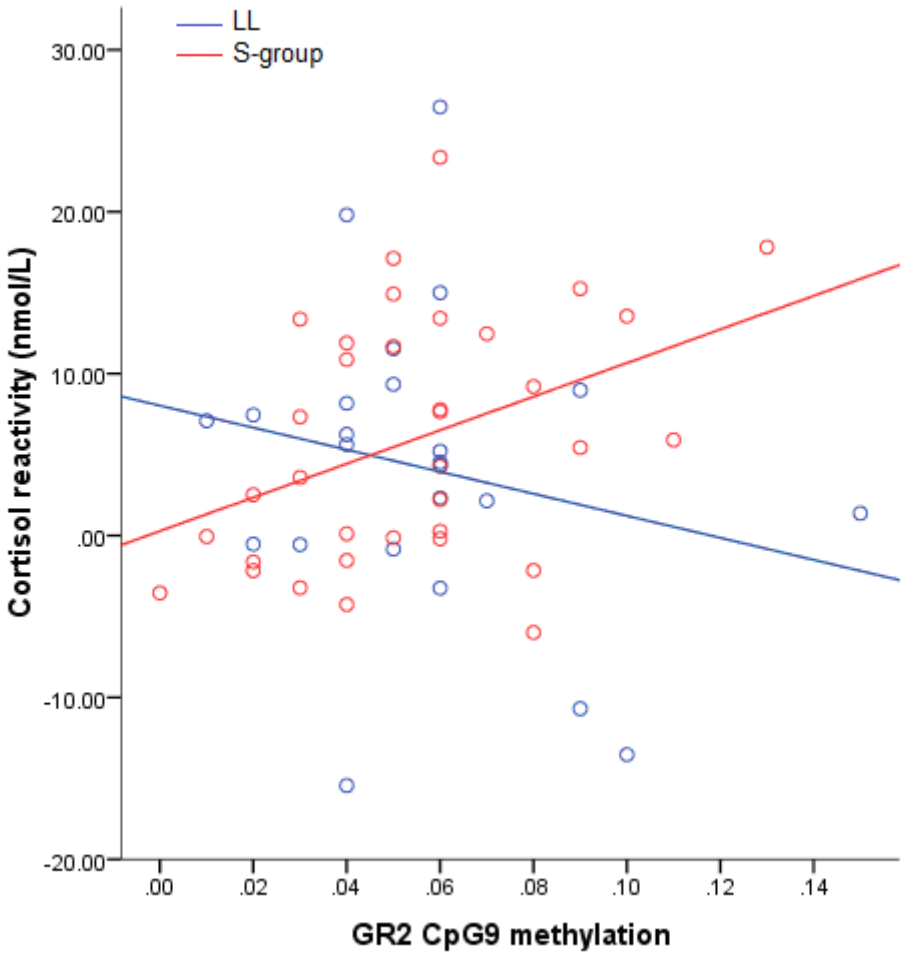


Figure 26. Association between cortisol reactivity and GR CpG9 methylation by 5-HTTLPR genotype.

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Appendix A

Consent Form

STONY BROOK
STATE UNIVERSITY OF NEW YORK

COMMITTEES ON RESEARCH INVOLVING HUMAN SUBJECTS

Established 1971

RESEARCH CONSENT FORM

Project Title: The Human Stress Response: Interaction of Life Stress History and Genetic Variation on Behavior. (NSF Grant title: "The Human Stress Response: Interaction of Life Stress History and Genetic Variation on Behavior, Brain Function, and the (Epi-)Genome"; NIA Grant title: "Gene-Environment Interactions in Loneliness and Stress Reactivity in Older Adults")
Principal Investigator: Turhan Canli, Ph.D.
Co-Investigators: Eli Hatchwell, M.D./Ph.D. (faculty), Arthur Stone, Ph.D. (faculty), Anett Müller (postdoctoral fellow and study coordinator), Dirk Moser (postdoctoral fellow). Other study personnel include graduate and undergraduate students.

Departments: Psychology, Pathology, Psychiatry

You are being asked to be a volunteer in a research study. This consent form contains important information to help you decide if you want to take part in this study. If you have any questions that are not answered in this consent form, please ask the member of the research staff who is reviewing the consent form with you for further information before you make your decision about taking part in this study.

PURPOSE

The purpose of this study is:

- You are invited to take part because you are a healthy adult and we are studying normal variations to social stress.
- The purpose of this study is to learn how individual differences in genes and in a person's life experience contribute to their response to social stress.

- A total of 200 younger (<65 years) and 200 older (65+ years) subjects are expected to participate in this study. These participants will either be enrolled at Stony Brook University or come from surrounding communities.

PROCEDURES

If you decide to be in this study, your part will involve:

- A visit to the General Clinical Research Center (GCRC) at the University Hospital where you will have an intravenous catheter placed in your arm to have some blood drawn (30 ml, which is approximately 2 tablespoons of fluid) over the course of the experiment for analysis of genetic information. You will then rest in a room for 20-45 minutes while completing various questionnaires. You will be shown how to collect samples of your saliva and will be asked to give at total up to 10 saliva samples during your study visit. You will then be asked to complete a series of tasks that may cause some psychological discomfort (not more than you would experience during a job interview). Afterwards, you will complete a few more questionnaires and interview questions for another 45-60 minutes. This visit will take about 4 hours.
- Your task performance will be videotaped. The interview in which you talk about your life experiences will be audiotaped in order to make sure that the researcher who conducted the interview correctly entered the information, after which the audio will be erased.
- Your saliva samples and blood, or biological materials extracted from your blood, may be kept for an indefinite amount of time.

RISKS / DISCOMFORTS

The following risks/discomforts may occur as a result of you being in this study:

- You may experience some distress from the laboratory tasks (similar to what a person experiences during a job interview). Also answering questions about past experiences might cause moderate psychological distress.
- The placement of the i.v. catheter and blood draw carries the risk of temporary pain and bruising where the needle enters the skin, and sometimes, fainting and/or infection.
- The genetic analyses to be conducted on your tissue in this study may pose future risks that are not known at this time.

BENEFITS

There is no benefit expected as a result of you being in this study. However, there is an indirect benefit, in that this work has the potential to reveal biological mechanisms of individual differences in traits and gene-environment interactions, which are currently poorly understood.

CREDIT TO SUBJECTS

If you participate in this study for course credit, you will receive 3 credits if you complete the study. If you do not complete the study, you will receive 1 credit for each 90 minutes of participation.

PAYMENT TO YOU

You will be paid $100 if you complete the study. If you do not complete the study, you will be paid $12.50 prorated per 30 minutes of participation. If you took public transport or a taxi cab to arrive at the GCRC, you will also be reimbursed for the cost of transportation.

CONFIDENTIALITY

Protecting Your Privacy in this Study

All the information we get about you will be kept private. We will do this by not writing down your name or anything else that could link you in any way to the answers you give us for our study. All the study data that we get from you will be kept locked up. If any papers and talks are given about this research, your name will not be used.

COSTS TO YOU

- There are no costs to you for participating in this study.

ALTERNATIVES

- Your alternative to being in this study is to simply not participate.

IN CASE OF INJURY

- If you are injured as a result of being in this study, please contact Dr. Turhan Canli at telephone # (631) 632-7803. The services of Stony Brook University Hospital will be open to you in case of such injury. However, you and/or your insurance company will be responsible for payment of any resulting treatment and/or hospitalization.

CONSEQUENCES OF WITHDRAWING

- If you withdraw before completion of study, you will not receive full subject pool credit (if you participate in this study for course credit) or you will only be paid for the time you spent in the study (if you participate in this study for payment).
- If you withdraw from the study, all biological samples (blood, DNA, and saliva samples) will be removed from the study analysis.

REMOVAL FROM STUDY

- You may be removed from the study if you fail to show up for the appointed time, fail to complete questionnaires, blood draw, saliva sample collection, or study tasks, or are non-compliant with instructions.

YOUR RIGHTS AS A RESEARCH SUBJECT

- Your participation in this study is voluntary. You do not have to be in this study if you don't want to be.
- You have the right to change your mind and leave the study at any time without giving any reason, and without penalty.
- Any new information that may make you change your mind about being in this study will be given to you.
- You will get a copy of this consent form to keep.
- You do not lose any of your legal rights by signing this consent form.

QUESTIONS ABOUT THE STUDY OR YOUR RIGHTS AS A RESEARCH SUBJECT

- If you have any questions, concerns, or complaints about the study, you may Dr. Canli, at telephone # (631) 632-7803.
- If you have any questions about your rights as a research subject or if you would like to obtain information or offer input, you may contact Ms. Judy Matuk, Committee on Research Involving Human Subjects, (631) 632-9036, OR by e-mail, judy.matuk@stonybrook.edu.

If you sign below, it means that you have read (or have had read to you) the information given in this consent form, and you would like to be a volunteer in this study.

Do you agree to allow use of the biological samples obtained from this study for use in future research, the purposes of which are unknown at this time? If you agree, any future studies using your sample will be subject to further regulatory review.

_____Yes _____ No Initial here:_____

You may be asked to come back to participate in other studies. Please check here if you would like to be contacted for future studies:

_____Yes _____ No Initial here:_____

Subject Name (printed) Subject Signature Date

Name of Person Obtaining Consent (printed) Signature of Person Obtaining Consent Date

Appendix B

TSST Instructions – for Experimenter:

Before taking the participant to the TSST:

- *Okay, now I will lead you over to the "testing" room, where you will begin the task portion of today's session.*
- *If a student: Please ask which job he/she would like to get after school.*
- *Alternatively, ask for the "dream job".*

On the way to the TSST room:

- "You mentioned that you could see yourself working in the field of …. Now imagine that you applied for a job, and were invited to a job interview at your dream company."

Please knock on the door once to notify the committee of your arrival and walk participant into the TSST room:

- *"The people you see here are panel members selected by your potential employer to evaluate all the applicants.*

- *Your job now is to explain to this panel why you are the right candidate for this job. The panel does have all your paper work, including your CV. So they are not interested in hearing about your academic and other achievements. Instead, they want to hear about your personality, about what makes you different from other people with the same qualifications.*

- *The panel is trained in evaluating your verbal and non-verbal behavior, and will be taking notes during your presentation. The presentation will also be videotaped for later analysis. Please look straight ahead during the talk.* (Point to the video camera.)

- *To prepare for this talk, you will now have 3 minutes to sit down at this desk to take some notes – which you will not be allowed to use later. After 3 minutes, the panel will ask you to fill out a questionnaire which lies right there on the table (upside down). And they will let you know when it's time to give your five minute presentation.*

- *It is important that you make a believable impression, because the committee members will ask additional questions in case of disagreement.*

- *After the presentation, the panel will have a second task for you.*
- *Do you have any questions?*
- *Keep in mind that you REALLY want to have this job!"*

Make sure that the participant has really understood the instructions, only then wish him/her good luck and leave the room.

- *"OK, I will now leave the room and pick you afterwards."*
- *"You can now start taking notes. Good luck!"*

Appendix C

Debriefing Form

Department of Psychology

Thank you very much for your participation today!

We would like to inform you of some details of the study. Your performance was not being evaluated or monitored, and your performance in this task ***does not at all*** reflect your speech or math ability. You did a wonderful job! *It is very important that you understand this.*

The reason why we use this task is we are interested in how the body responds to stress. The way we assess stress is to measure a naturally occurring stress hormone in your body called cortisol. The tasks were designed to be very difficult and stressful because we are interested in what happens to this hormone in your body under stress, which is why we have been collecting saliva samples from you.

Because the key to this task remaining a successful stressor relies very heavily on people actually believing that it is true, if people knew the true nature of the task before participating, they wouldn't feel very stressed, and then all our efforts would be in vain.

We would like to ask you to not talk about this task with your friends or people you know. This is really important since we still have to test a lot more subjects, and perhaps we might have scheduled people you know. Again we want to emphasize that your participation in this task by no means reflects your own performance or math ability, and we want to be sure you understand that.

Once again, we want to thank you very much for participating. *It would simply not be possible without your contribution to the study!*

If you have any questions please do not hesitate to contact us:

Dr. Turhan Canli

(631)-632-4209
canlilab2@gmail.com